STUDIES ON BIOETHANOL PRODUCTION FROM SELECTED AGRO-RESIDUES

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I.	Media composition

LIST OF ABBREVIATIONS

%	: Percentage	HMF	: Hydroxy methyl furfurol
µ	: Micron	IU	: International units
ADF	: Acid detergent fibre	L	: Litre
ADL	: Acid detergent lignin	LiP	: Lignin peroxidase
APEX	: Ammonia fibre explosion	M	: Molar
C_2H_5OH	: Ethanol	m	: Minute
$C_6H_{12}O_6$	: Glucose	mg	: Milligram
CD	: Critical difference	ml	: milliliter
cm	: Centimeter	mm	: Millimeter
CMC	: Carboxy methyl cellulose	MnP	: Manganese paraxidase
CO	: Carbon monoxide	NaOH	: Sodium hydroxide
CO_2	: Carbon dioxide	NDF	: Neutral detergent fibre
DNSA	: Di-nitro salicylic acid	NS	: Non-significant
FPU	: Filter paper units	SEm	: Standard error mean
g	: Grams	SHF	: Separate hydrolysis and fermentation
h	: Hour	SSF	: Simultaneous saccharification and fermentation
H_2SO_4	: Sulphuric acid	U	: Units
HCL	: Hydrochloric acid		

1. INTRODUCTION

Energy is an important factor for the economic development of a nation. The consumption of energy has increased due to urbanization, advanced technologies, industrial developments, increase in population *etc.* Petroleum is one source of non renewable liquid energy used extensively in the automobile sector. The present rate of consumption of petroleum products has resulted in depletion of petroleum reserves. Also, the burning of conventional automobile fuels emit lot of CO_2 and CO that contribute for global warming. Therefore, an alternative energy is required for automobiles.

India's proven oil resources are currently estimated at about five million barrels. A strong growth on oil demand has resulted in increased petroleum consumption by 75 per cent since last decade. India and China are expected to account for 17 per cent of global liquid fuel consumption surpassing total consumption of entire Europe by 2025 (Mark Gehlhar *et al.*, 2010). Howard *et al.* (2003) evaluated the fuel consumption and opined that the present rate of petrol consumption results in depletion of natural reserves. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050. An alternate energy source is essential to meet the ever growing demands of energy. Bio-ethanol is one such principal alternate fuel that can substitute gasoline.

Ethanol or ethyl alcohol (C_2H_5OH) is an important organic chemical because of its unique properties and therefore can be used widely for various purposes. Under ordinary conditions, ethanol is a volatile, flammable, clear, colourless liquid, miscible in both water and non-polar solvents (Lang *et al.,* 2001). Ethanol is a very attractive alternative fuel, not only because of the much lower contribution to greenhouse gases compared with fossil fuels, but also because of its versatility and advantageous physical properties. It can be used in a low level blend in unmodified vehicles or in high level gasoline blends for modified vehicles. Similar to high level gasoline blends it can be burnt in modified vehicles as neat ethanol. Because ethanol has a higher octane than gasoline, there is no need for additional, usually very toxic, octane boosters (Demain *et al.*, 2005). Furthermore, ethanol contains oxygen which facilitates better combustion in motor, reducing tailpipe carbon monoxide emission. Bioethanol forms an ecofriendly fuel.

The production of ethanol has two routes: synthetic and biological. The synthetic ethanol production is commonly carried out by a catalytic hydration of ethylene in vapour phase and often as a by-product of certain industrial operations. The ethanol produced from this process is mostly used as a solvent (60%) and chemical intermediate (40%). Fermentative ethanol production accounts for 93 per cent of the total ethanol production in the world. The ethanol is produced from fermentation of sugars extracted mostly from crops (Wyman, 1995). *Saccharomyces cerevisiae* is the most popular microorganism used for ethanol production due to its high ethanol yield and high tolerance to rather high ethanol concentration (Lynd and Zhang, 2002). However, several other yeasts namely *Kluyveromyces marxianus* (Sohn and Seu, 1994), *Pachysolen tannophilus* (Zayed and Meyer, 1996), *Candida shehate* (du-Preez *et al*., 1986) and *Pichia stipitis* (Ferrari *et al*., 1992) and bacteria *Zymomonas mobilis* (Kaur *et al*., 1993) are also capable of producing ethanol.

Several raw materials are being used for the bioethanol production. Easily fermentable sugar and starch feed stocks are the main feedstocks used for ethanol production nowadays. However, these crops are also food for human beings and animals, thus the expansion of production capacity of ethanol is limited by supply of these feed stocks. Therefore, abundantly available lignocellulosic materials in the form of agricultural residues are available as alternative feedstock for ethanol production (Hamelinck *et al*., 2005).

Solar energy received at earth is 2.5×10^{21} Btu per year and present human usage is only 2.0×10^{17} Btu per year. The energy stored as carbon *via* photosynthesis is ten times higher than human usage. Terrestrial plants produce 1.3×10^{10} M tons of dry wood per year which is equivalent to 7×10^{9} M tons coal per year (*i.e.* 2/3 of world requirement). Cellulose feed stock from agriculture and others contribute to about 180 million tons per year (Demain *et al.,* 2005). These lignocelluloses being inexpensive, plentiful and renewable in nature are available for conversion.

The current production of agro residues is 800 million tons, if 10 per cent of it is utilized for ethanol production, can produce 15 million tons of ethanol per year. Presently, most of the crop residues from cereals and about 50 per cent from pulses are being utilized as fodder. The residues of other crops *viz.* sunflower hulls, cotton stalks, corn stover, sugar cane trash, bagasse, sugar cane toppings, which are less used as cattle feed, can be diverted for ethanol production. It is estimated that, in India 127.27 million tons of biomass is presently available as surplus which will increase to about 226.01 million tons by 2015 (Pathak *et. al.*, 2004).

Several physical and chemical methods are employed for the pre-treatment of bagasse which include steam explosion, gamma radiation, use of alkali, acid and hydrogen peroxide treatment *etc.* Among these chemical treatments, NaOH solution was found effective and economical (Doran *et. al.*, 1994; Kodali and Pogaku, 2006 and Chen *et al.*, 2009).

Raw, untreated biomass is extremely recalcitrant to enzymatic digestion. Decades of research have demonstrated that biomass requires extensive processing and hydrolysis of the raw material into fermentable sugars, and its subsequent biological conversion into a myriad of fuels and chemicals. Therefore, a number of thermo chemical pre-treatment methods have been developed to improve digestibility (Wyman *et al.*, 2005).

Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin. Deriving fermentable sugars from lignocellulosic biomass and fermentation of derived sugars into ethanol by suitable means is a critical step in research and development. The present investigation was taken up to study different pre-treatment methods to derive maximum fermentable sugars to be fermented to bio- ethanol from different agro residues with the following objectives.

1. To achieve maximum delignification of selected agro-residues.
2. To release maximum reducing sugars through saccharification of delignified substrates.
3. To achieve maximum bioethanol production from the pre-treated substrates by different microbial combinations.
4. Pilot scale testing of potential agro-residue for bioethanol production.

2. REVIEW OF LITERATURE

The most common renewable fuel produced today is ethanol derived from corn grain (starch) and sugar cane (sucrose). It is expected that there will be limits to the supply of these raw materials in the near future; therefore, lignocellulosic biomass is considered as an attractive feed stock for future supplies of ethanol (Wyman *et al.,* 2005). Lignocellulosic biomass is one of the virtually inexhaustible and easily available renewable sources of energy. The abundantly available lignocelluloses require pre-treatment for obtaining fermentable sugars and conversion of the same to ethanol. The various types of pre-treatments employed for different lignocellulosics, saccharification strategies followed by microorganisms used for bio-ethanol production are being reviewed here.

2.1 Bioethanol

Simple alcohols are produced by three principal methods that are the backbone of aliphatic organic synthesis namely hydration of alkanes obtained from the cracking of petroleum, hydrolysis of cellulosic materials and fermentation of carbohydrates (Morrison and Boyd, 1983). Synthetic and biological means are the two routes for ethanol production. Wyman (1995), while reviewing the ethanol production methods categorised that the synthetic ethanol production is commonly carried out by a catalytic hydration of ethylene in vapour phase and often as a by-product of certain industrial operations. The ethanol produced from this process is mostly used as a solvent (60%) and chemical intermediate (40%). Fermentative ethanol production accounts for 93 per cent of the total ethanol production in the world. The ethanol is produced from fermentation of sugars extracted mostly from crops.

Ethanol or ethyl alcohol (C_2H_5OH) is an important organic chemical because of its unique properties and therefore can be used widely for various purposes. Under normal conditions, ethanol is a volatile, flammable, clear, colourless liquid, miscible in both water and non-polar solvents (Lang *et al.,* 2001). As reported by Lynd and Zhang (2002), *Saccharomyces cerevisiae* is the most popular microorganism used for ethanol production due to its high ethanol yield and high tolerance to high ethanol concentration. The ethanol produced is mostly used as fuels (92%), industrial solvents and chemicals (4%) and as beverages (4%).

Howard *et al.* (2003) had evaluated the fuel consumption and opined that the present rate of petrol consumption results in depletion of natural reserves. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels by 2050.

With the oil crisis of the 1970s, ethanol became established as an alternative fuel. Many countries started programs to study and develop fuels in an economic way from available raw materials (Demirbas, 2005). Subramanian *et al*. (2005) reported that the present availability of ethanol in India is 744 million litres which is sufficient only to 5 per cent blending with gasoline and not with diesel. Therefore, need to enhance the availability of ethanol to meet the auto-biofuel requirements.

2.2 Raw materials for bioethanol production

Several raw materials are being used for the bioethanol production *viz*. easily fermentable sugary feed stocks (molasses), starchy feed stocks (grains) and feed stocks containing complex sugars in the form of cellulose and hemicellulose.

2.2.1 Sugar feed stocks

Fermentation involves microorganisms that use the fermentable sugars for food and in the process produces ethanol and other by-products. These microorganisms can typically use the 6-carbon sugars, one of the most common being glucose. Therefore, biomass materials containing high levels of glucose or precursors of glucose are the easiest to convert to ethanol. One example of a sugar feedstock is sugarcane molasses. Other biomass feedstocks rich in sugars (known as saccharides) include sugar beet, sweet sorghum, and various fruits. However, these materials are all in the human food chain and, except for some processing residues, are generally too expensive to use for fuel ethanol production (Doelle and Doelle, 1990 and Yadav *et al.,* 1997).

2.2.2 Starchy feed stocks

Another potential ethanol feedstock is starch. Starch molecules are made up of long chains of glucose molecules. Thus, starchy materials can also be fermented after breaking starch molecules into simple glucose molecules. Examples of starchy materials commonly used around the world for ethanol production include cereal grains, potato, sweet potato, and cassava, but the starch being used is edible. This will lead to loss of valuable food for the purpose of ethanol production (Badger, 2002).

2.2.3 Cellulosic feed stocks

Like sugar materials, starchy materials are also in the human food chain and are thus expensive. Therefore, the next alternative is cellulosic materials. Jeffries (1985) reported that various forms of biomass resources in the world can be classified into wood residues, municipal solid waste, agriculture residues and dedicated energy crops. Several such feed stocks have been studied for their potentiality to yield ethanol by various researchers *viz*. rice straw (Tanaka *et al.,* 1985, Harpreet *et al*., 1998, Karimi *et al*., 2006 and Kodali and Pogaku, 2006), Bagasse (Saska and Ozer, 1995, Lavarack *et al*., 2002 and Singhania *et al*., 2006), Cotton stalks, (Kerem *et al*., 1992), Wheat straw (Koullas *et al*., 1993 and Zayed and Meyer, 1996), Alfalfa fibre (Sreenath *et al*., 2001), Sugar cane leaves (Harikrishna *et al*., 2001), sunflower hulls (Sharma *et al*., 2004) and Corn stover (Teymouri *et al*., 2005). Being abundant and outside the human food chain makes these cellulosic materials relatively inexpensive feed stocks for ethanol production (Howard *et al.,* 2003).

Biomass is a sustainable alternative to fossil energy carriers which are used to produce fuels, electricity, chemicals, and other goods. At the moment, the main biobased products are obtained by the conversion of biomass to basic products like starch, oil, and cellulose. In addition, some single chemicals and fuels are produced. Biorefineries are supposed to contribute to a more sustainable resource supply and to a reduction in greenhouse gas emissions. However, biobased products and fuels may also be associated with environmental disadvantages due to, *e.g.* land use or eutrophication of water. Uihlein and Schebek, (2009) performed a life cycle assessment of a lignocellulose feedstock biorefinery system and compared it to conventional product alternatives. The biorefinery was found to have the greatest environmental impacts in three categories: fossil fuel use, respiratory effects, and carcinogenics. However, the optimum variant (acid and heat recoveries) yields better results than the fossil alternatives, with the total environmental impacts being approximately 41 per cent lower than those of the fossil counterparts.

2.2.3.1 Composition of lignocellulosic feed stocks

The lignocellulosic materials are mainly composed of cellulose, hemicellulose, lignin and other minor components such as ash and protein (Alder, 1977). During cell growth, these cells are surrounded only by primary walls, which are mainly composed of polysaccharides and proteins, although phenolic substances can occur. However, when cell growth ceases, these cells synthesize a secondary wall that contains cellulose (40-50%), hemicelluloses (20-30%) and lignin (20-30%). The secondary walls of these cells usually account for more than 95 per cent by weight of the cell wall material (Palonen *et al.,* 1999). During secondary wall biosynthesis, the primary wall also becomes impregnated with lignin. The secondary walls are rigid and provide structural support. The impregnation of the walls with lignin makes them hydrophobic and more resistant against attack from microorganisms. The composition of cell walls varies depending on the plant species, tissue type, cell type, region within the cell wall and developmental stage of the cell. The cell wall composition can also be influenced by genetic variation within a species, growth conditions and age of the plant. This is important, because the cell wall composition and structure affect the saccharification process (Carpita and McCann, 2000).

Hamelinck *et al*. (2005) reviewed the composition of lignocellulosic biomass and reported that the exact biochemical composition of biomass depends on many factors such as area covered, use of fertilizers, time of harvesting and storage conditions. Generally the soft wood hemicelluloses yield more C6 sugars where as hard wood yields more C5 sugars. The bark and bark residues have relatively higher lignin content. The empherical formula for lignin is $C_9H_{10}O_2$ $(OCH_3)n$. Where, n= 1.4, 0.94 and 1.18 for hard wood, soft wood and grasses respectively.

Gadde, *et al*. (2009) reported that 97.19, 21.86, and 10.68 Mt of rice straw residue are produced in India, Thailand, and the Philippines, respectively and in India, 23 per cent of rice straw residue produced is surplus and is either left in the field as uncollected or to a large extent open-field burnt. About 48% of this residue produced is subjected to open-field burning in Thailand, and in the Philippines it is 95 per cent. The GHG emissions contribution through open-field burning of rice straw in India, Thailand, and the Philippines are 0.05, 0.18 and 0.56 per cent.

2.2.3.1.1 Cellulose

Cellulose is a linear polymer of hydro D-glucose units linked by β-1, 4 glucosidic bonds. Native cellulose exists in the form of micro fibrils, which are paracrystalline assemblies of several dozen (1→4) β-D-glucan chains with hydrogen bonds connected to one another. The cellulose micro fibrils are embedded in a matrix of non cellulosic polysaccharides, mainly hemicellulose and pectic substances. An important, relatively unusual feature of cellulose is its crystalline structure. The cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues) which undergo self-assembly at the site of biosynthesis (Brown and Saxena, 2000). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called micro fibrils, and these are in turn assembled into the familiar cellulose fibres. Regardless of their orientation, the chains are stiffened by both intra-chain and inter-chain hydrogen bonds. Adjacent sheets overlie one another and are held together by weak inter-sheet Van der Waals forces; despite the weakness of these interactions, their total effect over the many residues in the elementary fibril is considerable (Lynd *et al.,* 2002).

2.2.3.1.2 Hemicellulose

Hemicellulose is a complex, heterogeneous mixture of sugars and sugar derivatives that form a highly branched network. The monomers that comprise hemicellulose are hexoses (glucose, galactose, and mannose) and pentoses (arabinose and xylose). Some monomers are acetylated (Brigham *et al.,* 1996). Hemicellulose can be classified into three groups, namely, xylans, mannans, and galactans based on the polymer backbone that is very often homopolymeric with β-1,4 linkage. Xylan is by far the most important component because of its large quantities in the biomass (Hamelinck *et al*., 2005)

2.2.3.1.3 Lignin

Lignin is a three dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds. When the plant matures and the cell growth ceases, the middle lamella (the cement between the primary walls of adjacent cells) and the secondary cell wall (inside of primary wall) have a large degree of lignin. The lignin strengthens the cell structures by stiffening and holding the fibres of polysaccharides together (Sun and Cheng, 2002).

2.3 Substrate pre-treatment

The term “pre-treatment” is widely used in the process engineering literature to refer to a process step which converts lignocellulosic biomass from its native form, in which it is recalcitrant to cellulase enzyme systems, into a form for which enzymatic hydrolysis is effective. Most of the β -glucosidic bonds in naturally occurring lignocellulosic materials are inaccessible to cellulase enzymes by virtue of the small size of the pores in the multicomponent spatially heterogeneous biomass matrix as well as enzyme associated inaccessibility. In addition, cellulose in naturally occurring materials is closely associated with hemicellulose and other structural polysaccharides. Carbohydrate-rich micro fibrils are surrounded by a lignin seal rendering lignocellulosic materials amenable to enzymatic hydrolysis and overcoming both physical and chemical barriers which is done by pre-treatment (Fan *et al.,* 1982).

There are numerous pre-treatment methods or combinations of pre-treatment methods available. In general, pre-treatment techniques can be grouped into three categories: physical, chemical and biological. Physical pre-treatment methods include comminution, steam explosion and hydrothermolysis.

The most common chemical pre-treatment methods used for cellulosic feedstocks are dilute acid, alkaline, organic solvent, ammonia (ammonia fiber explosion AFEX), sulfur dioxide, carbon dioxide or other chemicals to make the biomass more digestible by the enzymes (Schell *et al.,* 2003 and Mosier *et al.,* 2005). Pre-treatment is required to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic chemical composition and structure, so that hydrolysis of carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. Pre-treatment affects the structure of biomass by solubilizing hemicellulose, reducing crystallinity and increasing the available surface area and pore volume of the substrate (Mosier *et al.,* 2005).

2.3.1 Mechanical pre-treatment

Mechanical pre-treatment basically refers to the mechanical and physical actions to clean and reduce the particle size of biomass and destroy its cell structure to make it accessible to further chemical or biological treatments (Hamelinck *et al.,* 2005).

2.3.1.1 Comminution

Biomass materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10 to 30 mm after chipping and 0.2 to 2 mm after milling or grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of spruce and aspen chips and improving the digestibility of the biomass than ordinary ball milling (Shafizadeh and Bradbury, 1979).

Effectiveness of ball milling (BM) and wet disk milling (WDM) of sugarcane bagasse and straw was compared and evaluated in terms of enzymatic hydrolysis and fermentation by Ana da Silva *et al*. (2010). At optimum conditions BM-treated bagasse and straw produced glucose of 78.7 and 77.6 per cent and xylose yields of 72.1 and 56.8 per cent, respectively. BM improved the enzymatic hydrolysis by decreasing the crystallinity, while the defibrillation effect favoured enzymatic conversion for WDM samples.

2.3.2 Chemical pre-treatment

The delignification of several lignocellulosic feed stocks has been achieved successfully by using acid and alkali by several researchers.

2.3.2.1 Acid pre-treatment

Concentrated acids such as sulphuric acid (H_2SO_4) and hydrochloric acid (HCl) have been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion (Sivers and Zacchi, 1995). This particular bottle neck was attempted to solve by using dilute acids.

Several experiments have been conducted with the use of dilute acid, particularly H_2SO_4 for the pre-treatment of various biomass feed stocks and many a times acid was coupled with hot water treatment or steam exposure at varying temperatures. Torget *et al.* (1991) pre-treated three hard woods (silver maple, Sycamore, black locust), corn cobs and corn stover with dilute sulphuric acid (0.45- 0.5% v/v) at 160^0 C for 5- 10 m. Water hyacinth with 1% H_2SO_4 was refluxed for 4 h by Premidevi and Singh (1995) and cellulose fibre from paper plant with 0.25% H_2SO_4 by Nikolov *et al*. (2000).

Saha and Bothast (1999) reported that the dilute acid pre-treatment of corn straw at high temperature pre-treatment had solubilised xylan and large portion of the glucose was degraded to hydroxyl methyl furfural (HMF) and xylose furfural, they were found to be inhibitory to ethanol production from microorganisms. Dilute-acid hydrolysates from lignocellulose are, to a varying degree, inhibitory to yeast. In particular Taherzadeh *et al.* (2000) had found that the hydrolyzates contained between 1.4 and 5 g L^{-1} of furfural and between 2.4 and 6.5 g L^{-1} of 5-hydroxy methyl furfural (HMF).

Hydrolysis of rice straw by dilute sulphuric acid at high temperature and pressure in two stages was studied by Karimi *et al*. (2006). The hydrolysis retention time (3-10 min), pressure (10-35 bars) and acid concentration (0-1%) were examined.

A maximum yield of 80.8 per cent xylose from depolymerization of xylan in the first stage hydrolysis and glucose yield of 25.8 per cent was obtained at 15 bars pressure, 10 min retention time and 0.5% acid concentration. In the second stage, best result was obtained at pressure 30 bars, retention time 3 min, where a total of 78.9 per cent xylan and 46.60 per cent glucan were converted to xylose and glucose respectively.

Prosopis juliflora (Mesquite) was pre-treated by using dilute H_2SO_4 (3.0%, v/v) which resulted in hydrolysis of hemicelluloses from lignocellulosic complex to pentose sugars along with other byproducts such as furfural, hydroxymethyl furfural (HMF), phenolics and acetic acid (Gupta *et al*., 2009). The enzymatic hydrolysis of the delignified substrate using 3.0 Units of FPase and 9.0 U of β-glucosidase per mL of citrate phosphate buffer (0.05 M, pH 5.0) they achieved maximum saccharification (82.8%) after 28 h incubation at 50 °C.

Dilute acid pre-treatment (DAP) is commonly employed prior to enzymatic deconstruction of cellulose to increase overall sugar and subsequent ethanol yields from downstream bioconversion processes. Typically optimization of pre-treatment is evaluated by determining hemicellulose removal, subsequent reactivity towards enzymatic deconstruction, and recoverable polysaccharide yields. The effect of dilute acid pre-treatment on the supramolecular and ultrastructure of lignocellulosic biomass was evaluated by Foston and Ragauskas, (2010). A series of dilute acidic pre-treatments, employing ∼0.10–0.20 mol M^{-3} H_2SO_4 at ∼160–180 °C, for varying residence times were conducted on *Populus* and switchgrass samples. The gel permeation chromatography analysis showed a reduction in the molecular weight of cellulose and change in its polydispersity index with increasing residence time, indicating that pre-treatment is actually degrading the cellulose chains. The ^{13}C cross polarization magic angle spinning (CPMAS) NMR spectroscopy showed that the crystallinity index increased with residence time where, the lateral fibril dimension (LFD) and lateral fibril aggregate dimension (LFAD) increased at the C_4 region in the carbon spectrum of the isolated cellulose.

Kuhad *et al*. (2010) have performed acid hydrolysis (3.0%, v/v H_2SO_4, 120°C for 45 min) of *Lantana camara* and observed that the hydrolysis had produced 187.14 mg g^{-1} total sugars along with fermentation inhibitors such as phenolics (8.2 mg g^{-1}), furfurals (5.1 mg g^{-1}) and hydroxy methyl furfurals (6.7 mg g^{-1}). Later, the toxic compounds from the acid hydrolysate was removed by sequential application of overliming (pH 10.0) and activated charcoal (1.5%, w/v) adsorption. The acid-pre-treated biomass of *L. camara* was further delignified through combined pre-treatment of sodium sulphite (5.0% w/v) and sodium chlorite (3.0% w/v) and achieved removal of 87.2 per cent lignin.

2.3.2.2 Alkali pre-treatment

The alkali pre-treatment is most frequently used to increase the digestibility of some lignocellulose substrates. This treatment was developed as a pulping process to produce high strength, long fibre paper products. Alkali pre-treatment was tried either solely or in combination for efficient delignification and thereby to make the pre-treated substrates accessible for further enzymatic saccharification.

Koullas *et al*. (1993) investigated alkaline delignification of wheat straw at 120^0 C and between 20^0 C and 36^0 C using either aqueous or organic alkaline solution and achieved 70-100 per cent hydrolysis. Bjerre *et al*. (1996) performed combined wet oxidation and alkaline hydrolysis of wheat straw. 20g NaOH L^{-1} at 170^0 C for 5-10 min, pre-treatment yielded about 85 per cent (w/w) yield of glucose convertible cellulose and no microbial inhibitors such as furfural and hydroxymethyl-furfural were found in the process. It was also tried by Lawther and Runcang (1996) on straw and other non-wood materials, which still constitute the major fibre source for paper production. Several biomass substrates were subjected to this treatment and responded differently.

Curreli *et al.* (1997) experimented a new mild alkaline/oxidative pre-treatment of wheat straw prior to enzymatic hydrolysis that starts with an alkaline (1% NaOH for 24h) step to solubilize the hemicellulose and prepare the substrate, then a second alkaline/oxidative step (1% NaOH and 0.3% H_2O_2 for 24h) solubilizes and oxidizes the lignin into low polluting compounds. The overall process was carried out at a low temperature (25-40°C) using a low concentration of chemicals, resulting in relatively low cost and waste liquor containing only a trace of pollutant derived from the lignin.

Sharma *et al*. (2004) pre-treated sunflower hulls (40 mesh size) with 0.5% NaOH w/v, followed by autoclaving at 15 psi for 1.5 h, the filtrate resulted in 57.6% saccharification with *Trichoderma reesei* Rut C30 cellulase enzyme and obtained 59.80% saccharification.

Kodali and Pogaku (2006) pre-treated rice bran with 5 N NaOH and then milled to 50 mesh (0.3 mm) size followed by 10 min steaming. They obtained cellulose enriched rice bran and reported this process to be an optimum pre-treatment method for rice bran. Raghavendra (2006) delignified paddy straw, wheat straw and sugarcane bagasse using alkali. The NaOH treatment at 3% (particle size 5 mm) with residential time of 8 h (ambient temp.), followed by autoclaving at 121°C, 15 psi for 1 h found efficient in releasing of maximum cellulose in paddy straw 0.56 g g^{-1} (56.0%), wheat straw 0.593 g g^{-1} (59.3%) and in bagasse 0.695 g g^{-1} (69.5%).

Four commonly used chemical pre-treatment processes namely dilute acid, lime, aqueous ammonia steeping followed by dilute acid hydrolysis, and sodium hydroxide, were evaluated by Chen *et al*. (2009) to provide comparative performance data on corn stover. The pre-treatment of corn stover with 2% NaOH substantially increased the lignin removal and enhanced the accessibility and digestibility of cellulose over other three chemical treatments.

Pre-treatment of switchgrass using aqueous ammonia or NaOH was investigated as a means to enhance the enzymatic digestibility was studied by Gupta and Lee (2010). To increase the effectiveness of pre-treatment, H_2O_2 was supplemented with ammonia or alkali. The composition of pre-treatment liquid indicated that hemicellulose was solubilized during alkaline treatment and existed either in the form of oligomers or as lignin–carbohydrate complex (LCC). LCC formation was prominent in ammonia treatment and in NaOH/H_2O_2 treatment, and that LCC formation protects the hemicelluloses sugars from degradation. Lignin analyses data indicated that NaOH–lignin had more uniform structure and higher O/C ratio than ammonia–lignin and NaOH–lignin had lower aromatic content but higher guaiacyl type structure than ammonia–lignin.

2.3.3 Combination of Physical and chemical pre-treatments

The combination of chemicals and heat treatments are known to delignify the agroresidues and other biomass materials. Some of the investigations are presented here.

Tanaka *et al.* (1985) investigated pre-treatment methods with n-butylamine (n-BA). In the first treatment rice straw was boiled at boiling point for 1 h under refluxing with 10% n-BA and in the second, autoclaved rice straw at 120^{0} C for 1h with 1% n-BA. They achieved delignification of 60% with overall cumulative recovery of 70% total sugars (cellulose and hemicelluloses) from both the pre-treatments. Torget and Hsu (1994) while evaluating two temperatures (140^{0} C and 170^{0} C) with dilute acid pre-hydrolysis of hybrid poplar using percolation process observed xylose solubilisation to about 92 per cent of the theoretical value with 2 per cent of the xylan being degraded to furfural.

Adrados *et al.* (2005) were pre-treated starch free wheat bran (5% DM) in microwave oven from 110^{0} to 210^{0} C for 20 to 60 min to determine optimal conditions, with or without H_2SO_4 (0.1, 0.2 and 0.4%) and enzymatically hydrolysed at pH 5 and temperature 50^{0} C for 72h. The highest yield of arabinose and xylose was achieved at 2% H_2SO_4 concentration and 130^{0} C for 10 minutes.

2.3.3.1 Steam explosion pre-treatment (autohydrolysis)

Steam explosion is the most commonly used method for pre-treatment of lignocellulosic materials. In this method, chipped biomass is treated with high-pressure saturated steam and then the pressure is swiftly reduced, which makes the materials undergo an explosive decompression.

Excoffier *et al*. (1991) studied steam explosion of wood chips impregnated with sulphuric acid (0.4%) and obtained the highest glucose recovery at 225^{0} C for 120s after 24h of *Trichoderma reesei* CL-847 cellulase enzymatic hydrolysis.

Nguyen *et al.* (1998) studied acid imprignated steam explosion (0.4% sulphuric acid, 200 - 230^{0}C, 1-5 min) and reported solubilisation of 90-95 per cent hemicelluloses from soft wood.

Liquid hot water pre-treatment was compared against steam explosion of bagasse by Laser *et al.* (2002). Liquid hot water pre-treated bagasse produced about 80% xylan recovery without formation of fermentation inhibitors. Further, the pre-treatment achieved about 80 per cent sugar conversion to ethanol by SSF in 14 days.

Soderstorm *et al*. (2003) steam exploded soft wood by two-step pre-treatment of temperatures between 180^0 C and 220^0 C for 2, 5 and 10 min residence time in presence of H_2SO_4 (1% and 2%). It was observed that H_2SO_4 impregnation improved overall sugar yield.

Mixed hemlock log fuel and pin chips (85:15 dry wt.) were converted into fermentable sugars by two stages dilute sulphuric acid hydrolysis. The first stage (190^0C for 150 s with 1.1% acid) and second stage (210^0 C for 115 s with 2.5% acid). The hydrolysis resulted in recovery of 13.6 g glucose, 10.5 g mannose and galactose and 2.8g xylose from 100g original dry sample (Kim *et al*., 2005).

Wood chips from six different Douglas-fir trees and a representative Lodgepole pine were steam pre-treated at a single pre-treatment condition (200 °C 4% SO_2 for 5 min) which had previously been shown to be effective for Spruce and Lodgepole pine chips by (Kumar *et al*., 2010). It was observed that all of the softwood samples responded with saccharification of more than 60 per cent of the cellulose after 72 h, at an enzyme loading of 20 FPU g^{-1} cellulose.

The dry and ensiled industrial hemp (*Cannabis sativa* L.), a biomass source was steam pre-treated prior to ethanol production by Sipos *et al.* (2010). For both materials, impregnation with 2% SO_2 followed by steam pre-treatment at 210° C for 5 min were found to be the optimal conditions leading to the highest overall yield of glucose recovery and polysaccharide conversion after the enzymatic hydrolysis step.

2.3.4 Biological pre-treatment

In recent years, biological pre-treatment has received emphasis because of its advantages such as low cost and environmental compatibility. Microorganisms produce multiple enzymes systems to degrade plant cell materials. Several microorganisms are capable of degrading lignin and thereby making the plant biomass accessible to saccharification of polysaccharides.

2.3.4.1 Microbial pre-treatment

The advance developments in the field of microbiology have led to the search of microorganisms that produce variety of lignin degrading enzymes. These microorganisms are capable to cause delignification of any recalcitrant substance particularly lignin in the agroresidues.

Lignin is the second most abundant deposit of organic carbon in the biosphere, being surpassed only by cellulose. It has been estimated that the amount of lignin in terrestrial plants approaches 4×10^{11} tons and that every year 3×10^{10} tons of the polymer are deposited in plant tissues. Therefore, lignin biodegradation is a key process for the recycling of carbon in nature. Lignin is a highly irregular biopolymer with an undefined structure. It is composed of oxygenated phenylpropanoid units which are linked among them through various types of C–C and C–O–C bonds. Due to these unusual features, the macromolecule is rather refractory to biodegradation.

The term white-rot fungi have been applied to certain ligninolytic basidiomycetes with a relatively high selectivity to degrade lignin in wood. This is the only group of microorganisms with the capacity to break down lignin extensively, all the way to carbon dioxide and water. These fungi produce a set of enzymes which are directly involved in lignin decay. Among these are a phenol-oxidase termed laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). Each species secretes a particular assortment of this enzymatic machinery to the medium in which it is growing. Thus, some strains produce LiP, MnP and laccase, others produce only MnP and laccase, or LiP plus laccase, *etc.* (Wariishi *et al*., 1992).

The changes in the major straw constituents were studied by the solid state fermentation with different lignolytic fungi. *Trametes versicolor* and *Pleurotus ostreatus* increased *in vitro* digestibility of wheat straw (Valmesada *et al*., 1991).

Trametes versicolor caused large simultaneous degradation and *Pleurotus ostreatus* produced preferential removal of lignin. The high biodegradability of wheat straw in spite of its low Syringyl / Guaiacyl lignin ratio was attributed to slight lignifications of most straw tissues. As the relative abundance of *p*-hydroxyphenyl (H), Guaiacyl (G) and Syringyl (S) units determine the lignin degradation and S rich lignins are more susceptible to fungal degradation than soft wood lignins and the extensive delignification of certain hard woods is due to high S/G lignin ratio.

Kerem *et al*. (1992) studied lignin degradation of cotton stalks with *Pleurotus ostreatus* and *Phanerochaete chrysosporium* using solid state fermentation and observed 55 per cent degradation of organic components by *Phanerochaete chrysosporium* while *Pleurotus ostreatus* showed 20 per cent degradation after 30 days of incubation.

Lignin degradation activities of two white rot fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium,* under solid state on cotton stalk was studied by Zohar *et al.* (1992). They reported that *Phanerochaete chrysosporium* grew vigorously, resulting in rapid, non selective degradation of organic component (55%) of cotton stalk within 15 days where as *Pleurotus ostreatus* grew slowly resulting in 20 per cent degradation in 30 days.

Microbial pre-treatment of wheat straw by using 19 white rot fungi was investigated by Hatakka (1993). Among the white rot fungi studied *Pleurotus ostreatus, Pleurotus* sp 535, *Pycnoporus cinnabarinus* 115 and *Ischnoderma benzoinum* 108 had increased the susceptibility of straw for enzymatic saccharification by *Trichoderma reesei* cellulase. The *Pycnoporus cinnabarinus* 115 pre-treatment resulted in conversion of 54.6 per cent of the residue to reducing sugars by enzymatic saccharification with *Trichoderma reesei* cellulase.

Masaaki and Takashi (1995) reported that wood meal treated with *Phanerochaete chrysosporium for* 28 days followed by steam explosion gave 76 per cent reducing sugars. Sawada *et al*. (1995) treated beech wood (32-60 mesh) with *Phanerochaete chrysosporium* for 28 days and then subjected for steam explosion at temperature 215^0 C for 6.5 min and obtained maximum saccharification of 74.8 per cent.

The fungal pre-treatment employ less severe chemical and temperature conditions thereby reducing sugar degradation and lowering inhibitory chemical concentrations compared to conventional thermo chemical pre-treatment (Keller *et al.,* 2003).

The degradation undergone by grape cluster stems (woody component of vine bagasse), an agro industrial waste, was investigated during the semi-solid-state cultivation of *Phanerochaete chrysosporium* BKM-F-1767 (ATCC 24725) by Rodriguez *et al.* (2003). For this, the content of lignin, cellulose and hemicellulose in grape cluster stems was determined before and after the enzymatic process. It was found that about 20 per cent of lignin, 48 per cent of hemicellulose and 5 per cent of cellulose were degraded during the process, by the ligninolytic enzymes (manganese-dependent peroxidase and lignin peroxidase) produced by such cultures responsible for the degradation of grape cluster stems. In parallel, semi-solid-state cultures of *Phanerochaete chrysosporium* grown on an inert support (cubes of nylon sponge), which is not susceptible to undergoing degradation during the enzymatic process, were used as reference cultures.

The production of lignin- and polysaccharide-degrading enzymes and changes of the chemical composition of litter were studied by Valaskova *et al*. (2007). The three isolates from a Quercus petraea forest, identified as *Gymnopus sp., Hypholoma fasciculare* and *Rhodocollybia butyracea* decomposed 38, 23 and 32 per cent of the substrate dry mass within 12 weeks of incubation respectively. *Gymnopus* sp. caused a substantial decrease in the lignin content. All fungi produced laccase and Mn-peroxidase (MnP) and none of them produced lignin peroxidase or other Mn-independent peroxidases. All fungi decreased the C/N ratio of the litter from 24 to 15–19. Analytical pyrolysis mass spectrometry of litter decomposed by this fungus showed changes in the litter composition similar to those caused by white-rot fungi during wood decay.

2.3.4.2 Microbial enzymatic pre-treatment

Karunanandaa *et al.* (1992) studied the biodegradability of crop residues colonized by white-rot fungi. Colonization of maize (*Zea mays* L.) and rice straw for 15 and 30 days by three fungi and a cellulase less mutant of *P. chrysosporium* were compared for dry matter (DM) digestibility. *Cyathus stercoreus* improved the *in vitro* cellulose digestibility of maize and rice straw by 37 and 45 per cent, respectively, and DM loss was only 3.3 per cent. The wild and mutant strains of *Phanerochaete* indiscriminately degraded both hemicellulose and cellulose. No direct correlation was found between lignin degradation and improved digestibility. Some species of *Pleurotus*, *Polyporus* and *Volvariella volvacea* were assessed by Mani and Marimuthu (1992) for their ability to degrade coir pith into good compost. *Pleurotus sajor-caju* was also found to metabolize the complex plant cell-walls.

Cellulase and laccase production were measured by Zadrazil *et al.* (1993) using cellulose and sawdust, respectively, as the Carbon sources. Endoglucanase production was the highest in *Pleurotus sajor-caju*. They reported that several white rot basidiomycetes metabolize the complex plant cell-walls by enzymatic depolymerization of lignin, cellulose and hemicellulose into sugars, simple phenols and other metabolites that are utilized by growing mycelia.

Agaricus bisporus, grown under standard composting conditions, was evaluated for its ability to produce lignin-degrading peroxidases by Bonnen *et al.* (1994). It was found that manganese peroxidase had a pH optimum of 5.4 to 5.5, with maximal activity during the initial stages of fruiting pin stage. The activity declined considerably with fruit body maturation (first harvest). Lignin peroxidase activity was not detected in the compost extracts.

Eggert *et al.* (1996) characterized white rot fungus *Pycnoporus cinnabarinus* with respect to its set of extracellular phenoloxidases. Laccase was produced as the predominant extracellular phenoloxidase in conjunction with low amounts of an unusual peroxidase. Neither lignin peroxidase nor manganese peroxidase was detected. They concluded that the principal feature of the *P. cinnabarinus* enzyme system was presence of a single predominant laccase and a lack of lignin- or manganese-type peroxidise.

The ligninolytic basidiomycetes *Pleurotus eryngii, Pleurotus ostreatus, Pleurotus pulmonarius* and *Pleurotus sajor-caju* did not exhibit detectable levels of manganese peroxidase (MP) when grown in liquid media with ammonium tartrate as N source. However, after examination of cells grown on different organic N-based media, high MP activity was obtained in peptone medium, up to nearly 3 Uml^{-1} in cultures of *P. eryngii* (Martinez *et al.*, 1996).

Ten white-rot fungi have been screened for the production of ligninase, manganese peroxidase and laccase by Nerud and Misurcova, (1996). It was observed that the fungi degraded lignin efficiently, but they differed significantly in the occurrence of individual ligninolytic enzymes. Based on the enzyme pattern produced under N-limited conditions they proposed that the fungi can be divided into the following four groups: 1. Ligninase-manganese peroxidase-laccase group, 2. Ligninase-manganese peroxidase group, 3. Manganese peroxidase-laccase group and 4. Laccase group.

Lignin depolymerisation by *Phanerochaete chrysosporium* includes extracellular enzymes such as lignin peroxidases (LiPs), manganese-dependent lignin peroxidases (MnPs), and a peroxide-generating enzyme, glyoxal oxidase (GLOX) (Gold and Alic, 1993, Broda *et al.,* 1996 and Cullen, 1997).

The degradation of lignocellulosic biomass of banana pseudostem was investigated by Ghosh *et al.* (1998) during solid state fermentation (SSF) by *Pleurotus ostreatus* and *Pleurotus sajor-caju*. Both organisms proved to be efficient degraders of banana pseudostem biomass.

Ganoderma lucidum, a white rot basidiomycete widely distributed worldwide, was studied for the production of the lignin-modifying enzymes laccase, manganese-dependent peroxidase (MnP), and lignin peroxidise (LiP) by D'souza, *et al.* (1999) using different substrates. They reported a high laccase level for shaken flask cultures at high nitrogen concentration (24 mM). Laccase production was readily seen in cultures grown with pine or poplar (100-mesh-size ground wood) as the sole carbon and energy source.

Cultures containing both pine and poplar showed 5- to 10-fold-higher levels of laccase than cultures containing pine or poplar alone.

Raghukumar *et al.* (1999) studied basidiomycetous fungus *Flavodon flavus* (Klotzsch) Ryvarden (strain 312). The strain produced three major classes of extracellular lignin-modifying enzymes (LMEs): manganese-dependent peroxidase (MNP), lignin peroxidise (LIP), and laccase. Low MNP and laccase activities were seen in high-nitrogen medium (24 mM), but no LIP activity was seen. Also,in media containing lignocellulosic substrates such as pine, poplar, or sugarcane bagasse as the sole source of carbon and nitrogen, relatively high MNP and moderate levels of laccases were seen, but LIP production either was minimal or not seen.

Kamitsuji *et al.* (2004) studied the incubation conditions of white rot fungus *Pleurotus ostreatus* and found that maximum growth and manganese peroxidise activity was achieved after eight days and 30 days respectively in peptone glucose yeast extract medium and in glucose yeast extract medium.

Liquid cultured and solid state fermentation cultured white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor* and the brown-rot fungus *Piptoporus betulinus* were assayed for the free and solid fraction-bound activity of lignocellulose-degrading enzymes using wheat straw by Valaskova and Baldrian (2006). They observed high laccase and Mn peroxidase activity in the cell free fraction of *P. ostreatus* and *T. versicolor* and low activity in *P. betulinus*. Also, the enzymes showed less stability under freeze-drying conditions.

Lee (2007) studied the lignin peroxidase of *Phanerochaete* fungus and observed the highest activity of 2800 U l^{-1} in cell free extract obtained from broth culture.

Chi *et al*. (2007) investigated the co-culturing of two white rot fungi on the production of lignin-degrading enzyme activities. Four species, *Ceriporiopsis subvermispora*, *Physisporinus rivulosus*, *Phanerochaete chrysosporium* and *Pleurotus ostreatus* were cultured in pairs to study the degradation of aspen wood and the production of lignin-degrading enzymes. Chemical analysis of decayed aspen wood blocks showed that co-culturing of *C. subvermispora* with *P. ostreatus* could significantly stimulate wood decay, when compared to monocultures. Other combinations of fungi were either slightly stimulating or not stimulatory. The pattern of lignin degradation was altered towards the acid insoluble part of lignin especially in co-cultures where *P. ostreatus* was included as a partner. Laccase was significantly stimulated only in the co-culture of *P. ostreatus* with *C. subvermispora*. Manganese peroxidase activity was stimulated in co-cultures of *P. ostreatus* with *C. subvermispora* or with *P. rivulosus*.

The capability of *Phanerochaete chrysosporium* for producing Lignin peroxidases (LiPs) and manganese peroxidases (MnPs) makes it a model organism of lignin-degrading enzymes production. Compiling and identifying the factors that affect LiP and MnP production by *P. chrysosporium*, Singh and Chen (2008) remarked that the major difficulty in using this organism for enzyme production is the instability of its productivity. This is largely due to the poor understanding of the regulatory mechanisms of *P. chrysosporium* responding to different nutrient sources in the culture medium, such as metal elements, detergents, lignin materials, *etc*.

Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. This enzyme is found in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. The laccases are the major ligninolytic enzymes which help in breakdown of lignin as well as in detoxification of phenolic compounds (Viswanath *et al*., 2008).

Production of ligninase enzymes by white rot fungi, *Datronia* sp. KAPI0039 and their application for removal of reactive dye was investigated by Vaithanomsat *et al*. (2010). It was found that the organism produced laccase and MnP enzymes but the LiP. Maximum laccase activity was detected in the early growth period at the end of 4 days cultivation (4502.2 U g^{-1} substrate) where as maximum MnP was detected (471.7 U g^{-1} substrate) after eight days of cultivation.

2.4 Saccharification

The hydrolysis of cellulose and hemicelluloses polysaccharides in to their respective monomers called as saccharification involves cellulolytic microorganisms or their enzymes namely, cellulase, hemicellulase and xylanases.

2.4.1 Cellulases

Three major types of enzymatic activities are found in cellulase system: (i) endoglucanases, also called as 1,4- β -D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4- β -D-glucan glucanohydrolases (cellodextrinases) (EC 3.2.1.74) and 1,4- β –D glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) β -glucosidases or β -glucoside glucohydrolases (EC 3.2.1.21).

Endoglucanases cut, at random, at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends (Bisaria and Ghose, 1981). Exoglucanases act on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose by peeling cellulose chains from the microcrystalline structure. The β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose.

Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze β -1, 4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β -1, 4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and base. The hydrolysis products can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end. The endoglucanase and exoglucanase activities of cellulases are extracellular whereas β -glucosidase activity is cell-associated where cellobiose must diffuse through the cell mass to be converted into glucose (Demain *et al.,* 2005).

2.4.2 Xylanase

Xylanase (EC 3.2.1.8) is the name given to a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is a major component of the cell wall of plants. Xylanases are present in fungi for the degradation of plant matter into usable nutrients (Wong *et al*., 1988).

2.4.3 Saccharification by microorganisms

The production of cellulases and xylanases by *Trichoderma viridae* L-333 was studied by Viesturs *et al.* (1996). Significant amounts of extracellular enzymes were obtained when submerged fermentation was performed in the fed-batch regime on cellulose- and xylan-based media with addition of sophorose and Tween-80. The culture filtrate of *T. viridae* was used for enzymatic treatment of recycling newspaper (TMP) fibres.

Zayed and Meyer (1996) examined *Trichoderma viridae* and *Aspergillus niger* for their ability to produce fermentable sugars from different cellulosic agro residues (straws of wheat, barley, corn). *Trichoderma viridae* most efficiently saccharified delignified wheat straw within 3 days at 25-30° C with a yield of reducing sugars of 27.0 g (54%) from 50 g delignified wheat straw (1% (w/v) NaOH autoclaved for 1 hr at 121^{0}C).

Aspergillus awamorii and *Trichoderma reesei* were used, separately and in mixed culture, by Friedrich *et al.* (1997) to convert apple distillery waste into microbial biomass. Overall, the use of mixed culture had considerable advantages, the positive properties of each species being retained. The effect of *Aspergillus* was evident in improved filtration and chemical oxygen demand reduction, as well as in the β-glucosidase synthesis. *Trichoderma*, on the other hand, contributed to good fibre degradation, protein enrichment of the biomass and cellulolytic, xylanolytic and pectinolytic activities in the filtrates

The production of cellulases by *Trichoderma citrinoviridae* on *Artemisia annua* (marc) and bioconversion of the same marc by produced cellulase system was studied by Chandra *et al.* (2010). Alkali hydrolysis with autoclaving was found to be most suitable for production of FPase, endoglucanase and β-glucosidase activities.

Optimum production of FPase, endoglucanase and β-glucosidase was obtained at 96 h, 96 h and 72 h of fermentation period, respectively. Substrate concentration of 1% with particle size between 200 μm and 475 μm gave the higher yields. Higher production of all the three enzymes was obtained with initial pH value of 5.5, temperature of 28 °C and 75% of mineral salt solution.

The effect of mild alkali and steam pre-treatments on fungal saccharification and sequential simultaneous-saccharification and fermentation (SSF) of corn fiber to ethanol was studied by Shrestha *et al.* (2010). The corn fiber was pre-treated with: (i) 2% NaOH (w/w) at 30 °C for 2 h and (ii) steaming at 100 °C for 2 h and obtained ethanol yields of 2.6 g, 2.9 g and 5.5 g per 100 g of corn fiber, respectively, for *Phanerochaete chrysosporium*, *Gloeophyllum trabeum* and *Trichoderma reesei* saccharification and sequential SSFs. The mild alkali pre-treatment resulted in higher glucose yields following fungal saccharification of corn fiber.

2.4.4 Saccharification by microbial crude enzymes

Gattinger *et al*. (1990) reported the production of cellulolytic enzymes on canola meal as a substrate by *Trichoderma reesei.* The enzyme extract produced maximum xylanase activity of 210 IU ml^{-1} in 9-12 days. The produced enzyme system also contained acetyl-xylan esterase, cellulase and xylosidase activities. Mishra *et al*. (1990) reported the extraction of non-debranching endo-β-D-xylanase from *Lentinus edodes* grown on a commercial oak wood. The enzyme extract could hydrolyse sugars from 40-50 per cent of the hydrolysable linkages in either glucuronoxylan or arabinoxylan. It showed optimal activity at pH 4.5-5.0 and specific activity of 310 units mg^{-1} protein at 40^0C. Excoffier *et al*. (1991) studied enzymatic hydrolysis of steam exploded wood chips impregnated with sulphuric acid (0.4%). The hydrolysis of pre-treated fibre with *Trichoderma reesei* CL-847 cellulase increased progressively with the severity of the steam treatment. The best yield in terms of glucose recovery after 24 h of enzymatic hydrolysis was 70 per cent potential glucose at 225^0C for 120s.

Alkali pre-treated wheat straw (2%, NaOH w/v) at 115^0 C for 10 min produced 41% reducing sugars after enzymatic saccharification with *Trichoderma reesei* cellulase (Hatakka 1993).

Van-Wyk (1999) saccharified used paper products (foolscap, filter paper, newspaper and microcrystalline cellulose by *Penicillium funiculosum* and *Trichoderma reesei* and reported the release of maximum glucose by *Penicillium funiculosum* from foolscap paper, i.e. 217%, 65% and 23% more glucose than news paper, office paper and filter paper respectively.

Koijam *et al.* (2000) studied cellulase production ability of *Phanerochaete chrysosporium* strain and *Cladosporium* sp. BK-II on rice husk, wheat bran and bagasse which are subjected for different pre-treatments. High cellulase activity was obtained with steam treated and alkali treated rice husk and wheat bran. The cellulases had wide range of pH stability (2.5 – 6.5 pH) with optimum pH between 4.5 and 5.0 at 50^0 C.

Further, they performed enzymatic hydrolysis in 250 ml Erlenmeyer flask. 2.5 g, 5.0g and 10.0 g cellulosic materials suspended in 100 ml of 0.5 M Citrate buffer at pH 4.8 containing 100 units of enzyme extract. The suspension was incubated for varying intervals of time 0- 48 h at 50^0 C at 150 rpm. On evaluation of parameters for optimum cellulase production, they found 30^0 C, pH 4.8, substrate concentration 2% (w/v), inoculums size 8% (v/v) and dissolved oxygen concentration 0.5 vvm to be the best conditions. By employing these conditions they hydrolysed rice husk, wheat bran and bagasse with crude extract of *Phanerochaete chrysosporium* strain and *Cladosporium* sp. BK-II and obtained maximum reducing sugars in 60 h incubation for 2.5 g, 5.0 g and 10.0 g substrate concentration. However, the amount of reducing sugars per gram of cellulose was highest at 2.5% (w/v) concentration. It was also observed that per cent saccharification depended on substrate concentration. The reducing sugar concentration showed a declining trend as the substrate concentration increased. The maximum reducing sugars released was 80 mg g^{-1} cellulose with *Phanerochaete chrysosporium* cellulase and 75 mg g^{-1} with *Cladosporium* sp. BK-II at 2.5% (w/v) concentration.

The carboxymethyl cellulase activity of *Aspergillus niger* Z-10 (wild type strain) was studied by Coral *et al*. (2002). The analysis of the enzyme preparation by SDS-PAGE showed two protein bands showing cellulolytic activity. The molecular weight of these bands was estimated to be around 83,000 and 50,000. The optimum temperature of the enzyme was observed to be around 40^0C. It was found that the enzyme's activity had a broad pH range between 3 to 9 and 41.2 per cent of the original activity which was retained after heat treatment at 90^0C for 15 min.

Baig *et al*. (2004) investigated the saccharification of banana agro-waste by cellulases of *Trichoderma lignorum.* The steam treated agro-residue yielded 1.34 mg ml^{-1} reducing sugars after 24 h. The substrate size of less than 120 µ yielded more sugars. Maximum sugars were released at pH 6.0 and temperature 40^0 C. Raghavendra (2006) studied the saccharification of alkali delignified substrates using efficient *Trichoderma reesei* and observed reducing sugar yield of 22.3 mg g^{-1} (2.2%), 25.56 mg g^{-1} (2.56%) and 26.26 mg g^{-1} (2.62%) respectively in delignified paddy straw, wheat straw and bagasse.

The cellulase production ability of *Trichoderma reesei* NRRL 11460 was investigated by Singhania *et al*. (2006) using Lignocellulosic residues. They observed maximum cellulase production of 154.58 Ug^{-1} dry solids when pre-treated sugarcane bagasse (PSCB) was used as substrate. The optimum conditions for cellulase production using PSCB were determined to be initial moisture content of 66 per cent, initial medium pH of 7.0 and incubation temperature of 28^0C and addition of 0.075 M NH_4NO_3 and 0.005 M cellobiose. The optimum incubation time for cellulase production was 72 h.

Partially purified enzyme system of *Trichoderma citrinoviridae* on *Artemisia annua* (marc), produced 46 per cent saccharification after 48 h of incubation on alkali hydrolyzed and autoclaved substrate which was 3.26 fold more than that of unpre-treated substrate Chandra *et al.* (2010).

Jeya *et al.* (2010) reported that *Agaricus arvensis* a new basidiomycetous fungus produced the highest endoglucanase (EG), cellobiohydrolase (CBH) and β-glucosidase (BGL) activities of 0.3, 3.2 and 8 U per mg-protein, respectively, with rice straw as the carbon source. Further, saccharification of the woody biomass (poplar) with *A. arvensis* cellulase as the enzyme source released a high level of fermentable sugars. A total reducing sugar level of 29 g L^{-1} (293 mg g^{-1}-substrate) was obtained at an enzyme concentration of 65 FPU g^{-1} substrate after optimization of the hydrolysis parameters.

2.4.5 Saccharification by commercial enzymes

Sattler *et al*. (1989) hydrolysed sigmacell 50 (20g L^{-1}) and steam pre-treated poplar wood with commercial cellulase at enzyme loading of 5, 10, 15, 37.5 75 and 100 FPU g^{-1} and determined soluble sugars in terms of glucose at 2, 5, 9, 13, 22, 24, 46 and 70 h time. Citrate buffer pH 4.8 (0.05 M) was used. It was found that 5 FPU g^{-1} resulted in more or less same value of cellulose components and also, the amorphous cellulose hydrolysed faster than crystalline cellulose as cellulose components are not identical with crystalline and amorphous fractions. The structural properties *viz*. adsorption properties, pore size and accessibility are important in enzyme hydrolysis. Also, observed that hydrolysis of cellulose with varying degrees of cellulases (celluclast) at a fixed time gave increased reducing sugars with increased enzyme dosage in poplar wood which was steam treated at 200^0C, 220^0C and 240^0C. The celluclast enzyme hydrolysis gave maximum digestibility in 20 h. The used enzymes properties are: celluclast, a brownish liquid had density of 1.989 g ml^{-1} and a protein content of 128.6 mg ml^{-1} liquid measuring 45 FPU and 6.5 units of glucosidase per gram liquid and Novozyme TN 188 (β-glucosidase) had 1.1884 g ml^{-1} density with activity of 520 units per gram liquid. The Novozyme TN 188 was supplemented with celluclast with a ratio of 1:1 since celluclast had low β-glucosidase activity.

Koullas *et al*. (1993) reported the linear relationship with the level of alkali for delignification and subsequent enzymatic saccharification of alkali pre-treated wheat straw at 120^0 C and between 20^0 C and 36^0 C using either aqueous or organic alkaline solution.

Kaar and Holtzapple (1998) studied the effect of tween 20 and tween 80 on enzymatic hydrolysis of cellulose by cellulase enzymes. The pre-treated biomass (corn stover) was transferred to culture flask, added citrate buffer and sodium azide solutions and neutralized to 4.8 pH with glacial acetic acid.

The samples were pre heated for 10 min in the incubator shaker, tween was added followed by immediate addition of the enzyme. After hydrolysis, samples were heated in a boiling water bath for 10 min to denature the enzyme, 4 ml of sample centrifuged and clear supernatant analysed by DNS assay. They reported that addition of tween during cellulase enzyme hydrolysis of pretereated biomass enhanced the enzymatic activity. The recommended tween loading was 0.15 g per gram dry biomass, where the critical relationship was the loading of the tween on the biomass and not the tween concentration in solution. The tween prevented the thermal denaturation of the enzymes and appears to act as enzyme stabilizer, lignocelluloses disrupter and enzyme effecter. The 72 h enzymatic conversion of pre-treated corn stover using 5 FPU cellulase per gram dry biomass at 50^0 C in presence of tween 20 or tween 80 as part of the medium (0.85 g g^{-1} for cellulose, 0.66 g g^{-1} for xylan and 0.75 g g^{-1} for total poly saccharides) improved cellulose, xylose and total poly saccharides conversion by 42, 40 and 42 per cent, respectively.

Clarkin and Clesceri (2002) evaluated commercial cellulases (BH20, Epicote, FC+) using enzymatic and physical methods of pre-treatment and found that the reducing sugars released by the cellulase of *Trichoderma viridae* ranged from 135.37 to 244.48 mg per day.

Sharma *et al*. (2004) hydrolysed pre-treated sunflower hulls (40 mesh size) with *Trichoderma reesei* Rut C30 cellulase enzyme and obtained 59.80 per cent saccharification.

Wen *et al*. (2004) have optimum enzyme loading to hydrolyse lignocellulosic part of animal manure into fermentable sugars. The use of 13 FPU cellulose g^{-1} substrate and 5 IU β-galactosidase g^{-1} substrate at 46^0 C and pH 4.8. Tween-80 at 2% resulted in 20 per cent increase in glucose yield. Substrate concentration of 50.0g L^{-1} favoured glucose yield. Maximum glucose yield obtained was 11.32 g 100 g^{-1} manure, corresponding to 40% cellulose conversion.

Sun and Cheng (2005) hydrolysed dilute H_2SO_4 pre-treated rye straw and Bermuda grass at 5% solid loading with cellulase and β-glucosidase at 50^0 C and 100 rpm for 48 h in a water bath shaker. The Citrate buffer, 4.8 pH was added with sodium azide (0.3% v/v) to inhibit microbial infections. It was observed that solid residue pre-treated at 121^0 C for 30 min produced much less glucose after 48 h saccharification than that pre-treated for 60 min. The glucose obtained at 60 and 90 m pre-treatment was similar. With the increase in dilute acid concentration the digestibility also increased rapidly and produced increased glucose yield.

The effect of enzyme concentration on acid cum steam pre-treated lignocelluloses (wood chips) was investigated by Kumar *et al*. (2010). It was observed that 60 per cent of the cellulose was saccharified after 72 h, at an enzyme loading of 20 FPU g^{-1} cellulose. However, when the enzyme loading was reduced to 5 FPU, less than 27 per cent of the cellulose was hydrolysed. When the steam pre-treated substrates were subsequently delignified they were almost completely hydrolysed, at both high, 20 FPU g^{-1} cellulose (less than 12 h) and low, FPU g^{-1} (within 72 h) enzyme loadings. Although optimized steam pre-treatment could result in greater than 90 per cent glucose recovery, in order to obtain complete hydrolysis of the cellulosic component at reduced enzyme loadings a delignification step will likely be required.

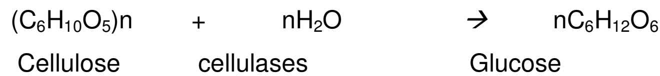
Qing *et al.* (2010) reported that xylose, xylan, and xylo-oligomers dramatically decrease conversion rates and yields of saccharification. Xylooligomers were more inhibitory than xylan or xylose in terms of a decreased initial hydrolysis rate and a lower final glucose yield even for a low concentration of 1.67 mg ml^{-1}. At a higher concentration of 12.5 mg ml^{-1}, xylooligomers lowered initial hydrolysis rates of Avicel by 82 per cent and the final hydrolysis yield by 38 per cent. A comparison among glucose sugars and xylose sugars also showed that xylooligomers were more powerful inhibitors than well-established glucose and cellobiose. Typically, the enzymatic hydrolysis rate of lignocellulosic biomass is fast initially but then slows down more rapidly than can be explained by just consumption of substrate. Although several factors including enzyme inhibition, enzyme deactivation, a drop in substrate reactivity, or nonproductive binding of enzyme to lignin could be responsible for this loss of effectiveness.

2.5 Fermentation

The industrial fermentation of lignocellulose hydrolysate to ethanol requires microorganisms, which have a broad substrate range and which produce ethanol with high yield and productivity. Baker's yeast is the most commonly used microorganism for industrial ethanol production. It has also been shown to efficiently ferment lignocellulosic hydrolysate to ethanol.

The cellulose-based bioethanol production involves two steps:

(1) Enzymatic hydrolysis (saccharification):



$(C_6H_{10}O_5)n$ + nH_2O → $nC_6H_{12}O_6$

Cellulose cellulases Glucose

(2) Fermentation:

$C_6H_{12}O_6$ → $2C_2H_5OH + 2CO_2$

Glucose microbes Ethanol

2.5.1 Hydrolysis and fermentation as two step processes

Karsch *et al*. (1983) evaluated the potential of *Zymomonas mobilis* and *Saccharomyces cerevisiae* for ethanol production from glucose both aerobically and anaerobically. It was found that *Zymomonas mobilis* exhibited 94 per cent efficiency in ethanol production compared to 88 per cent efficiency by *Saccharomyces cerevisiae*, both under anaerobic conditions with corresponding biomass production of 2.5 g L^{-1} and 6.5 g L^{-1} and total acid content of l6 mM l^{-1} and 12 mM l^{-1} respectively.

du-Preez *et al*. (1986) investigated the fermentation of various sugars liberated during hydrolysis of lignocellulosics by *Candida shehatae* and *Pichia stipitis*. Both the yeasts produced ethanol from D-glucose, D-mannose, D-galactose and D-xylose. However, only *Pichia stipitis* fermented D-cellobiose, producing 6.5 g L^{-1} ethanol from 20 g L^{-1} cellobiose within 48 h. No ethanol was produced from L-arabinose, L-rhamnose or xylitol. *Pichia stipitis* fermented glucose 30-50 per cent more rapidly than xylose, whereas *Candida shehatae* fermented both sugars in similar rates.

Doelle and Doelle (1990) investigated the performance of *Zymomonas mobilis* for bioethanol production from sugarcane molasses containing total sugar content of 48-50 per cent (w/v) and 35-42 per cent (w/v). It was observed that molasses concentrations up to 250 g L^{-1} (1:3 dilutions) were successfully fermented within 24h and the addition of sucrose to a final sugar concentration of 15% (w/v) resulted in production of 10 per cent (v/v) ethanol with conversion efficiency up to 96 per cent.

Wide range in activities between cellobiohydrolase and carboxymethyl cellulase (CMCase) had affected the ethanol production. The presence of β-glucosidase at 0.7- 0.8 unit ml^{-1} growth medium had produced maximum ethanol between 7.6 and 9.1 g L^{-1} (Christakopoulas *et al*. 1990).

Pichia stipitis NPRLY 71224 was evaluated for ethanol fermentation of *Eucalyptus* wood hemicellulose hydrolysate by Ferrari *et al*. (1992).

Joshi *et al*. (1990) had fermented wood hydrolysate to ethanol by two strains of *Fusarium oxysporum.* An optimum reducing sugar concentration of 54.8 g L^{-1} medium, pH 5.5 and 30^0 C produced ethanol content of 12.30 g L^{-1} and 11.70 g L^{-1} by *F. oxysporum* D-140 and *F. oxysporum* NCIM-1072 respectively during 96 h fermentation. The maximum production of ethanol under optimum cultural conditions and in presence of yeast extract and minerals was 13.2 g L^{-1} medium by *F. oxysporum* D-140 after 108 h fermentation.

Kaur *et al*. (1993) immobilized *Zymomonas mobilis* ATCC 10988 using sodium alginate at 2% (w/v*)* with cell load 100 mg 10 ml^{-1} normal saline and obtained ethanol yield after fermentation up to 49 g L^{-1} ethanol with residual sugar 2 g L^{-1}.

van Zyl *et al*. (1993) investigated the influence of D-ribose as a co-substrate on the uptake and metabolism of the non-growth substrate D-xylose by *Saccharomyces cerevisiae* ATCC 26602.

They observed strong inhibition of xylose and ribose transport by glucose if one of them was present along with glucose but in the presence of mixture of xylose and ribose, glucose did not inhibit xylose transport, this indicated the existence of both the catabolic pathways for ribose and xylose metabolism.

Sohn and Seu (1994) screened thermo tolerant *Kluyveromyces marxianus* yeast strain for ethanol production and obtained 86.6 g L^{-1} from 20 per cent glucose solution at 40^0 C in 96 h.

Bioconversion of wheat straw to ethanol by employing the *Trichoderma viridae* and the yeast *Pachysolen tannophilus* was studied by Zayed and Meyer (1996) and observed that, pre-treatment of wheat straw with 1% (w/v) NaOH autoclaved for 1 hr at 121^0C and saccharified by using *Trichoderma viridae.* After heat inactivation of fungal activities the sugars were converted to ethanol by the oxygen tolerant yeast, *Pachysolen tannophylus*. 70 g of wheat straw yielded 50 g delignified straw (71.4%), which was saccharified to 27.0 g reducing sugars (38.6%), fermentation of sugars yielded 11.8 g ethanol (16.9%).

Premidevi and Singh (1995) reported that presence of glucose stimulated *Candida shehatae* in xylose fermentation to ethanol and obtained an ethanol yield of 38 per cent from (1% H_2SO_4 refluxed hydrolysate for 4 h) water hyacinth.

Harpreet *et al*. (1998) reported ethanol yield of 34% by *Saccharomyces cerevisiae* from alkali pre-treated, *Trichoderma reesei,* cellulase saccharified rice straw.

Stevenson and Weimer (2002) isolated and characterized fungal strain *Trichoderma* (A 10), capable of fermenting cellulose to ethanol from cow dung. The strain produced 0.4 g (0.04%) of ethanol per litre. This strain did not grow on any substrate under anaerobic condition but could ferment micro crystalline cellulose or several other sugars to ethanol. A maximum of 2g ethanol per litre (0.2%) was produced under submerged cultures in minimal medium. Soderstorm *et al*. (2003) obtained maximum ethanol yield by separate saccharification and fermentation as against simultaneous saccharification and fermentation (SSF) of soft wood hydrolysate that was pre-treated with two step steam explosion in presence of sulphuric acid.

Sharma *et al*. (2004) obtained maximum ethanol yield of 0.454 g g^{-1} (45.4%) from enzymatically hydrolysed sunflower hulls yielding 40.0 g per litre reducing sugars by fermentation with *Saccharomyces cerevisiae* var. *ellipsoideus* under optimum conditions of pH 5.0, temperature 30.0^o C in 24 h time.

Raghavendra (2006) reported ethanol production of 49.13 mg g^{-1} (4.91%), 54.27 mg g^{-1} (5.47%) and 57.67 mg g^{-1} (5.76%) by *Pachysolen tannophylus* (NCIM-3445) respectively from delignified, saccharified paddy straw, wheat straw and bagasse respectively.

Chen *et al*. (2009) observed that 2% NaOH pre-treatment corn stover had substantially increased the lignin removal and enhanced the accessibility and digestibility of cellulose to enzyme hydrolysis. The hydrolysis yield of NaOH- pre-treated corn stover reached 81.2 per cent by 48 h at 8.0% substrate concentration and cellulase dosage of 20 FPU g^{-1} substrate. Chemical analysis showed that the enzymatic hydrolysate had higher content of fermentable sugars and less inhibitors. The results showed that NaOH-pre-treatment followed by enzymatic bioconversion of corn stover is suitable for subsequent fermentation process to produce ethanol.

Fermentation of acid hydrolysate of water-hyacinth (*Eichhornia crassipes*), a free floating aquatic plant has been investigated for ethanol production by Kumar *et al.* (2009) using *Pichia stipitis*. It was found that 72.83 per cent of xylose was converted to ethanol with a yield of 0.425 g g^{-1} and productivity of 0.176 g L^{-1} h^{-1}.

The fermentation of acid and enzymatic hydrolysates of *Prosopis juliflora* (Mesquite), containing 18.24 g L^{-1} and 37.47 g L^{-1} sugars was studied by Gupta *et al*. (2009), using *Pichia stipitis* and *Saccharomyces cerevisiae.* The fermentation produced 7.13 g L^{-1} and 18.52 g L^{-1} of ethanol with corresponding yield of 0.39 g/g and 0.49 g/g, respectively from *Pichia stipitis* and *Saccharomyces cerevisiae*.

Kuhad *et al*. (2010) fermented the enzyme hydrolysed, acid pre-treated (3.0%, v/v H_2SO_4, 120 °C for 45 min) *Lantana camara* hydrolysate with *Pichia stipitis* and *Saccharomyces cerevisiae* and observed ethanol content of 5.16 and 17.7 g L^{-1} with corresponding yields of 0.32 and 0.48 g g^{-1} after 24 and 16 h, respectively for *Pichia stipitis* and *Saccharomyces cerevisiae.*

Nikolic *et al*. (2010) studied the ethanol fermentation of enzymatically obtained corn meal hydrolyzates by free and immobilized *Saccharomyces cerevisiae* var. *ellipsoideus* yeast in a batch system. It was found that the immobilized cell system was superior to the free cell system for higher ethanol tolerance and productivity and lower substrate inhibition. Initial glucose concentrations of 150 and 176 g L^{-1} for free and immobilized system, initial inoculum concentration of 2% (v v^{-1}) and fermentation time of 38 h were the optimum conditions determined for both immobilized and free yeasts.

Shrestha *et al.* (2010) obtained 7.7 g ethanol 100 g^{-1} corn fiber which was pre-treated with 2% NaOH (w/w) at 30 °C for 2 h followed by microbial saccharification and saccharification with commercial cellulase enzyme – Spezyme-CP. They reported that solid-substrate fermentation of corn fiber with fungi reduced the dosage of commercial cellulase enzymes during SSF.

2.5.2 Co-fermentation

Abate *et al.* (1996) evaluated mixed cultures of *Zymomonas mobilis* and *Saccharomyces cereviseae* for production of ethanol and obtained higher with mixed cultures than single culture.

Amutha and Gunasekaran (2000) fermented liquefied cassava starch by monoculture and co-culture using *Saccharomyces diastaticus* and *Zymomonas mobilis* and reported that *Saccharomyces diastaticus* had produced an ethanol yield of 0.24 g g^{-1} while the mixed culture fermentation had produced 0.34 g g^{-1} ethanol.

Ethanol production from raw unhydrolysed starch by co-culture using *Saccharomyces diastaticus* and *Saccharomyces cereviseae*- 21 was studied by Varma *et al*. (2000). They reported 48% higher (24.8 g L^{-1}) ethanol production from co-culture over mono-culture by *Saccharomyces diastaticus* (16.8 g L^{-1}).

Sreenath and Jeffries (2000) screened more than 40 yeast strains of *Pichia stipitis* and *Candida shehate* to determine their fermentation rates on mixed sugars and observed that all the tested strains fermented both glucose and xylose. They obtained ethanol concentrations of 34.8 ± 2.42 g L^{-1} with *Pichia stipitis* and 34.0 ± 1.67 g L^{-1} with *Candida shehate.*

Ana da Silva *et al*. (2010) ball milling pre-treated bagasse and sugarcane straw were fermented by using C6-fermenting and C6/C5 fermenting strains of *Saccharomyces cerevisiae*. It was observed that ethanol yields from total fermentable sugars by C6-fermenting strain reached 89.8 and 91.8 per cent for bagasse and straw hydrolysates, respectively, and 82 and 78 per cent by C6/C5 fermenting strain.

2.5.3 Simultaneous Saccharification and Fermentation (SSF)

Performance of four woody crops (aspen, two populus hybrids snd sweetgum), three herbaceous crops (switch grass, weeping Love grass and *Sericea lespedeza*) and three agricultural residues (corn cobs, corn stover and wheat straw) (pre-treated) for SSF employing enzymes, cellulose and β-glucosidase (8:1ratio) and *Saccharomyces cereviseae*. Excellent results were obtained for corn cobs, followed by corn stover, wheat straw, weeping grass, woody crops and switch grass (Wyman *et al*., 1992). The SSF was studied in 250 ml flasks containing 100 ml fermentation broth of 7.5% (w/v) lignocelluloses, which was amended with 1% yeast extract, 2% peptone (w/v), lipid mixture, ergosterol (5 mg L^{-1}) and oleic acid (30 mg L^{-1}) to improve the ethanol yield, and penicillin and streptomycin at 10 mg L^{-1}, to minimise the bacterial contamination. The medium was inoculated with 10% yeast and incubated at 37^0C under shaken conditions.

Walsum *et al*. (1996) studied the conversion of liquid hot water (LHW) pre-treated lignocellulosics to ethanol by simultaneous saccharification and fermentation (SSF) using *Saccharomyces cerevisiae* in presence of *Trichoderma viridae*.

They observed that SSF of sugarcane bagasse, aspen chips (3 mm) and hardwood floor (70 mesh) had resulted in 90 per cent conversion to ethanol in 2-5 days at enzyme loadings of 15-30 FPU g^{-1}. Reducing the particle size of the pre-treated substrates did not affect the conversion to ethanol. The hydrolysate produced from the LHW pre-treatment showed slight inhibition of growth of *Saccharomyces cerevisiae.*

Harikrishna *et al*. (2001) performed simultaneous saccharification and fermentation (SSF) of pre-treated and *Trichoderma reesei* cellulase derived hudrolysate (sugar cane leaves) using two different yeast cultures. It was found that *Kluyveromyces fragilis* NCIM 3358 performed better and produced high yields of ethanol (2.5-3.5% w/v) than *Saccharomyces cerevisiae* NRRL-Y-132 (2.0-2.5% w/v) in SSF process. Also, increased ethanol yields were obtained when the cellulase was supplemented with β-glucosidase.

Cellulose can be enzymatically hydrolysed by cellulose enzyme and fermented to ethanol as simultaneous saccharification and fermentation. *Trichoderma reesei* cellulases, which constitute the most active preparations, have optimal activity at pH 4.5 and temperature 55^0 C. For *Saccharomyces* cultures, SSF are typically controlled at pH 4.5 and temperature 37^0 C (Dien *et al*., 2003).

Kodali and Pogaku (2006) obtained maximum cellulase activity (16.7% cellulose solubilization) by simultaneous saccharification and fermentation with 5N sodium hydroxide pre-treated rice bran using fungal strain *Scopulariopsis pranu.*

Choi *et al*. (2010) performed simultaneous saccharification and fermentation of liquefied cassava (*Manihot esculenta*) starch in a 5 L lab-scale jar fermenter at 32 °C for 66 h with an agitation speed of 2 Hz using two ethanol-producing novel strains of *Saccharomyces cerevisiae*, CHY1011 and CHFY0901. Under these conditions, *S. cerevisiae* CHY1011 and CHFY0901 yielded a final ethanol concentration of 89.1 ± 0.87 g L^{-1} and 83.8 ± 1.11 g L^{-1}, a maximum ethanol productivity of 2.10 ± 0.02 g L^{-1} h^{-1} and 1.88 ± 0.01 g L^{-1} h^{-1}, and a theoretical yield of 93.5 ± 1.4% and 91.3 ± 1.1%, respectively.

Shen and Agblevor, (2010) studied the kinetics of the simultaneous saccharification and fermentation (SSF) of cellulose to ethanol using a highly refined standard cellulose (microcrystalline cellulose, (Avicel PH101)), with a prior hydrolysis phase (semi-simultaneous saccharification and fermentation (SSF). They observed that the 24-h pre-hydrolysis + 48-h SSF (SSF 24) produced higher yield and higher productivity of ethanol than other models. For the continuous operation in the SSF, the productivity of SSF 24 was much higher than that of SSF 12 though the ethanol concentrations of both cases did not differ significantly.

Simultaneous saccharification and fermentation experiments carried out with optimised pre-treatment conditions resulted in ethanol yields of 163 g kg^{-1} ensiled hemp (dry matter) (71% of the theoretical maximum) and 171 g kg^{-1} dry hemp (74%), which corresponds to 206–216 l Mg^{-1} ethanol based on initial dry material from the dry and ensiled industrial hemp (*Cannabis sativa* L.), which was steam impregnated with 2% SO_2 followed by steam pre-treatment at 210 ° C for 5 min (Sipos *et al.,* 2010).

2.6 Pilot scale production of bioethanol

Ballerini *et al*. (1994) studied large scale ethanol production. They performed hydrolysis of the steam exploded poplar in 25 M^3 stirred reactor using cellulase enzyme of *Trichoderme reesei*. The pre-treated substrate at 5-6% feed stock concentration when incubated for 72 h at 50^0 C and 4.8 pH with 16 FPU g^{-1} substrate produced 96.2 per cent hydrolysis of polysaccharide. The hydrolysate had 93.6 per cent glucose, 3.5 per cent cellobiose 2.9 per cent xylose. Upon fermentation with *Saccharomyces cereviseae* ATCC 26603 produced 160- 190 kg ethanol from 1000 kg poplar wood.

Sharma *et al*. (2004) observed maximum ethanol content of 0.449 (44.9%) and 0.446 (44.6%) g g^{-1}, respectively from sunflower hulls in scaled up fermentation of 1 and 15 L under optimum conditions.

3 MATERIAL AND METHODS

Ethanol is the most common renewable fuel, presently produced from sugar or grain and this raw material may not be sufficient for future production on large scale as these crops are also food for human beings and animals. Therefore, production of ethanol will most certainly have to be based on production from lignocellulosic materials. These lignocellulosic materials are cheap renewable sources, available in large quantities.

Lignocellulosic feedstock includes agricultural residues (sugarcane bagasse, sugarcane trash, corn cobs, corn stover wheat straw, rice straw, grasses, *etc.*), forest residues and other low-value biomass such as municipal wastes. Therefore, the use of lignocellulosic materials for ethanol production is very promising. Sugarcane and corn are the widely cultivated crops in Karnataka and in India. The demand for increased crop production is also generating increased quantities of their residues, which are generally burnt in the field as a means of disposal or either used as cattle feed or composted to organic manure. However, the rich cellulose and hemicelluloses content present in these residues could be exploited for bioethanol production.

In this regard the investigations were carried out to achieve maximum saccharification of selected pre-treated agro residues and further, their conversion to bioethanol in the Department of Agricultural Microbiology, University of Agricultural Sciences, Dharwad and the details of materials used and methods employed during the course of investigation are explained in this chapter.

The substrates used for investigation

Sl. No	Substrates	Source
1	Sugarcane bagasse	Malaprabha Sahakari Sugar Factory, M. K. Hubli, Belgaum
2	Sugarcane trash (Var. CO-8014)	From fields of Basavaraj Patil, Yettinagudda village, Dharwad
3	Sugarcane tops (Var. CO-8014)	From fields of Basavaraj Patil, Yettinagudda village , Dharwad
4	Corn stover (Var. Arjun)	MARS, UAS, Dharwad
5	Corn husk (Var. Arjun)	MARS, UAS, Dharwad

Mold cultures used in the study

Sl. No	Name of the culture	Source
1	*Phanerochaete chrysosporium*	Bioconversion Lab, AICRP on RES, UAS, Dharwad
2	*Pleurotus florida*	Bioconversion Lab, AICRP on RES, UAS, Dharwad
3	UASD- LF1	Department of Agricultural Microbiology, UAS, Dharwad
4	*Aspergillus sydowii*	Department of Agricultural Microbiology, UAS, Dharwad
5	*Trichoderma reesi*	NCIM, Pune
6	*Trichoderma viridae*	NCIM, Pune
7	*Aspergillus awamorii*	NCIM, Pune

Yeast and bacterial cultures used in the study

Sl. No	Name of the culture	Source
1	*Saccharomyces cerevisiae* NCIM-3455 (Yeast)	NCIM, Pune
2	*Kluyveromyces marxianus* NCIM-3465 (Yeast)	NCIM, Pune
3	*Pachysolen tannophylus* NCIM-3445 (Yeast)	NCIM, Pune
4	*Pichia stipitis* NCIM-3498 (Yeast)	NCIM, Pune
5	*Candida shehatae* NCIM-3500 (Yeast)	NCIM, Pune
6	*Zymomonas mobilis* NCIM-2428 (Bacteria)	NCIM, Pune

3.1 Maintenance of the culture

The mold cultures used for the pre-treatment of the substrates and saccharification of delignified substrates were maintained on potato dextrose agar medium. The yeast cultures used for ethanol production were maintained on MGYEP Agar (Malt Extract Glucose Yeast Extract Peptone) medium and *Zymomonas mobilis* on the *Zymomonas* Selective Agar medium (Appendix I).

3.2 Preparation of the substrate for pre-treatment

The fresh substrates *viz*., sugarcane bagasse, sugarcane trash, sugarcane tops, corn stover and corn husk were brought to the laboratory, chopped into small pieces, dried at 60^0C in a hot air oven for 12 h and powdered by dry milling (M/s. Willey mill, India) to obtain different particle sizes of 500 μ (0.5 mm) and 1000 μ (1.0 mm) and 10,000 μ (10 mm), filled into polyethylene bags and stored at room temperature for further use (Karimi *et al*., 2006). The different particle sizes of the substrates are shown in Plate 1.

3.3 Methods of pre-treatment

3.3.1 Physical pre-treatment

The physical pre-treatments were conducted for all the substrates *viz.,* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover of three different particle sizes 0.5 mm, 1.0 mm and 10 mm with three replications.

3.3.2 Chemical pre-treatment using alkali

Ten grams of the oven dried substrates were transferred separately in to 250 ml capacity Erlenmeyer flasks. All the substrates of above mentioned particle sizes in triplicates were treated with 2.50% and 3.0% alkali, NaOH (w/v) and incubated for 8 h. The quantity of alkali added was approximately 50 ml 10 g^{-1} dry substrate which was sufficient enough to moisten entire substrate except in case of bagasse where additional 10 ml was used.

3.3.3 Combination of physical, chemical and heat pre-treatment

The above mentioned chemically treated substrates (eight hours of reaction period) were subjected for heat treatment in autoclave at two different temperatures individually, one at 121^0C at 15 lb pressure for one h and another at 125^0C corresponding to 20.5 lb pressure for one hour separately. After the heat pre-treatment, substrates were washed with tap water followed by distilled water to remove the alkali content (until the pH was close to 7.0). Otherwise, the pH of the substrates was neutralized with acetic acid. The residue obtained after the treatment was dried in a hot air oven at 60^0C to constant weight. The weight of the pre-treated substrates were recorded and preserved in air tight poly vinyl chloride containers at room temperature for further analysis.

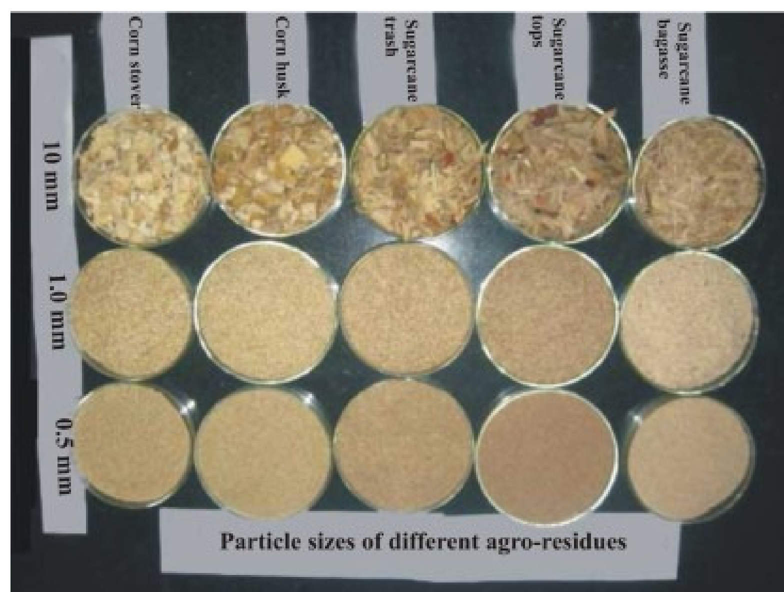


Plate 1. Agro-residues of different particle sizes used in the study

3.4 Pre-treatment by autohydrolysis

The autohydrolysis pre-treatments were conducted for all the substrates *viz.,* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover of three different particle sizes 0.5 mm, 1.0 mm and 10 mm with three replications.

3.4.1 Pre-treatment by autohydrolysis without acid

The substrates of different particle sizes were subjected for autohydrolysis with three replications at 180^0C and 200^0C for 10 minutes. The unit was deviced locally consisting of a small cylinder of inside height 10" and inside diameter 4" made of high quality stainless steel. The bottom end of the cylinder was provided with a gasket lid for working and there were two inlet and outlet ports to regulate steam entry and steam exhaust. Ten grams of each substrate was filled in to temperature resistant polyethylene bags and placed inside the autohydrolysis unit. The source of steam was from the boilers of a distillery (M/s. S. L. N. Distillery, Garag). The unit was fixed at the steam pipeline where the temperature was 180^0C and 200^0C (based on the pressure gauge installed in the steam pipeline). The steam having the designated 180^0C and 200^0C temperature was passed through inlet. Once the inside temperature had attained 180^0C and 200^0C, the substrates were treated for 10 minutes and the steam entry was suddenly stopped by letting cold water into the explosion unit to cause explosion reaction of the substrates. The pre-treated substrates were dried in an oven to constant weight at 60^0C and after recording the dry weight preserved in air tight poly vinyl chloride containers for further analysis at room temperature. The unit used for autohydrolysis is shown in Plate 2a and 2b.

3.4.2 Autohydrolysis with dilute acid H_2SO_4

Ten grams of each substrate and all the three particle sizes, in triplicates were soaked in 1.0% H_2SO_4 (w/v) for two hours in 250 ml beakers, filled in to polyethylene bags (temperature resistant) and placed inside the autohydrolysis unit. The pre-treatment with 1.0% H_2SO_4 was performed as per the method described in pre-treatment by autohydrolysis (Soderstorm *et al.*, 2003).

3.5 Biological pre-treatment

The biological pre-treatments were conducted for all the substrates *viz.,* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover of three different particle sizes 0.5 mm, 1.0 mm and 10.00 mm with three replications along with uninoculated control.

3.5.1 Biological pre-treatment with lignolytic fungi

Ten grams of oven dried substrates of three particle sizes maintaining three replications were transferred separately in to 250 ml capacity Erlenmeyer flasks. Each substrate was wetted with nutrient solution on the basis of its water holding capacity (approximately @ 5.0 ml g^{-1} dry substrate). The nutrient solution consisted of $(NH_4)_2 SO_4$ (10. 0 $g^{-1}L$), KH_2PO_4 (3.0 $g^{-1}L$), $MgSO_4 \cdot 7H_2O$ (0.50 $g^{-1}L$) and $CaCl_2 \cdot H_2O$ (0.50 g^{-1} L), (Singhania *et al*., 2006 and Valaskova *et al.*, (2007)). The lignolytic cultures maintained on PDA were used as inocula for the substrate pre-treatment. The respective fungal cultures were grown in potato dextrose broth, 50 ml in 150 ml Erlenmeyer flasks for five days on rotary shaker (150 rpm) at 28 ± 1^0 C. The mycelia were homogenised and the homogenate having 20×10^8 CFU per ml was inoculated to the substrates aseptically @ 5.0% and incubated for 10, 20 and 30 days at 28 ± 1^0 C. (Valmaseda *et al*., 1991). One un-inoculated control also was maintained for all the substrates and all the particle sizes. After an interval of 10, 20 and 30 days solid state fermentation, the growth of fungi was arrested by autoclaving at 121^0 C for 15 min. Later, the samples were dried in oven at 60^0 C till it attains constant weight. The weight of the pre-treated substrates were recorded and preserved in air tight poly vinyl chloride containers for further analysis at room temperature.

3.5.2 Biological pre-treatment with crude enzymes of lignolytic fungi

The selected lignolytic cultures namely *Phanerochaete chrysosporium, Pleurotus ostreatus* and UASD-LF1 (white rot fungi) were used in the study.

3.5.2.1 Preparation of crude enzyme extract

To prepare inoculums, 10 agar culture discs (10 mm from the edge of a 7-day-old agar culture) of individual cultures were inoculated into 250 ml Kirks medium. (Appendix) (Kirk *et al*., 1978). The Kirks basal liquid medium contained the following nutrients (per liter): glucose (3.0 g), KH_2PO_4 (1.0 g), NaH_2PO_4 (0.26 g), $(NH_4)_2SO_4$ (2.4 mM, 0.317 g), $MgSO_4.7H_2O$ (0.5 g), $CuSO_4.7H_2O$ (0.05 Mg), 2,2-dimethylsuccinic acid (2.2 g), $CaCl_2 . 2H_2O$ (74 mg), $ZnSO_4. 7H_2O$ (6 mg), $FeSO_4 . 7H_2O$ (5 mg), $MnSO_4. 4H_2O$ (5 mg), $CoCl_2 . 6H_2O$ (1 mg) and vitamin solution (500 µl). The pH of the medium was adjusted to 4.5 with 1 N NaOH. 1 mM veratryl alcohol was added to the basal medium to stimulate production of peroxidases and incubated at 30^0C on a rotary shaker (150 rpm) for 5 days. After the incubation the culture broth was centrifuged at 10,000 rpm for 20 min to sediment mycelia, spores and solids. The supernatant was diluted ten times in sodium tartarate buffer, pH 4.5 and used as the enzyme source (Koullas *et al*., 1993).

3.5.2.2 Pre-treatment by crude enzymes

Ten grams of oven dried substrates of three particle sizes were transferred separately in to 250 ml capacity Erlenmeyer flasks. The respective fungal crude enzymes were added to each flask at the rate of 100 ml per 10 g substrate and incubated for 10, 20 and 30 days at 28 ±1^0 C. One un-inoculated control was also maintained for all the substrates and particle sizes. After an interval of 10, 20 and 30 days of incubation, the samples were dried in oven at 60^0 C till constant weight was obtained. The weight of the pre-treated substrates were recorded and preserved in air tight poly vinyl chloride containers at room temperature for further analysis.

Plate 2a: Locally fabricated steel unit used for autohydrolysis of substrates

Plate 2b : Autohydrolysis of substrates with 200^0C steam temperature at the S. L. N. Distillary, Garag

3.6 Saccharification of pre-treated substrates

The alkali pre-treatment @ 3.0% NaOH for 8 hours incubation followed by autoclaving at 121^0C at 15 lbs pressure for 1 h resulted in recovery of maximum cellulose and hemicelluloses and maximum delignification in 0.5 mm particle size. Therefore the alkali pre-treated substrates were further subjected for saccharification studies using different methods as described below.

3.6.1 Microbial saccharification using cellulolytic fungi

The delignified substrates *viz.,* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover of particle size 0.50 mm, obtained after alkali pre-treatment @ 3.0% NaOH for 8 hours incubation followed by autoclaving at 121^0C, 15 lbs pressure for 1h were subjected for saccharification study using proven cellulolytic fungal cultures *viz., Trichoderma reesei, T. Viridae, Aspergillus awamorii, A. sydowii* and *Phanerochaete chrysosporium* in triplicates.

Ten grams oven dried substrates (five) were transferred separately in to 250 ml capacity Erlenmeyer flasks. Each substrate was wetted with nutrient solution (Singhania *et al.,* 2006) on the basis of its water holding capacity (approximately 5.0 ml g^{-1} dry substrate) and inoculated with each of the respective five fungal cultures at different inoculums levels such as 2, 4 and 6% aseptically and incubated for different days *viz.,* two, four, six and eight days at 30 $\pm 1^0$ C (Valmaseda *et al.*, 1991). The fungal cultures maintained on potato dextrose agar slants were separately grown on potato dextrose broth, 50 ml in 150 ml Erlenmeyer flasks for five days in rotary shaker (150 rpm) at 30 ± 1^0C. The mycelia was homogenised and used as inoculums. The homogenized inoculum had 20×10^8 CFU per ml. After an interval of 2, 4, 6 and 8 days solid state fermentation, the growth of fungi was ceased by autoclaving at 121^0 C for 15 min (Koijam *et al.,* 2000) and the samples were dried in an oven at 60^0 C to constant weight and analysed for reducing sugars by DNSA method as described by Miller (1959). For the buffer, distilled water and equal amount of enzyme were added. The sugar concentration if any was subtracted from the substrate and enzyme blank. The per cent saccharification was calculated by the formula given below.

$$\% \text{ Saccharification} = \frac{\text{Reducing sugars (mg g}^{-1}) \times 0.9 \times 100}{\text{Initial cellulose (mg g}^{-1})}$$

3.6.1.1 Factors influencing cellulase activity

3.6.1.1a Effect of different temperatures on cellulase production

The five different fungal cultures were inoculated separately in to Mandel's basal salt solution at pH 6.0 (Mandel and Weber, 1969) (Appendix I) for cellulase enzyme production. The cellulose of this medium formed the control. The individual cultures were inoculated to the 100 ml of autoclaved basal salt solution @ 5%, taken in 250 ml Erlenmeyer flask incubated for eight days on rotary shaker at 150 rpm at different temperatures *viz.,* 25^0C, 30^0C, 35^0C and 40^0C. After eight days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to sediment mycelia, spores and solids. The supernatant enzyme extract was analysed for cellulase activity in terms of filter paper units (Koullas *et al.*, 1993).

3.6.1.1b Effect of different pH on cellulase production

The five different fungal cultures were inoculated separately in to Mandel's basal salt solution (Mandel and Weber, 1969) for cellulase enzyme production. The cellulose of this medium formed the control. The individual cultures were inoculated to the 100 ml of autoclaved basal salt solution @ 5%, taken in 250 ml Erlenmeyer flask incubated for eight days on rotary shaker at 150 rpm at different pH *viz.,* 5.0, 5.5, 6.0 and 6.5, pH at 30^0C temperature. After eight days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to sediment mycelia, spores and solids. The supernatant enzyme extract was analysed for cellulase activity in terms of filter paper units.

3.6.1.1c Effect of different carbon source on cellulase production

The five different fungal cultures were inoculated separately in to Mandel's basal salt solution (Mandel and Weber, 1969) for cellulase enzyme production. The cellulose of this medium formed the control. The carbon source, was replaced with different alkali pre-treated agro-residues namely Sugarcane bagasse, Sugarcane tops, Sugarcane trash, Corn husk and Corn stover for cellulase production. The individual cultures were inoculated to the 100 ml of autoclaved basal salt solution @ 5%, taken in 250 ml Erlenmeyer flask incubated for eight days on rotary shaker at 150 rpm at 30^0C and pH 6.0. After eight days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to sediment mycelia, spores and solids. The supernatant was analysed for cellulase activity in terms of filter paper units.

3.6.1.2 Extraction of crude cellulase enzyme

The five different fungal cultures were inoculated separately in to Mandel's basal salt solution (Mandel and Weber, 1969) for cellulase enzyme production. The individual cultures were inoculated to the 100 ml of autoclaved basal salt solution @ 5%, taken in 250 ml Erlenmeyer flask incubated for eight days on rotary shaker at 150 rpm at 30^0 C at 6.0 pH on cellulose as carbon source, since these conditions produced highest cellulase activity for all the cellulolytic cultures. After eight days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to sediment mycelia, spores and solids. The supernatant was collected and used as the source of enzyme and stored at 4^0C for immediate future use. (Mandel's basal salt solution, Appendix I)

The culture filtrates obtained from eight days grown fungi at 30^0C and pH 6.0 were tested for cellulase activity (FPU) to determine the optimum conditions of temperature and pH. They were analysed for filter paper activity, CMCase activity β–glucosidase activity, xylanase activity and the protein content of the crude enzyme extracts.

3.6.2 Saccharification using crude cellulase enzymes

3.6.2.1 Treatment of substrate with crude cellulase enzyme

The pre-treated substrates (Sugarcane bagasse, Sugarcane tops, Sugarcane trash, Corn husk and Corn stover) were subjected for crude enzyme hydrolysis in 250 ml Erlenmeyer flask. Oven dried, alkali and temperature pre-treated samples (2.50 g, 5.0 g and 7.50 g) were suspended in varying quantities of 0.05 M Citrate buffer of pH 4.8 such that after the addition of enzyme extract the final volume of solution was 100 ml. (Sattler *et al*., 1989 and Koijam *et al.,* 2000). The substrate and buffer solution was autoclaved at 121^0 C for 15 min and added with crude enzyme extract of different cellulolytic fungi *viz., Trichoderma reesei, Trichoderma viridae, Aspergillus awamorii, Aspergillus sydowii* and *Phanerochaete chrysosporium* separately at 10, 20 and 30 FPU g^{-1} with 5% substrate concentration. Further, the optimized crude cellulase (20 FPU) of different fungi was used for saccharification substrate at different solid concentrations (2.5, 5.0 and 7.5). The enzyme extract of different fungi had different cellulase activity. The final volume of buffer and enzyme extract was 100 ml solution. The suspension was incubated for varying intervals of time 0 to 48 h at 50^0 C at 150 rpm. Then the reaction of hydrolysis was ceased to proceed by holding flasks in boiling water for 10 min to denature the enzyme. Solution of 4 ml was withdrawn, centrifuged at 10,000 rpm for 10 min and the supernatant was used for the estimation of reducing sugars and per cent hydrolysis. The amount of reducing sugars was estimated by DNSA method. The per cent saccharification (Kaar and Holtzapple, 1998) was calculated by the formula given below.

$$\% \text{ Saccharification} = \frac{\text{Reducing sugars (mg g}^{-1}) \times 0.9 \times 100}{\text{Initial cellulose (mg g}^{-1})}$$

3.6.3 Treatment of substrate with commercial cellulase enzyme

The pre-treated substrates *viz.*, sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were hydrolysed by using commercial cellulase and β–glucosidase and xylanase enzymes.

Oven dried, alkali and temperature pre-treated samples (2.50 g, 5.0 g and 7.50 g) were suspended separately in 250 ml Erlenmeyer flask containing varied quantities of 0.05 M Citrate buffer, pH 4.8 (Koijam *et al.,* 2000), autoclaved and added with 10, 15 and 20 FPU g^{-1} cellulase enzyme (MAPS India, Ahmadabad) separately. The β–glucosidase (SRL chemicals) enzyme at 10 IU g^{-1} and xylanase enzyme at 5 U g^{-1} (M/s. Sigma Industry – Courtesy, Godavari Sugar Mills, Sameerwadi) were also supplemented commonly making the ratio at 10:10:5, 15:10:5 and 20:10:5 for cellulase, β-glucosidase and xylanase, respectively to enhance the rate of cellulose hydrolysis and to overcome the inhibition of glucose formation by cellobiose (Sattler *et al.,* 1989 and Sun and Cheng, 2005). The optimized commercial enzyme concentration of 15 FPU per g cellulase, 10 IU β-glucosidase and 5 U xylanase was tested at different concentrations of substrates (2.5%, 5.0% and 7.5%). The final volume of buffer and enzyme mixture was 100 ml solution. The cellulose enzyme of M/s. MAPS India, Ahmedabad had cellulase activity of 166 FPU ml^{-1}, the β–glucosidase of M/s. SRL Chemicals had β–glucosidase activity of 19.6 U mg^{-1} and the xylanase of M/s. Sigma Chemicals had xylanase activity of 170 U g^{-1}. The suspension was incubated for varying intervals of time 0 to 24h at 50^0 C at 150 rpm. Then the reaction of hydrolysis was ceased to proceed by holding flasks in boiling water for 10 min to denature the enzyme. Solution of 4 ml was withdrawn, centrifuged at 10000 rpm for 10 min and the supernatant was used for the estimation of reducing sugars and per cent hydrolysis (Kaar and Holtzapple, 1998). The amount of reducing sugars was estimated by Di-Nitro Salicylic Acid (DNSA) method. The per cent saccharification was calculated by the formula given below.

$$\%\ \text{Saccharification} = \frac{\text{Reducing sugars (mg g}^{-1}) \times 0.9 \times 100}{\text{Initial cellulose (mg g}^{-1})}$$

3.6.5 Enzyme assays

Enzyme assays such as filter paper activity, CMCase activity, β-glucosidase activity and xylanase activity were performed to know the sacchrification potential of different enzyme extracts and commercial enzymes used during the investigations on bioethanol production from agro-residues. The methods followed for the determinations are explained below.

3.6.5.1 Filter paper activity

To 50 mg of Whatman No. 1 filter paper strip (1 cm x 6 cm) taken in a test tube, 0.80 ml of sodium citrate buffer (0.05 M, pH 4.8) and 0.80 ml of appropriately diluted enzyme or enzyme extract were added. The reaction system was incubated for 10 min at 50^0 C. After the incubation, 2.40 ml DNSA was added and the tubes were kept under room temperature for 15 min so as to lower the temperature of reaction mixture to room temperature. Appropriate enzyme blank and reagent blank was also included in the estimations. The reducing sugars were estimated by following DNSA as method described before. The enzyme activity was expressed in terms of filter paper units (FPU). One unit of filter paper activity was defined as the amount of enzyme required to liberate one µ mole reducing sugar (expressed as glucose equivalents) in one minute under reaction conditions. The enzyme activity was calculated by using the following formula.

$$\text{Enzyme activity (U ml}^{-1}) = \frac{\text{OD x µg sugar (from the standard graph) x Dilution Factor}}{\text{0.1 x Mol. Wt of glucose x Incubation time (min) x Aliquot of enzyme (ml)}}$$

3.6.5.2 CMCase activity

Carboxymethyl cellulose (CMC) of 0.50 g was dissolved in 49.50 ml of sodium citrate buffer (0.05 M, pH 4.8) by vigorous stirring until the CMC was completely dissolved. This solution had the cellulose concentration of one mg per ml. To 0.80 ml of CMC solution (1 mg ml^{-1}), 0.80 ml of appropriately diluted enzyme or enzyme extract was added. The reaction system was incubated for 10 min at 50^0 C. After the incubation, 2.40 ml DNSA was added and the tubes were kept under room temperature for 15 min so as to lower the temperature of reaction mixture to room temperature. Appropriate enzyme blank and reagent blank was also included in the estimations. The reducing sugars were estimated by following DNSA method described for reducing sugars estimation.

The enzyme CMCase activity was expressed in units. One unit of cellulase activity was defined as the amount of enzyme required to liberate one μ mole of reducing sugar (expressed as glucose equivalents) in one minute under reaction conditions. The enzyme activity was calculated by using the following formula.

Enzyme OD x μg sugar (from the standard graph) x Dilution Factor

activity = ---

($U ml^{-1}$) 0.1 x Mol. Wt of glucose x Incubation time (min) x Aliquot of enzyme (ml)

3.6.5.3 β-glucosidase activity

Appropriately diluted enzyme of 0.2 ml was added to 0.2 ml of 0.01 M, p- nitrophenyl-β-D- glucopyranoside (PNPG) and 1.6 ml of sodium citrate buffer (0.05 M, pH 4.8). The reaction system was incubated for 30 minutes at 50^0C. The reaction was stopped by addition of 4 ml NaOH- glycine buffer (0.2 M, pH 10.6). One unit of β-glucosidase activity was defined as the amount of enzyme required to liberate one micro mole p- nitrophenol in one minute under assay conditions. The values are expressed in IU. One IU is the amount of enzyme required to release two micromoles of glucose from one micromole of cellobiose.

Enzyme OD x μ moles of p-nitrophenol (from the standard graph) x Dilution Factor

activity = ---

($U ml^{-1}$) 0.1 x Mol. Wt of glucose x Incubation time (min) x Aliquot of enzyme (ml)

3.6.5.4 Xylanase activity

Appropriately diluted enzyme of 0.8 ml was added to 0.8 ml of 1% birch wood xylan (Hi-Media Chemicals, Pune), prepared in sodium citrate buffer (0.05 M, pH 4.8). The reaction system was incubated for 10 minutes at 50^0 C. After the incubation, 2.40 ml DNSA was added and the tubes were kept under room temperature for 15 min so as to bring down the temperature of reaction mixture to room temperature. The reducing sugars were estimated by following DNSA method as described earlier. The enzyme xylanase activity was expressed in units. One unit of xylanase activity was defined as the amount of enzyme required to liberate one μ mole of reducing sugar (expressed as glucose equivalents) in one minute under reaction conditions. The enzyme activity was calculated by using the following formula.

Enzyme OD x μg sugar (from the standard graph) x Dilution Factor

activity = ---

($U ml^{-1}$) 0.1 x Mol. Wt of xylose x Incubation time (min) x Aliquot of enzyme (ml)

3.7 Fermentation of the hydrolysate

The saccharification studies had yielded maximum reducing sugars and the highest per cent saccharification with commercial cellulase enzyme at 15 FPU g^{-1}, 10 IU g^{-1} β–glucosidase and 5 U xylanase at 5% solid concentration for all the five substrates. Hence, the commercial enzyme hydrolysed substrates were used for further ethanol fermentation studies.

3.7.1 Fermentation medium

The solution containing 5% solids in 0.05 M Citrate buffer, which was subjected for commercial enzyme hydrolysis was added with nutrients (urea 0.64%, KH_2PO_4 0.2% and $MgSO_4.7H_2O$ 0.1%) to form a fermentation medium (Yu and Zhang, 2004). The medium was fermented to bioethanol with five different yeast cultures *viz., Saccharomyces cereviseae, Kluyveromyces marxianus, Pachysolen tannophilus, Pichia stipitis* and *Candida shihatae* and one bacterium culture (*Zymomonas mobilis*) individually, in combinations and simultaneous saccharification and fermentation separately. The details of the fermentation are discussed below.

3.7.1.1 Fermentation by individual cultures

The medium was fermented to bioethanol with five different yeast cultures *viz., Saccharomyces cereviseae, Kluyveromyces marxianus, Pachysolen tannophilus, Pichia stipitis* and *Candida shihatae* and one bacterium culture (*Zymomonas mobilis*) individually.

The 24 h grown broth cultures (50 ml in 100 ml Erlenmeyer flask) having approximately 30×10^6 cells ml^{-1} was inoculated to cellulase hydrolysed fermentation medium @ 4%. The inoculated substrates were incubated under aerobic condition for 24h at 37 ± 2^0 C. After 24h, anaerobic condition was created by plugging flasks with rubber cork, making provision for trapping carbon dioxide. The flasks were incubated for four to six days till the carbon dioxide ceased to evolve. The amount of ethanol formed in the broth was estimated colorimetrically as described by Caputi *et al.* (1968) and the residual reducing sugars were estimated by following DNSA method (Miller, 1959).

3.7.1.2 Fermentation by mixed cultures (Co-cultures)

The medium was fermented to bioethanol with five different yeast cultures *viz., Saccharomyces cereviseae, Kluyveromyces marxianus, Pachysolen tannophilus, Pichia stipitis* and *Candida shihatae* and one bacterium culture (*Zymomonas mobilis*) individually such that the pentose utilizing *Pichia stipitis* and *Candida shihatae* formed combinations with hexose fermenting *Saccharomyces cereviseae, Kluyveromyces marxianus, Pachysolen tannophilus* as dual inoculums and pentose utilizing *Pichia stipitis* and *Candida shihatae* forming triple inoculum combinations with *Saccharomyces cereviseae* and *Zymomonas mobilis*.

The 24 h old broth cultures (50 ml in 100 ml Erlenmeyer flask) having approximately 30×10^6 cells ml^{-1} was inoculated to cellulase hydrolysed fermentation medium @ 2% each as per the combinations. The inoculated substrates were incubated under aerobic condition for 24 h at 37 ± 1^0 C. The treatments with three cultures were inoculated at 1.33 per cent each as per the combinations of cultures. After 12 h, anaerobic condition was created by plugging flasks with rubber cork, making provision for trapping carbon dioxide. The flasks were incubated for four days till the carbon dioxide ceased to evolve. The amount of ethanol formed in the broth was estimated colorimetrically and the residual reducing sugars were estimated by following DNSA method.

3.7.1.3 Simultaneous Saccharification and Fermentation (SSF)

The pre-treated substrates *viz.,* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover at 5% solids were suspended in 250 ml Erlenmeyer flask containing varied quantities of 0.05 M Citrate buffer, pH 4.8, added with nutrients (urea 0.64%, KH_2PO_4 0.2% and $MgSO_4.7H_2O$ 0.1%), autoclaved and added with 15 FPU g^{-1} cellulase enzyme, 10 IU g^{-1} β–glucosidase and 5 U xylanase making final volume of the solution to 100 ml. The pre hydrolysate was incubated at 37^0 C for 6h on shaker cum water bath, since this was the temperature employed for fermentation by yeasts and bacteria (Wyman *et al.,* 1992). This hydrolysing medium was inoculated with five different yeast cultures *viz., Saccharomyces cereviseae, Kluyveromyces marxianus, Pachysolen tannophilus, Pichia stipitis* and *Candida shihatae* and one bacterium culture (*Zymomonas mobilis*) separately at 4% (v/v). The simultaneous saccharification and aerobic fermentation was carried out for 24 h at 37^0 C, followed by anaerobic fermentation for four days at 37 ± 2^0 C till the carbon dioxide ceased to evolve. The amount of ethanol formed in the broth was estimated colorimetrically and the residual reducing sugars were estimated by following DNSA method.

3.8 Pilot scale study on production of bioethanol

The alkali pre-treated (3.0% NaOH for 8 hours followed by autoclaving at 121^0C at 15 lbs pressure for 1h) and enzymatically saccharified bagasse (commercial cellulase enzyme at 15 FPU g^{-1}, 10 IU g^{-1} β–glucosidase and 5 U xylanse at 5% solid concentration) was fermented using laboratory fermentor.

3.8.1 Fermentation

Twenty litre fermentation medium consisting of 1 kg pre-treated and saccharified bagasse (5% solids) was added with nutrients (urea 0.64%, KH_2PO_4-0.2% and $MgSO_4.7H_2O$ 0.1%). The medium was fermented to bioethanol using *Saccharomyces cereviseae* and *Pichia stipitis* dual yeast cultures. Twenty four hour grown broth cultures having approximately 30×10^6 cells ml^{-1} was inoculated @ 2% each.

The inoculated substrates were incubated under aerobic condition for 12 h at 37 ± 1^0C. After 12 h, anaerobic condition was created and incubated for four days. The amount of ethanol formed in the broth was estimated colorimetrically.

3.9 Analysis of the substrates

The pre-treated substrates with various methods *viz.,* alkali and temperature pre-treated substrates, autohydrolysis pre-treated substrates with and without dilute acid- 1.0% H_2SO_4, wet ground sugarcane tops and biologically pre-treated substrates with lignolytic fungi and crude enzymes of lignolytic fungi after drying to constant weight were subjected for analysis of various composition of the substrates.

The weight of the substrates after pre-treatment were measured by using a sensitive balance (M/s. Essay Taroka) and expressed in terms of g g^{-1}.

These samples were used for estimation of cellulose, hemicellulose and lignin contents as follows. The percent increase or decrease in weight (variation) of the components of substrates after the pre-treatment was calculated. Also, the total sugars in terms of cellulose and hemicelluloses (g g^{-1}) obtained in different agro-residues due to different pre-treatments were determined.

$$\% \text{ weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

3.9.1 Chemical analysis - Estimation of cellulose, hemicellulose and lignin

The cellulose, hemicelluloses and lignin contents of the raw substrates and pre-treated substrates were estimated by determining Neutral detergent fibre, Acid detergent fibre and Acid detergent lignin as per method developed by Goering and Van Soest (1975). The cellulose, hemicelluloses and lignin contents were expressed in grams. The details of the method followed are explained below.

3.9.1.1 Neutral detergent fibre (NDF)

Preparation of neutral detergent solution

Disodium ethylene diamine tetra acetate of 18.16 g and 6.81g of sodium borate decahydrate were dissolved by heating in 200 ml of distilled water taken in a beaker. To this, solution containing 30 g of sodium lauryl sulphate dissolved in about 200 ml distilled water and 10 ml of 2-ethoxy ethanol was added and followed by addition of about 100 ml of solution containing 4.5g of disodium hydrogen phosphate. Finally the volume was made up to one litre and pH was adjusted to 7.0.

Procedure

Raw or pre-treated substrate of 0.5 g was transferred to 500 ml refluxing flask. 100 ml of cold neutral detergent solution; 2 ml of decahydronaphthalene (Decalin) and 0.5g of sodium sulphite were added. The mixture was heated to boiling and then the heat was regulated to avoid foaming and refluxed for one hour. After cooling, the sample was filtered through a previously weighed 50 ml capacity gooch crucible of G-1 grade under suction using vacuum pump. The residue remained in the gooch crucible was washed with hot water repeatedly followed by two washings with acetone. The crucible containing residue was dried at 100^0 C for 8h in a hot air oven. Finally, the crucibles were cooled in a desiccator and dry weight was recorded. The per cent NDF was calculated by the formula given below.

$$\% \text{ NDF} = \frac{Y - X}{W} \times 100$$

Where, Y = weight of crucible + NDF

X = weight of empty crucible

W = weight of the sample

3.9.1.2 Acid detergent fibre

Preparation of Acid detergent solution

One litre of 1N sulphuric acid was prepared and in this solution, 20 g of cetyl trimethyl ammonium bromide was dissolved.

Procedure

Raw or pre-treated substrate of 0.5 g was transferred to a 500 ml refluxing flask. To this, 100 ml of acid detergent solution and 2 ml of decahydronaphthalene were added. This mixture was heated to boiling and the heat was regulated to avoid foaming and refluxed for one hour. After one hour of refluxing, the mixture was cooled and filtered through a previously weighed 50 ml capacity gooch crucible of G-1 grade under suction using vacuum pump. The sample in the crucible was washed with hot water to remove acid followed by two washings with acetone. The crucibles were dried at 100^0C for 8h in a hot air oven. Finally, the crucibles were cooled in a desiccator and dry weight was recorded. The per cent ADF was calculated by the formula given below.

$$\% \text{ ADF} = \frac{Y - X}{W} \times 100$$

Where, Y = weight of crucible + ADF

X = weight of empty crucible

W = weight of the sample

3.9.1.3 Acid detergent lignin

Preparation of acid

72% H_2SO_4 (w/v) : Concentrated sulphuric acid of 73.5 ml was added to a beaker containing distilled water of 26.5 ml.

Procedure

Crucibles containing ADF (acid detergent fibre) were kept in 100 ml Petri dish and the contents of crucibles were filled with cooled 72% H_2SO_4. The contents were stirred with a glass rod to break lumps of residue if any. As the acid drains out, fresh acid was refilled in to the crucibles and frequent stirring was given. After 3 h of acid digestion along with intermittent stirring, the contents were wash filtered with hot water by vacuum suction to retain the residue and to remove the used acid. Then the crucibles with residues were dried at 100^0C for 8h. After this, the crucibles were cooled in a desicator and weighed (L). After weighing, the contents in crucibles were ashed in a muffle furnace at 500^0 C for 2 h. After the furnace temperature had decreased, the crucibles were taken out, cooled in a desiccator and the weight (A) of the ash was recorded. The per cent cellulose, hemicellulose and lignin contents were calculated by the formula given below.

% Hemicellulose =% NDF -% ADF

$$\% \text{ Cellulose} = \frac{Y - L}{W} \times 100$$

$$\% \text{ Lignin} = \frac{L - A}{W} \times 100$$

Where, Y = weight of ADF + crucible

L = weight of crucible + lignin

A = weight of crucible + ash

W = weight of the sample

3.9.2 Estimation of reducing sugars

The amount of reducing sugars present in the saccharified samples (hydrolysate) was estimated by dinitrosalicylic acid (DNSA) method (Miller, 1959).

Reagents used

Standard glucose stock solution

D-glucose of 360 mg (oven dried at 105^0 C to constant weight) was dissolved in distilled water and the volume made up to 100 ml. The solution had the glucose concentration of 3.60 mg ml^{-1}.

Glucose working standard solution

The glucose stock solution of 20 ml was taken out and volume was made up to 100 ml with distilled water in a 100 ml volumetric flask to obtain the glucose concentration of 720 µg ml^{-1}.

Dinitrosalisilic acid reagent (DNSA):

One gram of 3, 5-dinitrosalycylic acid (DNSA) was suspended in 50 ml distilled water. To this, 10 ml of 16% sodium hydroxide solution was gradually added while mixing the solution. After complete dissolution of DNSA in the solution, 30 g Potassium sodium tartarate was added and the volume was made up to 100 ml using distilled water.

Procedure

Appropriate sample aliquots between 0.25 ml and 0.50 ml from the sample was pipetted out and transferred to test tubes. For standard graph, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml of glucose working standard solution was pipetted out and transferred in to series of test tubes (for blank one ml of distilled water was used). The volume was made up to 1.6 ml with distilled water. To, this 2.40 ml of DNSA reagent was added. The prepared tubes were covered with polyethylene sheet and kept in boiling water bath for 10 minutes exactly. Immediately after 10 minutes incubation, the tubes were taken out, cooled in ice cold water to attain room temperature and the optical density was measured at 540 nm using Systronics UV/Vis Spectrophotometer-117.

3.9.3 Estimation of protein

Reagents required

Lowry's reagent : Solution A, containing 2% sodium carbonate in 0.1 N sodium hydroxide and Solution B, containing 0.5% copper sulphate in 1% sodium potassium tartarate were prepared separately and mixed in the ratio of 50:1 before use (Lowry *et al.,* 1951).

Standard stock solution of protein : Bovine serum albumin (BSA) of 100 mg was dissolved in distilled water and the volume made up to 100 ml to get the concentration of protein at 1 mg ml^{-1}.

Protein working standard solution : Standard stock solution of 10 ml protein was pippetted in to 100 ml volumetric flask and the volume was made up to 100 ml with distilled water. The solution had the protein concentration of 0.1 mg ml^{-1}.

Folin's phenol solution: Ready solution was obtained from Hi-Media Chemicals, Pune.

Procedure

Enzyme extract of 0.5 ml was transferred to test tube and the volume made up to 5 ml with distilled water. For standard graph, one to five ml of working standard protein solution was transferred to different test tubes and volume was made up to 5 ml with distilled water. To, all the tubes 4.5 ml of lowry's reagent was added and allowed to react for 10 minutes.

Then, 0.5 ml of folin's phenol reagent was added and after 10 minutes, the optical density was measured at 620 nm using UV/Vis Spectrophotometer-117 (M/s. Systronics make, Japan). The amount of protein in the sample was calculated and expressed in mg ml^{-1}.

3.9.4 Estimation of ethanol

The ethanol content of the fermented medium was estimated colorimetrically as per the method described by Caputi *et al*, (1968).

Preparation of reagents

Potassium dichromate ($K_2Cr_2O_7$) 0.23N : Potassium dichromate of 34 g (oven dried at 105^0 C) was dissolved in 500 ml of distilled water. To this 325 ml of concentrated sulphuric acid was added and the volume was made up to 1000 ml with distilled water.

Preparation of ethanol stock solution : Analytical grade ethanol (789 mg ml^{-1}) of 12.6 ml was mixed in little amount of distilled water and the final volume made up to 100 ml using distilled water, this solution had the concentration of 100 mg ethanol ml^{-1}.

Procedure

Three ml of representative sample from each treatment was diluted with 30 ml distilled water and transferred to 250 ml round bottom flask, connected to the condenser and the sample was distilled at 74 -75^0 C. The distillate was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ reagent, which was kept at the receiving end, till the total volume of 45 ml was obtained. Similarly, for standards ranging from 20-100 mg ethanol were mixed with 25 ml of $K_2Cr_2O_7$ separately. The distillate containing alcohol was collected till the total volume of 45 ml was obtained. These, samples and standards were kept in water bath at 60^0 C for 20 min and were cooled immediately. The final volume was made up to 50 ml with distilled water and the optical density was measured at 600 nm using UV/Vis. Spectrophotometer-117 (M/s. Systronics make, Japan). The standard curve was plotted amount of ethanol in the sample was calculated using the graph and expressed in g g^{-1}.

3.10 Statistical analysis

The results obtained in the investigations on bioethanol production were statistically analysed using factorial, completely randomised design as described by Panse and Sukhatme (1985).

4. EXPERIMENTAL RESULTS

Bioethanol is considered to be one of the potential energy sources to reduce the world's dependence on non-renewable energy sources. Lignocellulosic biomass is seen as an alternative feedstock for future bioethanol production in place of conventional feedstocks such as sugarcane and corn grain. For the bioconversion of lignocellulosic biomass to bioethanol certain pre-treatment methods are necessary to increase the accessibility of cellulose and to improve formation of fermentable sugars.

In the present investigation on bioethanol production from selected agro-residues, different pre-treatment methods (alkali, acid, steam, combination of physical and chemical pre-treatments, microbial pre-treatment and crude lignolytic enzyme pre-treatment) were conducted to increase the accessibility of cellulose. Further, the saccharification of cellulose was achieved employing cellulolytic fungi and their crude cellulose enzymes and also commercial cellulose enzymes. This was followed by fermentation of derived sugars to produce bioethanol. The results obtained from these treatments are presented in this chapter.

4.1 Initial chemical composition of agro-residues

The initial chemical composition of the five agro-residues selected for the bioethanol production study varied differently with respect to cellulose, hemi cellulose and lignin content (Table 1).

In general, the cellulose, hemi cellulose and lignin contents of the five substrates ranged between 0.327 and 0.353, 0.227 and 0.293 and 0.140 and 0.160 g per g, respectively.

Sugarcane bagasse contained the highest cellulose (0.353 g g^{-1}) and hemi cellulose (0.293 g g^{-1}), whereas corn stover was found to contain the lowest cellulose content of 0.327 g per g. Sugarcane tops had the lowest hemi cellulose content of 0.227 g per g. The highest lignin content of 0.160 g per g was observed in sugarcane trash and the lowest lignin content of 0.140 g per g both in sugarcane bagasse and corn husk.

4.2 Effect of alkali and temperature pre-treatment

4.2.1 Weight loss

The data pertaining to the loss in weight of substrates due to pre-treatment is presented in Table 2. Significant variations were observed due to individual effects such as combination of physical and chemical methods of pre-treatment, different substrates and their particle size. However, the interaction effects of these factors were found to be non significant.

With regards to the effect of combination of physical and chemical methods of pre-treatment on weight loss, all the treatments differed significantly with each other.

The maximum mean loss in weight 0.488 g per g was observed in the pre-treatment with 3.0% NaOH treatment (8 h incubation at room temperature) and exposure to 125^{0}C temperature for 1 h. This was significantly superior over all other combinations of alkali and temperature treatments. The pre-treatment with 2.5% NaOH (8 h incubation at room temp.) followed by autoclaving at 121^{0}C for 1 h recorded the least weight loss (0.454 g g^{-1}).

Among the five substrates subjected for different treatments sugarcane bagasse recorded the highest mean weight loss (0.480 g g^{-1}). It was on par with corn husk (0.479 g g^{-1}), but significantly superior over corn stover (0.471 g g^{-1}), corn trash (0.467 g g^{-1}) and sugarcane tops (0.457 g g^{-1}).

The particle size of the substrate also significantly influenced weight loss and even the three particle sizes evaluated differed significantly from each other.

The particle size of 0.5 mm recorded significantly the highest weight loss (0.531 g g^{-1}) as compared to particle size 1.0 mm (0.512 g g^{-1}) and 10.00 mm (0.371 g g^{-1}). The effect of alkali pre-treatment (8 h) coupled with temperature exposure at 121^{0}C (1 h) on 0.5 mm particle size is presented in Plate 3.

Table 1: Initial chemical composition of agro-residues used for bioethanol production studies

Sl. No	Substrate	Cellulose ($g\ g^{-1}$)	Hemicellulose ($g\ g^{-1}$)	Lignin ($g\ g^{-1}$)
1	Sugarcane bagasse	0.353	0.293	0.140
2	Sugarcane tops	0.333	0.227	0.157
3	Sugarcane trash	0.343	0.247	0.160
4	Corn husk	0.333	0.253	0.140
5	Corn stover	0.327	0.260	0.153

Table 2: Effect of alkali and temperature pre-treatments on loss of weight of substrates

Treatments	Particle size (mm)	Substrates (g g^{-1})					Mean
		Sugarcane bagasse	**Sugarcane tops**	**Sugarcane trash**	**Corn husk**	**Corn stover**	
2.50 % NaOH + 121^{0}C temp.	0.5	0.520	0.501	0.504	0.525	0.514	**0.454**
	1.0	0.502	0.487	0.486	0.499	0.492	
	10.0	0.372	0.339	0.355	0.362	0.352	
3.00 % NaOH + 121^{0}C temp.	0.5	0.526	0.515	0.511	0.530	0.523	**0.465**
	1.0	0.511	0.493	0.509	0.520	0.519	
	10.0	0.380	0.345	0.363	0.369	0.360	
2.50 % NaOH + 125^{0}C temp	0.5	0.541	0.523	0.536	0.542	0.534	**0.476**
	1.0	0.525	0.504	0.514	0.528	0.525	
	10.0	0.390	0.360	0.372	0.382	0.371	
3.00 % NaOH + 125^{0}C temp.	0.5	0.568	0.532	0.548	0.555	0.547	**0.488**
	1.0	0.527	0.512	0.525	0.538	0.531	
	10.0	0.400	0.371	0.383	0.403	0.386	
Mean		**0.480**	**0.457**	**0.467**	**0.479**	**0.471**	
Mean	0.5	**0.530**					
	1.0	**0.512**					
	10.0	**0.371**					

	SEm $\pm$	CD at 1%
Treatments (A)	0.001	0.005
Substrates (B)	0.002	0.006
Particle size (C)	0.001	0.005
A x B	0.003	NS
A x C	0.002	NS
B x C	0.003	NS
A x B x C	0.005	NS

Plate 3. The various agro-residues of 0.5 mm particle size pre-treated with 3% NaOH and autoclaved at 121^{0}C which yielded maximum amount of reducing sugars

With respect to alkali and heat pre-treatments, the highest weight loss was observed in the treatment with 3.0% NaOH (8 h incubation at room temperature) followed by autoclaving at 125^{0}C (1 h) for the particles of size 0.5 mm in all the substrates, sugarcane bagasse 0.568 g per g. sugarcane tops 0.532 g per g, sugarcane trash 0.548 g per g, corn husk 0.555 g per g, and corn stover 0.547 g per g. The lowest weight loss was observed in the treatment with 2.50% NaOH (8 h incubation at room temperature) followed by autoclaving at 121^{0}C (1h) for the particle size of 10.00 mm in all the substrates, (sugarcane bagasse 0.372 g per g, sugarcane tops g per g, sugarcane trash g per g, corn husk g per g and corn stover (g g^{-1}).

4.2.2 Cellulose content

The data on cellulose content of different substrates as influenced by different pre-treatments are presented in Table 3.

Pre-treatment with NaOH and exposure to different temperature significantly influenced the cellulose content. The highest mean cellulose content of 0.562 g per g was recorded with 3.0% NaOH (8 h incubation at room temperature) followed by autoclaving at 121^{0}C temperature (1 h), which was on par with 2.5% NaOH and exposure to 125^{0}C. These treatments were significantly superior over other two treatments with respect to all particle sizes in cellulose content. The least cellulose recovery of 0.512 g per g across different substrates was recorded with 2.50% NaOH plus exposure to 121^{0}C temp.

Various substrates evaluated also recorded significant differences in cellulose content. The highest mean cellulose content was obtained with sugarcane bagasse 0.565 g per g, which was significantly superior over all other substrates. The other substrates are at par with each other with respect to cellulose content, which ranged between 0.541 g per g in case of corn stover and 0.544 g per g in case of sugarcane trash. The cellulose content was also influenced significantly due to the particle size. The maximum cellulose content of 0.592 g per g was observed in case of 0.5 mm particle size, which was significantly superior over particles with a size of 1.0 mm (0.568 g g^{-1}) and 10.0 mm (0.481 g g^{-1}).

The interaction between the treatments at different levels of alkali coupled with temperature and substrate differed significantly, where in treatment with 2.50% alkali and 125^{0}C exposure temperature recorded the highest cellulose content for bagasse (0.576 g g^{-1}), sugarcane trash (0.564 g g^{-1}), sugarcane tops (0.560 g g^{-1}) and corn stover (0.558 g g^{-1}). treatments with 3.0% NaOH and 121^{0}C exposure temperature produced highest cellulose content for corn husk (0.564 g g^{-1}). However, these two treatments were found to be on par with each other for their respective substrates.

Table 3: Effect of alkali and temperature pre-treatments on cellulose content

Treatments	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
2.50 % NaOH + 121^{0}C temp.	0.5	0.587 (66.29)	0.540 (62.16)	0.533 (55.39)	0.540 (62.16)	0.527 (61.16)	0.512
	1.0	0.580 (64.31)	0.520 (56.16)	0.513 (49.56)	0.533 (60.06)	0.507 (55.05)	
	10.0	0.473 (33.99)	0.453 (36.04)	0.453 (32.07)	0.460 (38.14)	0.453 (38.53)	
3.00 % NaOH + 121^{0}C temp.	0.5	0.633 (79.32)	0.613 (84.08)	0.613 (78.72)	0.620 (86.19)	0.613 (87.46)	0.562
	1.0	0.593 (67.99)	0.587 (76.28)	0.573 (67.06)	0.587 (76.28)	0.580 (77.37)	
	10.0	0.500 (41.64)	0.473 (42.04)	0.480 (39.94)	0.487 (46.25)	0.473 (44.65)	
2.50 % NaOH + 125^{0}C temp.	0.5	0.627 (77.62)	0.613 (84.08)	0.620 (80.76)	0.607 (82.28)	0.607 (85.63)	0.562
	1.0	0.607 (71.95)	0.580 (74.17)	0.580 (69.10)	0.573 (72.07)	0.580 (77.37)	
	10.0	0.493 (39.66)	0.487 (46.25)	0.493 (43.73)	0.473 (42.04)	0.487 (48.93)	
3.00 % NaOH + 125^{0}C temp.	0.5	0.593 (67.99)	0.587 (76.28)	0.600 (74.93)	0.587 (76.28)	0.587 (79.51)	0.553
	1.0	0.587 (66.29)	0.567 (70.27)	0.567 (65.31)	0.573 (72.07)	0.580 (77.37)	
	10.0	0.507 (43.63)	0.493 (48.05)	0.500 (45.77)	0.480 (44.14)	0.493 (50.76)	
Mean		**0.565**	**0.543**	**0.544**	**0.543**	**0.541**	
Mean	0.5	0.592					
	1.0	0.568					
	10.0	0.481					

Figures in parentheses indicate per cent increase in cellulose over the initial cellulose content of raw substrates

Note : Initial cellulose content ($g\ g^{-1}$) Sugarcane bagasse (0.353), Sugarcane tops (0.333), Sugarcane trash (0.343), Corn husk (0.333) and Corn stover (0.327)

Contd…..

Two way interaction A x B

Treatments \ Substrate	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
2.50 % NaOH + 121^{0}C temp.	0.549	0.504	0.500	0.511	0.496	**0.512**
3.00 % NaOH + 121^{0}C temp.	0.576	0.558	0.556	0.564	0.556	**0.562**
2.50 % NaOH + 125^{0}C temp.	0.576	0.560	0.564	0.551	0.558	**0.562**
3.00 % NaOH + 125^{0}C temp.	0.562	0.549	0.556	0.547	0.553	**0.553**
Mean	**0.565**	**0.543**	**0.544**	**0.543**	**0.541**	

Two way interaction A x C

Treatments \ Particle size (mm)	0.5 mm	1.0 mm	10 mm	Mean
2.50 % NaOH + 121^{0}C temp.	0.545	0.531	0.459	**0.512**
3.00 % NaOH + 121^{0}C temp.	0.619	0.584	0.483	**0.562**
2.50 % NaOH + 125^{0}C temp.	0.615	0.584	0.487	**0.562**
3.00 % NaOH + 125^{0}C temp.	0.591	0.575	0.495	**0.553**
Mean	**0.592**	**0.568**	**0.481**	

	SEm $\pm$	CD at 1%
Treatments (A)	0.002	0.007
Substrates (B)	0.002	0.008
Particle size (C)	0.002	0.006
A x B	0.004	0.015
A x C	0.003	0.012
B x C	0.004	NS
A x B x C	0.007	NS

The three way interactions of treatments with different alkali coupled with temperature and particle size also differed significantly. Where, 3.0% alkali and 121^0C exposure temperature and particle size of 0.5 mm recorded the highest cellulose content (0.619 g g^{-1}), followed by 2.50% NaOH and 125^0C temperature exposure (0.615 g g^{-1}). These two treatments were on par with each other and found to be significantly superior over all other treatments.

The interactions of substrates with the particle sizes and combined interaction of all three factors were not significant. However, the highest cellulose content was observed in the treatment combination of 3.0% NaOH coupled with 121^0C temperature and with a particle size 0.5 mm for bagasse (0.633 g g^{-1}), corn husk (0.620 g g^{-1}), sugarcane tops (0.613 g g^{-1}) and corn stover (0.613 g g^{-1}). All these substrates exhibited the highest per cent increase in cellulose content over their initial cellulose content (bagasse 79.32, corn husk 86.19%, sugarcane tops 84.08% and corn stover 87.46%). The treatment 2.5% NaOH coupled with 125^0C temperature exposure showed the highest cellulose content of 0.620 g per g in case of sugarcane trash with a per cent increase in cellulose content at 80.76 per cent. These two treatments were numerically close to each other.

4.2.3 Hemi-cellulose content

Hemicellulose content of the substrate as influenced by individual treatment effects such as different alkali concentrations coupled with different exposure temperatures, substrates and particle sizes were found to decrease significantly (Table 4).

The treatment with 2.5% NaOH coupled with 121^0C temperature of exposure showed mean highest hemi cellulose content (0.222 g g^{-1}), which was significantly superior over values recorded in all other physico-chemical treatments. Significantly the lowest hemi-cellulose content was observed in the treatment 3.0% NaOH (8 h incubation at room temperature) which was subjected to autoclaving at 125^0C temperature (0.166 g g^{-1}).

Among the various substrates, sugarcane trash showed significantly the highest mean hemi cellulose content of 0.197 g per g, which was on par with that of bagasse and corn stover (0.196 g g^{-1}). The least hemi cellulose content was observed in sugarcane tops (0.181 g g^{-1}).

Particle size of 10.0 mm showed maximum mean hemi cellulose content of 0.214 g per g, which was significantly higher over 1.0 mm (0.187 g g^{-1}) and 0.5 mm (0.175 g g^{-1}).

The interaction between concentrations of alkali coupled with different temperatures of exposure and particle size were found to be significant with respect to hemi cellulose content. Treatment with 2.50% NaOH coupled with 121^0C temperature autoclaving showed the significantly highest hemi cellulose content for particle size 10.0 mm (0.235 g g^{-1}), which was on par with 1.0 mm particle size (0.221 g g^{-1}) and significantly higher over all other physico-chemical treatments and particle sizes. The treatment receiving 3.0% NaOH coupled with 125^0C exposure for particle of size 0.5 mm showed significantly least hemi cellulose content (0.143 g g^{-1}).

The interactions between the treatments alkali coupled with heat and substrates, treatment combinations of substrates and particle size and combination of all the three factors did not differ significantly. Although, these treatments were non-significant, the highest hemi cellulose content was observed in the treatment 2.50% NaOH coupled with 121^0C temperature exposure in case of particle size 10.00 mm for all the substrates. Sugarcane bagasse (0.247 g g^{-1}), sugarcane tops (0.220 g g^{-1}), sugarcane trash (0.233 g g^{-1}), corn husk (0.240 g g^{-1}) and corn stover (0.233 g g^{-1}) with a least reduction in hemi cellulose content of 15.70, 3.08, 5.67, 5.14 and 10.38 per cent, respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover. The lowest hemi cellulose content was observed in the temperature 3.00% NaOH coupled with 125^0C temperature exposure for the particle size 0.50 mm for all the substrates, sugarcane bagasse (0.147 g g^{-1}), sugarcane tops (0.133 g g^{-1}), sugarcane trash (0.153 g g^{-1}), corn husk (0.133 g g^{-1}) and corn stover (0.147 g g^{-1}) had shown highest reduction in hemi cellulose content of 49.83, 41.41, 38.06, 47.43 and 43.46 per cent, respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover.

Table 4: Effect of alkali and temperature pre-treatments on hemi cellulose content

Treatments	Particle size (mm)	Substrates ($g\ g^{-1}$) Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
2.50 % NaOH + 121⁰C temp.	0.5	0.220 (24.91)	0.193 (14.98)	0.213 (13.77)	0.207 (18.18)	0.213 (18.08)	0.222
	1.0	0.233 (20.48)	0.207 (8.81)	0.227 (8.10)	0.220 (13.04)	0.220 (15.38)	
	10.0	0.247 (15.70)	0.220 (3.08)	0.233 (5.67)	0.24 (05.14)	0.233 (10.38)	
3.00 % NaOH + 121⁰C temp.	0.5	0.180 (38.57)	0.173 (23.79)	0.193 (21.86)	0.180 (28.85)	0.193 (25.77)	0.199
	1.0	0.193 (34.13)	0.187 (17.62)	0.200 (19.03)	0.193 (23.72)	0.207 (20.38)	
	10.0	0.220 (24.91)	0.200 (11.89)	0.213 (13.77)	0.227 (10.28)	0.220 (15.38)	
2.50 % NaOH + 125⁰C temp	0.5	0.167 (43.00)	0.153 (32.60)	0.173 (29.96)	0.153 (39.53)	0.167 (35.77)	0.181
	1.0	0.173 (40.96)	0.167 (26.43)	0.187 (24.29)	0.167 (33.99)	0.173 (33.46)	
	10.0	0.213 (27.30)	0.193 (14.98)	0.207 (16.19)	0.213 (15.81)	0.213 (18.08)	
3.00 % NaOH + 125⁰C temp.	0.5	0.147 (49.83)	0.133 (41.41)	0.153 (38.06)	0.133 (47.43)	0.147 (43.46)	0.166
	1.0	0.160 (45.39)	0.153 (32.60)	0.167 (32.39)	0.147 (41.90)	0.160 (38.46)	
	10.0	0.193 (34.13)	0.187 (17.62)	0.193 (21.86)	0.207 (18.18)	0.207 (20.38)	
Mean		**0.196**	**0.181**	**0.197**	**0.191**	**0.196**	
Mean	0.5	0.175					
	1.0	0.187					
	10.0	0.214					

Figures in parentheses indicate per cent decrease in hemicellulose over the initial hemicellulose content of raw substrates

Note : Initial hemicellulose content ($g\ g^{-1}$) Sugarcane bagasse (0.293), Sugarcane tops (0.227), Sugarcane trash (0.247), Corn husk (0.253) and Corn stover (0.260)

Two way interaction A x C

Treatments \ Particle size (mm)	C1	C2	C3	Mean
2.50 % NaOH + 121⁰C temp.	0.209	0.221	0.235	**0.222**
3.00 % NaOH + 121⁰C temp.	0.184	0.196	0.216	**0.199**
2.50 % NaOH + 125⁰C temp.	0.163	0.173	0.208	**0.181**
3.00 % NaOH + 125⁰C temp.	0.143	0.157	0.197	**0.166**
Mean	**0.175**	**0.187**	**0.214**	

	SEm ±	CD at 1%
Treatments (A)	0.002	0.009
Substrates (B)	0.003	0.010
Particle size (C)	0.002	0.008
A x B	0.005	NS
A x C	0.004	0.015
B x C	0.005	NS
A x B x C	0.009	NS

4.2.4 Lignin content

The amount of lignin content present in the substrate due to individual treatment effects such as alkali and temperature, substrates and particle size varied significantly (Table 5). It was observed that lignin content had decreased from its initial value.

The treatment 2.5% alkali coupled with 121^0C temperature showed the highest mean lignin content of 0.062 g per g, which was significantly superior over all other physico-chemical treatments. The least lignin content was observed in case of 3.0% NaOH coupled with 125^0C temperature exposure (0.046 g g^{-1}).

The substrates sugarcane trash and corn stover were found to contain the highest mean lignin of 0.058 g per g each after the treatment. These values were observed to be on par with corn husk (0.056 g g^{-1}) and sugarcane tops (0.053 g g^{-1}), but had significantly higher lignin content over bagasse (0.047 g g^{-1}).

Significant differences in the lignin content were also observed in case of particle size. Highest lignin content was observed in particle size of 10.00 mm (0.071 g g^{-1}), which was significantly higher over 1.00 mm (0.050 g g^{-1}) and 0.50 mm (0.043 g g^{-1}) particle size.

All the two factor and three factor interactions were non-significant for lignin content, but the highest lignin content was observed in the treatment receiving 2.50% NaOH along with 121^0C temperature exposure for all the substrates at 10.00 mm particle size. However, these combinations of alkali with heat and particle size for various substrates showed least reduction in lignin content of 52.14, 49.04, 50.00, 42.80 and 43.14 per cent, respectively. The lowest lignin content was observed in case of 0.50 mm particle size treated 3.00% NaOH plus 125^0C temperature exposure, where the lignin content and per cent reduction observed were 0.027 g per g and 80.71% in sugarcane bagasse, 0.033 g per g and 78.98% in sugarcane tops, 0.040 g per g and 75.00% in sugarcane trash, 0.033 g per g and 76.43% in corn husk and 0.040 g per g and 73.76% in corn stover.

4.3 Effect of autohydrolysis with and without acid pre-treatment

4.3.1 Weight loss

The weight loss of the substrates as influenced by individual treatments such as different autohydrolysis with and without acid, different substrates and particle sizes differed significantly (Table 6). The interaction of factors with one another as well as their combinations also differed significantly.

Autohydrolysis at 200^0C for 10 min in presence of 1.0% H_2SO_4 resulted in highest mean weight loss of substrate. The loss of weight with this treatment was significantly the highest (0.465 g g^{-1}) over other three treatments such as autohydrolysis at 18^0C in presence of 1% H_2SO_4 (0.436 g g^{-1}), autohydrolysis at 200^0C (0.151 g g^{-1}) and autohydrolysis at 180^0C (0.129 g g^{-1}) temperature.

Corn husk showed the highest mean weight loss among the substrates, which was on par with sugarcane bagasse (0.301 g g^{-1}) but significantly higher over sugarcane bagasse (0.294 g g^{-1}), corn stover (0.290 g g^{-1}) and sugarcane trash (0.289 g g^{-1}) with respect to weight loss.

The particle size differed significantly with respect to weight loss. The highest mean weight loss was observed in the particle size 0.50 mm (0.331 g g^{-1}), which was significantly higher over particle size of 1.0 mm (0.301 g g^{-1}) and 10.00 mm (0.255 g g^{-1}).

The interaction between autohydrolysis (with and without acid) and substrates indicated a maximum weight loss for the treatment with 1.0% H_2SO_4 coupled with autohydrolysis at 200^0C for all the substrates, sugarcane bagasse (0.476 g g^{-1}), sugarcane tops (0.458), sugarcane trash (0.457 h/h), corn husk (0.458 g g^{-1}) and corn stover (0.458 g g^{-1}). This treatment was significantly highest over other three treatments, also all the treatments differed significantly with one another. The lowest weight loss of the substrates was recorded in the treatment autohydrolysis alone at 180^0C, where the observed weight loss was 0.124 for bagasse, 0.134 for sugarcane tops, 0.120 for sugarcane trash, 0.133 for corn husk and 0.131 g per g for corn stover.

Table 5: Effect of alkali and temperature pre-treatments on lignin content

Treatments	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	**Sugarcane tops**	**Sugarcane trash**	**Corn husk**	**Corn stover**	
2.50 % NaOH + 121^0C temp.	0.5	0.047 (66.43)	0.047 (70.06)	0.053 (66.88)	0.053 (61.14)	0.053 (65.36)	0.062
	1.0	0.053 (62.14)	0.053 (66.24)	0.060 (62.50)	0.060 (57.14)	0.060 (60.78)	
	10.0	0.067 (52.14)	0.080 (49.04)	0.080 (50.00)	0.080 (42.86)	0.087 (43.14)	
3.00 % NaOH + 121^0C temp.	0.5	0.033 (76.43)	0.040 (74.52)	0.047 (70.63)	0.047 (66.43)	0.047 (69.28)	0.054
	1.0	0.040 (71.43)	0.047 (70.06)	0.053 (66.88)	0.053 (62.14)	0.053 (65.36)	
	10.0	0.060 (57.14)	0.073 (53.50)	0.073 (54.38)	0.073 (47.86)	0.073 (52.29)	
2.50 % NaOH + 125^0C temp	0.5	0.040 (71.43)	0.040 (74.52)	0.047 (70.63)	0.047 (66.43)	0.047 (69.28)	0.055
	1.0	0.047 (66.43)	0.047 (70.06)	0.053 (66.88)	0.053 (62.14)	0.053 (65.36)	
	10.0	0.060 (57.14)	0.073 (53.50)	0.073 (54.38)	0.073 (47.86)	0.073 (52.29)	
3.00 % NaOH + 125^0C temp.	0.5	0.027 (80.71)	0.033 (78.98)	0.040 (75.00)	0.033 (76.43)	0.040 (73.86)	0.046
	1.0	0.033 (76.43)	0.040 (74.52)	0.047 (70.63)	0.040 (71.43)	0.047 (69.28)	
	10.0	0.053 (62.14)	0.067 (57.32)	0.067 (58.13)	0.060 (57.14)	0.067 (56.21)	
Mean		**0.047**	**0.053**	**0.058**	**0.056**	**0.058**	
Mean	0.5	0.043					
	1.0	0.050					
	10.0	0.071					

Figures in parentheses indicate per cent decrease in lignin over the initial lignin content of raw substrates

Note : Initial lignin content ($g\ g^{-1}$) Sugarcane bagasse (0.140), Sugarcane tops (0.157), Sugarcane trash (0.160), Corn husk (0.140) and Corn stover (0.153)

	SEm ±	**CD at 1%**
Treatments (A)	0.002	0.006
Substrates (B)	0.002	0.007
Particle size (C)	0.001	0.005
A x B	0.004	NS
A x C	0.003	NS
B x C	0.003	NS
A x B x C	0.006	NS

Table 6: Effect of autohydrolysis with and without acid pre-treatments on loss of weight of substrates

Treatments	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Autohydrolysis at 180^0C	0.5	0.143	0.147	0.137	0.147	0.143	0.129
	1.0	0.127	0.143	0.120	0.137	0.133	
	10.0	0.113	0.113	0.103	0.117	0.117	
Autohydrolysis at 200^0C	0.5	0.173	0.173	0.160	0.177	0.170	0.151
	1.0	0.153	0.153	0.147	0.157	0.157	
	10.0	0.130	0.123	0.127	0.130	0.133	
1.0 % H_2SO_4 + Autohydrolysis at 180^0C	0.5	0.495	0.489	0.486	0.516	0.496	0.436
	1.0	0.447	0.445	0.438	0.465	0.433	
	10.0	0.404	0.365	0.375	0.378	0.317	
1.0 % H_2SO_4 + Autohydrolysis at 200^0C	0.5	0.517	0.507	0.502	0.530	0.509	0.465
	1.0	0.469	0.465	0.483	0.485	0.458	
	10.0	0.441	0.403	0.387	0.419	0.409	
Mean		**0.301**	**0.294**	**0.289**	**0.305**	**0.290**	
Mean	0.5	0.331					
	1.0	0.301					
	10.0	0.255					

Two way interaction A x B

Particle size (mm) / Treatments	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
Autohydrolysis at 180^0C	0.128	0.134	0.120	0.133	0.131	**0.129**
Autohydrolysis at 200^0C	0.152	0.150	0.144	0.154	0.153	**0.151**
1.0 % H_2SO_4 + Autohydrolysis at 180^0C	0.448	0.433	0.433	0.453	0.416	**0.436**
1.0 % H_2SO_4 + Autohydrolysis at 200^0C	0.476	0.458	0.457	0.478	0.458	**0.465**
Mean	**0.301**	**0.294**	**0.289**	**0.305**	**0.290**	

Contd…..

Two way interaction A x C

Treatments \ Particle size	0.5 mm	1.0 mm	10.0 mm	Mean
Autohydrolysis at 180^{0}C	0.143	0.132	0.113	**0.129**
Autohydrolysis at 200^{0}C	0.171	0.153	0.129	**0.151**
1.0 % H_2SO_4 + Autohydrolysis at 180^{0}C	0.496	0.445	0.368	**0.436**
1.0 % H_2SO_4 + Autohydrolysis at 200^{0}C	0.513	0.472	0.411	**0.465**
Mean	**0.331**	**0.301**	**0.255**	

Two way interaction B x C

Particle size \ Substrates	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
0.5 mm	0.332	0.329	0.321	0.342	0.330	**0.331**
1.0 mm	0.299	0.302	0.297	0.311	0.295	**0.301**
10.0 mm	0.272	0.251	0.248	0.261	0.244	**0.255**
Mean	**0.301**	**0.294**	**0.289**	**0.305**	**0.290**	

	SEm $\pm$	CD at 1%
Treatments (A)	0.002	0.006
Substrates (B)	0.002	0.007
Particle size (C)	0.001	0.005
A x B	0.004	0.013
A x C	0.003	0.010
B x C	0.003	0.011
A x B x C	0.006	0.023

The interaction between autohydrolysis (with and without acid) and particle size resulted in maximum weight loss in particles with 0.50 mm. Treatment with 1.00% H_2SO_4 plus autohydrolysis at 200^0C showed the highest loss of weight (0.513 g g^{-1}) followed by particles with 1.00 mm (0.472 g g^{-1}) and 10.00 mm (0.411 g g^{-1}). This treatment was significantly superior over all other treatments and all the treatments differed significantly with one another.

The interaction between particle size and substrates indicated a significant variation in loss of weight. The highest loss of weight was observed in 0.50 mm particle size for corn husk (0.342 g g^{-1}), which was significantly the highest over other substrates such as sugarcane bagasse (0.332 g g^{-1}), corn stover (0.330 g g^{-1}), sugarcane tops (0.329 g g^{-1}) and sugarcane trash (0.321 g g^{-1}). The loss of weight observed in 0.50 mm was significantly highest for all the substrates with 1.00 mm and 10.00 mm particle size.

Combined interaction of all the three factors resulted in significantly highest loss of weight in substrate for 0.50 mm particle size treated with 1.0% H_2SO_4 coupled with autohydrolysis at 200^0C temperature. The loss of weight for sugarcane bagasse was 0.517, sugarcane tops 0.507, sugarcane trash 0.502, corn husk 0.530 and corn stover 0.507. This treatment combination was found to be significantly higher t over other treatment combinations. Significantly the lowest loss of weight was observed in the treatment combination of autohydrolysis at 180^0C for particle size 0.50 mm in all the substrates.

4.3.2 Cellulose content

The data pertaining to cellulose content due to pre-treatment is presented in Table 7. The cellulose content varied differently due to individual factors such as autohydrolysis (with and without acid), substrates and particle sizes.

Autohydrolysis with 1.0% H_2SO_4 at 200^0C resulted in the recovery of the highest amount of mean cellulose content (0.583 g g^{-1}). This treatment was significantly superior over autohydrolysis with 1.0% H_2SO_4 at 180^0C (0.503 g g^{-1}) and over autohydrolysis in absence of acid at both 200^0C (0.417 g g^{-1}) and 180^0C (0.378 g g^{-1}) temperature.

Among the substrates subjected for pre-treatment, the higher mean cellulose recovery was recorded in sugarcane bagasse (0.489 g g^{-1}). Sugarcane bagasse showed significantly higher differences with respect to cellulose content over rest of the substrates. The cellulose content in other four substrates varied between 0.468 and 0.463 g per g, these substrates were at par with each other.

The main factor, particle size also differed significantly. Particle size of 0.50 mm resulted in the highest cellulose content of 0.498 g per g, which was significantly higher over those with 1.00 mm (0.480 g g^{-1}) and 10.00 mm (0.432 g g^{-1}) particle size.

The interaction between autohydrolysis either in presence or in absence of acid at different temperatures and particle sizes were also observed to vary significantly. The combination of 1.0% H_2SO_4 and autohydrolysis at 200^0C steam temperature resulted in the recovery of highest cellulose content in 0.50 mm particle sized substrates (0.629 g g^{-1}), which was significantly superior over 1.00 mm (0.601 g g^{-1}) and 10.00 mm (0.517 g g^{-1}) particle size. This treatment was also found to be significantly higher in cellulose content over other treatments such as those with 1% H_2SO_4 plus autohydrolysis at 180^0C and autohydrolysis in absence of acid either at 200^0C or at 180^0C irrespective of the particle sizes.

The two way interaction between the treatments autohydrolysis with and without acid and substrate, substrates and particle sizes and combined interaction of all the three factors were observed to be non-significant.

The three way interaction although found to be insignificant for cellulose content. It could be noted that the highest cellulose content was found in the treatment 1.0% H_2SO_4 coupled with autohydrolysis at 200^0C temperature in the particle size 0.5 mm. At this combination the cellulose content and the per cent increase in cellulose of the substrates observed was 0.647 g per g and 83.29 per cent in sugarcane bagasse, 0.627 g per g and 88.29 per cent in sugarcane tops, 0.627 g per g and 82.80 per cent in sugarcane trash, 0.620 g per g and 86.19 per cent in corn husk and 0.627 g per g and 91.71 per cent in corn stover, respectively.

Table 7: Effect of autohydrolysis with and without acid pre-treatments on cellulose content of substrates

Treatments	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Autohydrolysis at 180^0C	0.5	0.427 (20.96)	0.387 (16.22)	0.393 (14.58)	0.387 (16.22)	0.380 (16.21)	0.378
	1.0	0.407 (15.30)	0.373 (12.01)	0.387 (12.83)	0.373 (12.01)	0.373 (14.07)	
	10.0	0.373 (05.67)	0.353 (06.01)	0.353 (02.92)	0.353 (06.01)	0.353 (07.95)	
Autohydrolysis at 200^0C	0.5	0.460 (30.31)	0.433 (30.03)	0.433 (26.24)	0.433 (30.03)	0.427 (30.58)	0.417
	1.0	0.447 (26.63)	0.413 (24.02)	0.420 (22.45)	0.413 (24.02)	0.413 (26.30)	
	10.0	0.407 (15.30)	0.393 (18.02)	0.400 (16.62)	0.387 (16.22)	0.373 (14.07)	
1.0 % H_2SO_4 + Autohydrolysis at 180^0C	0.5	0.553 (56.66)	0.520 (56.16)	0.527 (53.64)	0.527 (58.26)	0.533 (63.00)	0.503
	1.0	0.533 (50.99)	0.507 (52.25)	0.507 (47.81)	0.513 (54.05)	0.513 (56.88)	
	10.0	0.473 (33.99)	0.453 (36.04)	0.460 (34.11)	0.467 (40.24)	0.460 (40.67)	
1.0 % H_2SO_4 + Autohydrolysis at 200^0C	0.5	0.647 (83.29)	0.627 (88.29)	0.627 (82.80)	0.620 (86.19)	0.627 (91.71)	0.583
	1.0	0.613 (73.65)	0.593 (78.08)	0.600 (74.93)	0.593 (78.08)	0.607 (85.63)	
	10.0	0.533 (50.99)	0.507 (52.25)	0.513 (49.56)	0.513 (54.05)	0.520 (59.02)	
Mean		**0.489**	**0.463**	**0.468**	**0.465**	**0.465**	
Mean	0.5	0.498					
	1.0	0.480					
	10.0	0.432					

Figures in parentheses indicate per cent increase in cellulose over the initial cellulose content of raw substrates

Note : Initial cellulose content ($g\ g^{-1}$) Sugarcane bagasse (0.353), Sugarcane tops (0.333), Sugarcane trash (0.343), Corn husk (0.333) and Corn stover (0.327)

Two way interaction A x C

A \ C	0.5 mm	1.0 mm	10.0 mm	Mean
Autohydrolysis at 180^0C	0.395	0.383	0.357	**0.378**
Autohydrolysis at 200^0C	0.437	0.421	0.392	**0.417**
1.0 % H_2SO_4 + Autohydrolysis at 180^0C	0.532	0.515	0.463	**0.503**
1.0 % H_2SO_4 + Autohydrolysis at 200^0C	0.629	0.601	0.517	**0.583**
Mean	**0.498**	**0.480**	**0.432**	

	SEm $\pm$	CD at 1%
Treatments (A)	0.002	0.007
Substrates (B)	0.002	0.008
Particle size (C)	0.002	0.006
A x B	0.004	NS
A x C	0.003	0.013
B x C	0.004	NS
A x B x C	0.008	NS

The lowest cellulose content with the least increase in cellulose content was observed in the treatment autohydrolysis at 180^{0}C temperature for the particle size 10.00 mm in all the substrates. This combination ranged in cellulose content from 0.353 to 0.373 g per g with a per cent increase in cellulose content from 2.92 to 7.95 per cent.

4.3.3 Hemicellulose content

The data concerned to the hemi cellulose content of the pre-treatment substrates indicated a significant variation due to individual effects such as autohydrolysis in presence or absence of acid, substrates and their particle size. The interaction between the factors autohydrolysis in presence or absence of acid and substrates as well as the factors autohydrolysis in presence or absence of acid and particle size also showed significant variations. The interaction between substrates and particle size and three way interaction of all the three factors found to be insignificant (Table 8).

The treatment with 1% H_2SO_4 and autohydrolysis at 200^{0}C temperature showed significantly the lowest mean hemi cellulose content (0.148 g g^{-1}). This treatment was significantly lower to the treatment with 1.00% H_2SO_4 followed by autohydrolysis at 180^{0}C (0.170 g g^{-1}) and the treatments steam explosion in absence of acid either at 200^{0}C (0.212 g g^{-1}) or 180^{0}C (0.228 g g^{-1}).

The various substrates subjected for autohydrolysis pre-treatments differed significantly with one another. The highest mean hemi cellilose content was observed in bagasse (0.210 g g^{-1}), which was significantly superior over all other substrates. Where, the hemi cellulose content ranged between the lowest (0.174 g g^{-1}) in sugarcane tops and the highest (0.210 g g^{-1}) in sugarcane bagasse.

The lowest mean hemi cellulose content for the particle size was observed in case of 0.5 mm particle size which was significantly lower than those with 1.00 mm (0.185 g g^{-1}) and 10.00 mm (0.209 g g^{-1}) particle size.

The combined interaction effect of autohydrolysis in presence or absence of acid and substrates resulted in significant variation for hemi cellulose content. Treatment with 1.0% H_2SO_4 and autohydrolysis at 200^{0}C recorded significantly the lowest hemi cellulose content 0.169 g per g for sugarcane bagaase, 0.142 forsugarcane tops, 0.144 for sugarcane trash, 0.142 for corn husk and 0.144 g per g for corn stover. These treatments were found to be significantly lower than the treatments with 1.0% H_2SO_4 plus autohydrolysis at 180^{0}C and autohydrolysis without acid. Significantly the highest hemi cellulose content in the range of 0.204 in case of sugarcane tops and 0.251 g per g in case of sugarcane bagasse were observed for the substrates receiving only autohydrolysis at 180^{0}C.

The interaction between autohydrolysis in presence or absence of acid and particle size indicated significantly lower hemi cellulose content for 0.50 mm particle size (0.132 g g^{-1}) followed by 1.00 mm (0.140 g g^{-1}) in the treatment 1% H_2SO_4 plus autohydrolysis at 200^{0}C. Higher hemi cellulose content of 0.219 (0.50 mm), 0.227 (1.00 mm) and 0.237 g per g (10.00 mm) was obtained with the treatment autohydrolysis at 180^{0}C temperature.

The three way interactions were however non-significant for hemi cellulose content, it indicated that the per cent decrease in hemi cellulose was the highest in the particle size, which was subjected for 1% H_2SO_4 plus autohydrolysis at 200^{0}C. A maximum reduction in the hemi cellulose content of 47.78, 44.05, 48.58, 49.80 and 51.15 per cent was recorded in this combination for the substrates sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover, respectively. The least reduction in hemi cellulose content with a range from 5.67 to 8.87 per cent was observed with the treatment autohydrolysis at 180^{0}C temperature for the particle size 10.00 mm in all the substrates.

4.3.4 Lignin content

The quantity of lignin content present in the substrates after the pre-treatment with autohydrolysis in presence or absence of acid is presented in Table 9. The individual factors such as autohydrolysis with or without acid, substrates and particle sizes differed significantly with one another, whereas the two way interactions and three way interactions among the factors did not vary significantly.

Table 8: Effect of autohydrolysis with and without acid pre-treatments on hemi cellulose content of substrates

Treatments	Particle size (mm)	Substrates (g g^{-1})					Mean
		Sugarcane bagasse	**Sugarcane tops**	**Sugarcane trash**	**Corn husk**	**Corn stover**	
Autohydrolysis at 180^0C	0.5	0.240 (18.09)	0.193 (14.98)	0.213 (13.77)	0.220 (13.04)	0.227 (12.69)	0.228
	1.0	0.247 (15.70)	0.207 (08.81)	0.220 (10.93)	0.227 (10.28)	0.233 (10.38)	
	10.0	0.267 (08.87)	0.213 (06.17)	0.23 (05.67)	0.23 (07.91)	0.240 (07.69)	
Autohydrolysis at 200^0C	0.5	0.213 (27.30)	0.180 (20.70)	0.193 (21.86)	0.207 (18.18)	0.213 (18.08)	0.212
	1.0	0.227 (22.53)	0.187 (17.62)	0.207 (16.19)	0.213 (15.81)	0.220 (15.38)	
	10.0	0.247 (15.70)	0.200 (11.89)	0.213 (13.77)	0.23 (07.91)	0.233 (10.38)	
1.0 % H_2SO_4 + Autohydrolysis at 180^0C	0.5	0.173 (40.96)	0.140 (38.33)	0.153 (38.06)	0.140 (44.66)	0.147 (43.46)	0.170
	1.0	0.180 (38.57)	0.153 (32.60)	0.167 (32.39)	0.153 (39.53)	0.153 (41.15)	
	10.0	0.220 (24.91)	0.187 (17.62)	0.193 (21.86)	0.193 (23.72)	0.200 (23.08)	
1.0 % H_2SO_4 + Autohydrolysis at 200^0C	0.5	0.153 (47.78)	0.127 (44.05)	0.127 (48.58)	0.127 (49.80)	0.127 (51.15)	0.148
	1.0	0.167 (43.00)	0.133 (41.41)	0.133 (46.15)	0.133 (47.43)	0.133 (48.85)	
	10.0	0.187 (36.18)	0.167 (26.43)	0.173 (29.96)	0.167 (33.99)	0.173 (33.46)	
Mean		**0.210**	**0.174**	**0.186**	**0.187**	**0.192**	
Mean	0.5	0.176					
	1.0	0.185					
	10.0	0.209					

Figures in parentheses indicate per cent decrease in hemicellulose over the initial hemicellulose content of raw substrates

Note : Initial hemicellulose content (g g^{-1}) Sugarcane bagasse (0.293), Sugarcane tops (0.227), Sugarcane trash (0.247), Corn husk (0.253) and Corn stover (0.260)

Contd…..

Two way interaction A x B

Treatments \ Substrates	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
Autohydrolysis at 180^{0}C	0.251	0.204	0.222	0.227	0.233	**0.228**
Autohydrolysis at 200^{0}C	0.229	0.189	0.204	0.218	0.222	**0.212**
1.0 % H_2SO_4 + Autohydrolysis at 180^{0}C	0.191	0.160	0.171	0.162	0.167	**0.170**
1.0 % H_2SO_4 + Autohydrolysis at 200^{0}C	0.169	0.142	0.144	0.142	0.144	**0.148**
Mean	**0.210**	**0.174**	**0.186**	**0.187**	**0.192**	

Two way interaction A x C

Treatments \ Particle size	0.5 mm	1.0 mm	10.0 mm	Mean
Autohydrolysis at 180^{0}C	0.219	0.227	0.237	**0.228**
Autohydrolysis at 200^{0}C	0.201	0.211	0.225	**0.212**
1.0 % H_2SO_4 + Autohydrolysis at 180^{0}C	0.151	0.161	0.199	**0.170**
1.0 % H_2SO_4 + Autohydrolysis at 200^{0}C	0.132	0.140	0.173	**0.148**
Mean	**0.176**	**0.185**	**0.209**	

	SEm $\pm$	CD at 1%
Treatments (A)	0.002	0.006
Substrates (B)	0.002	0.007
Particle size (C)	0.001	0.005
A x B	0.004	0.014
A x C	0.003	0.010
B x C	0.003	NS
A x B x C	0.006	NS

Table 9: Effect of autohydrolysis with and without acid pre-treatments on lignin content of substrates

Treatments	Particle size (mm)	Substrates (g g^{-1})					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Autohydrolysis at 180^0C	0.5	0.120 (14.29)	0.133 (15.29)	0.147 (08.13)	0.113 (19.29)	0.133 (13.07)	0.136
	1.0	0.127 (09.29)	0.140 (10.83)	0.147 (08.13)	0.127 (09.29)	0.133 (13.07)	
	10.0	0.133 (05.00)	0.147 (06.37)	0.153 (04.38)	0.133 (05.00)	0.147 (03.92)	
Autohydrolysis at 200^0C	0.5	0.107 (23.57)	0.113 (28.03)	0.113 (29.38)	0.107 (23.57)	0.113 (26.14)	0.119
	1.0	0.113 (19.29)	0.120 (23.57)	0.120 (25.00)	0.113 (19.29)	0.120 (21.57)	
	10.0	0.127 (09.29)	0.133 (15.29)	0.140 (12.50)	0.120 (14.29)	0.127 (16.99)	
1.0 % H_2SO_4 + Autohydrolysis at 180^0C	0.5	0.053 (62.14)	0.067 (57.32)	0.073 (54.38)	0.053 (61.14)	0.060 (60.78)	0.073
	1.0	0.060 (57.14)	0.073 (53.50)	0.080 (50.00)	0.067 (52.14)	0.073 (52.29)	
	10.0	0.073 (47.86)	0.093 (40.76)	0.093 (41.88)	0.087 (37.86)	0.093 (39.22)	
1.0 % H_2SO_4 + Autohydrolysis at 200^0C	0.5	0.027 (80.71)	0.033 (78.98)	0.047 (70.63)	0.040 (71.43)	0.040 (73.86)	0.050
	1.0	0.033 (76.43)	0.047 (70.06)	0.053 (66.88)	0.053 (62.14)	0.047 (69.28)	
	10.0	0.053 (62.14)	0.067 (57.32)	0.067 (58.13)	0.073 (47.86)	0.073 (52.29)	
Mean		**0.086**	**0.097**	**0.103**	**0.091**	**0.097**	
Mean	0.5	0.085					
	1.0	0.092					
	10.0	0.107					

Figures in parentheses indicate per cent decrease in lignin over the initial lignin content of raw substrates

Note : Initial lignin content (g g^{-1}) Sugarcane bagasse (0.140), Sugarcane tops (0.157), Sugarcane trash (0.160), Corn husk (0.140) and Corn stover (0.153)

	SEm ±	**CD at 1%**
Treatments (A)	0.002	0.006
Substrates (B)	0.002	0.007
Particle size (C)	0.001	0.005
A x B	0.004	NS
A x C	0.003	NS
B x C	0.003	NS
A x B x C	0.006	NS

The treatment with 1% H_2SO_4 plus autohydrolysis at 200^{0}C steam temperature resulted in significantly lower mean lignin content (0.050 g g^{-1}) followed by treatments with 1% H_2SO_4 plus autohydrolysis at 180^{0}C (0.073 g g^{-1}), autohydrolysis either at 200^{0}C (0.119 g g^{-1}) or at 180^{0}C (0.136 g g^{-1}).

Lower mean lignin content of 0.086 g per g was observed in case of substrate sugarcane bagasse which was on par with corn stover (0.091 g g^{-1}) but significantly lower than sugarcane tops and corn stover (0.097 g g^{-1}) and sugarcane trash (0.103 g g^{-1}).

The particle size also differed significantly with each other. The lowest and significantly the least mean hemi cellulose content (0.085 g g^{-1}) was observed with particle size of 0.50 mm, followed by 1.00 mm (0.092 g g^{-1}) and 10.00 mm (0.107 g g^{-1}). These particle sizes differed significantly over one another.

The combined interaction of all the three factors was insignificant but a maximum reduction in lignin content was observed in the treatment with 1% H_2SO_4 plus autohydrolysis at 200^{0}C temperature for all the substrates, where in particle size 0.50 mm, reduction in lignin content of 80.71, 78.98, 70.63, 71.43 and 73.80 per cent, respectively was recorded for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover.

4.4 Effect of lignolytic fungi inoculation pre-treatment

4.4.1 Weight loss

The data pertaining to loss in weight of substrates due to treatment by lignolytic fungi is presented in Table 10. Significant variations were observed due to individual effects such as microbial pre-treatment for different days, different substrates and their particle size. The interaction of each of these factors with other as well as their combined interactions were also significant with respect to weight loss.

With regards to effect of lignolytic fungi inoculation in weight loss, the lignolytic fungi showed significant differences at different incubation periods. The mean maximum loss of weight was observed with *P. chrysosporium* at 30 days incubation period with a value of 0.125 g per g. It was found to be on par with *P. florida* (0.124 g g^{-1}) and significantly superior over UASD-LF1 (0.119 g g^{-1}) at 30 days incubation period with regards to weight loss. The weight loss observed at 30 days incubation was found to be significantly superior over 20 days and 10 days incubation in all the lignolytic fungi. The mean lowest weight loss was observed with control treatment (0.08 g g^{-1}) at all incubation periods studied.

Among the different substrates pre-treated with lignolytic fungi, corn husk showed the mean maximum weight loss, 0.093 g per g. It was found to be significantly highest over all other substrates. The sugarcane bagasse with mean weight loss of 0.054 g per g resulted in significantly lowest weight loss. All the particle sizes of the substrates exhibited significant differences with each other in mean weight loss. The particle size 0.50 mm recorded significantly maximum mean weight loss of 0.095 g per g as compared to 1.0 mm (0.085 g g^{-1}) and 10.0 mm (0.066 g g^{-1}) particle size.

The interaction between different days to incubation with lignolytic fungi and substrates showed significant differences in weight loss. *P. chrysosporium* showed the highest weight loss of 0.143 g per g at 30 days incubation in case of corn husk. It was found to be on par with corn stover (0.136 g g^{-1}), sugarcane trash (0.135 g g^{-1}) with the same combination of fungi and incubation period as well as with *P. florida* (0.135 g g^{-1}) at 30 days incubation period in corn husk but significantly superior over rest of the combinations of lignolytic fungi and substrates at all incubation periods. *P chrysosporium* recorded the highest weight loss in sugarcane tops (0.133 g g^{-1}), sugarcane trash (0.135 g g^{-1}), corn husk (0.143 g g^{-1}) and corn stover (0.136 g g^{-1}) at 30 days incubation, however they were on par with *P. florida* and UASD-LF1 in respective substrates at 30 days incubation but for corn husk, where the *P. chrysosporium* differed significantly over other two lignolytic fungi with respect to weight loss. In sugarcane bagasse, the highest weight loss was observed with *P. florida* (0.101 g g^{-1}), it was found to be significantly superior over *P. chrysosporium* and UASD-LF1 with respect to weight loss. The weight loss observed in lignolytic fungi at 30 days incubation was found to be superior over 20 days and 10 days incubation period for all the substrates.

Table 10: Effect of lignolytic fungi on weight loss of substrates

Lignolytic fungi	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.052	0.130	0.123	0.135	0.125	**0.099**
	1.0	0.049	0.107	0.115	0.115	0.124	
	10.0	0.032	0.085	0.088	0.109	0.102	
Pleurotus florida (10 days)	0.5	0.061	0.126	0.103	0.131	0.121	**0.092**
	1.0	0.054	0.099	0.091	0.117	0.107	
	10.0	0.035	0.080	0.070	0.099	0.085	
UASD-LF1 (10 days)	0.5	0.053	0.121	0.095	0.109	0.100	**0.081**
	1.0	0.045	0.097	0.086	0.093	0.092	
	10.0	0.029	0.077	0.061	0.083	0.079	
Control (10 days)	0.5	0.010	0.007	0.010	0.010	0.010	**0.008**
	1.0	0.010	0.010	0.010	0.010	0.010	
	10.0	0.002	0.007	0.003	0.003	0.001	
Phanerochaete chrysosporium (20 days)	0.5	0.064	0.117	0.142	0.139	0.136	**0.109**
	1.0	0.063	0.109	0.133	0.128	0.128	
	10.0	0.039	0.098	0.108	0.117	0.111	
Pleurotus florida (20 days)	0.5	0.101	0.139	0.139	0.132	0.126	**0.108**
	1.0	0.081	0.117	0.128	0.117	0.112	
	10.0	0.058	0.087	0.096	0.101	0.087	
UASD-LF1 (20 days)	0.5	0.104	0.137	0.140	0.127	0.125	**0.108**
	1.0	0.084	0.123	0.127	0.113	0.113	
	10.0	0.061	0.082	0.097	0.099	0.081	
Control (20 days)	0.5	0.010	0.007	0.010	0.010	0.010	**0.008**
	1.0	0.010	0.010	0.010	0.010	0.010	
	10.0	0.002	0.007	0.003	0.003	0.001	
Phanerochaete chrysosporium (30 days)	0.5	0.095	0.151	0.159	0.159	0.144	**0.125**
	1.0	0.079	0.137	0.150	0.143	0.139	
	10.0	0.059	0.113	0.095	0.127	0.126	
Pleurotus florida (30 days)	0.5	0.129	0.142	0.156	0.150	0.141	**0.124**
	1.0	0.100	0.134	0.146	0.137	0.129	
	10.0	0.074	0.099	0.101	0.118	0.111	
UASD-LF1 (30 days)	0.5	0.117	0.143	0.152	0.147	0.137	**0.119**
	1.0	0.093	0.128	0.139	0.133	0.120	
	10.0	0.063	0.103	0.097	0.116	0.096	
Control (30 days)	0.5	0.010	0.007	0.010	0.010	0.010	**0.008**
	1.0	0.010	0.010	0.010	0.010	0.010	
	10.0	0.002	0.007	0.003	0.003	0.001	
Mean		**0.054**	**0.088**	**0.089**	**0.093**	**0.088**	
Mean	0.5	0.095					
	1.0	0.085					
	10.0	0.066					

Contd

Two way table for interaction (A × B).....

Lignolytic fungi \ Substrates	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
Phanerochaete chrysosporium (10 days)	0.044	0.107	0.109	0.120	0.117	**0.099**
Pleurotus florida (10 days)	0.050	0.102	0.088	0.116	0.104	**0.092**
UASD-LF1 (10 days)	0.042	0.098	0.081	0.095	0.090	**0.081**
Control (10 days)	0.007	0.008	0.008	0.008	0.007	**0.008**
Phanerochaete chrysosporium (20 days)	0.055	0.108	0.128	0.128	0.125	**0.109**
Pleurotus florida (20 days)	0.080	0.114	0.121	0.117	0.108	**0.108**
UASD-LF1 (20 days)	0.083	0.114	0.121	0.113	0.106	**0.108**
Control (20 days)	0.007	0.008	0.008	0.008	0.007	**0.008**
Phanerochaete chrysosporium (30 days)	0.078	0.133	0.135	0.143	0.136	**0.125**
Pleurotus florida (30 days)	0.101	0.125	0.134	0.135	0.127	**0.124**
UASD-LF1 (30 days)	0.091	0.125	0.129	0.132	0.118	**0.119**
Control (30 days)	0.007	0.008	0.008	0.008	0.007	**0.008**
Means for substrates	0.054	0.088	0.089	0.093	0.088	

Contd…..

Two way table for interaction (A × C)

Lignolytic fungi \ Particle size	0.5 mm	1.0 mm	10 mm	Mean
Phanerochaete chrysosporium (10 days)	0.113	0.102	0.083	**0.099**
Pleurotus florida (10 days)	0.108	0.094	0.074	**0.092**
UASD-LF1 (10 days)	0.096	0.082	0.066	**0.081**
Control (10 days)	0.009	0.010	0.003	**0.008**
Phanerochaete chrysosporium (20 days)	0.119	0.112	0.095	**0.109**
Pleurotus florida (20 days)	0.128	0.111	0.086	**0.108**
UASD-LF1 (20 days)	0.127	0.112	0.084	**0.108**
Control (20 days)	0.009	0.010	0.004	**0.008**
Phanerochaete chrysosporium (30 days)	0.142	0.129	0.104	**0.125**
Pleurotus florida (30 days)	0.144	0.129	0.100	**0.124**
UASD-LF1 (30 days)	0.139	0.122	0.095	**0.119**
Control (30 days)	0.009	0.010	0.003	**0.008**
Mean	0.095	0.085	0.066	

Two way table for interaction (B × C)

Particle size \ Substrates	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
0.5 mm	0.067	0.102	0.103	0.105	0.099	**0.095**
1.0 mm	0.056	0.090	0.095	0.094	0.091	**0.085**
10 mm	0.038	0.070	0.069	0.082	0.073	**0.066**
Mean	**0.054**	**0.088**	**0.089**	**0.093**	**0.088**	

	SE±	CD 1 %
Lignolytic fungi (A)	0.001	0.004
Substrates (B)	0.001	0.002
Particle size (C)	0.000	0.002
A x B	0.002	0.008
A x C	0.002	0.006
B x C	0.001	0.004
A x B x C	0.004	0.014

Plate 4a. Biologically pre-treated substrates with various fungi

Plate 4b. Biologically pre-treated substrates with *Phanerochaete chrysosporium* at 30 days incubation period

Significant differences in weight loss were observed due to combined effect of lignolytic fungi and particle size of substrates. The highest weight loss of substrates was observed in *P. florida* (0.144 g g^{-1}) for the particle size 0.5 mm and it was on par with *P. chrysosporium* (0.142 g g^{-1}) and UASD-LF1 (0.139 g g^{-1}) at 30 days incubation period. The weight loss recorded at 30 days incubation with different lignolytic fungi for the particle size 0.50 mm was found to significantly superior over particle size of both 1.0 mm and 10.0 mm. Also, the weight loss observed at 30 days incubation for different particle sizes were found to be superior over 20 days and 10 days incubation for respective particle sizes with regards to weight loss.

The weight loss as influenced by particle size of different substrates indicated significant differences. The highest weight loss was observed in corn husk (0.105 g g^{-1}) for the substrate sugarcane trash (0.103 g g^{-1}) and sugarcane tops (0.102 g g^{-1}). It was found to be significantly superior over corn stover (0.099 g g^{-1}), sugarcane bagasse (0.067 g g^{-1}) with respect to weight loss in particle size of 0.50 mm. The weight loss recorded in 0.5 mm particle size of different substrates were highly significant over 1.0 mm and 10.0 mm particle sizes within the respective substrates. The 10.0 mm particle size had resulted in significantly lowest weight loss for respective substrates.

The combined effect of lignolytic fungi inoculation, different substrates and their particle sizes had indicated significant variations with regards to weight loss. The significant weight loss, 0.159 g per g was recorded by *P. chrysosporium* in both sugarcane trash and corn husk at 30 days of incubation. It was found to be on par with sugarcane tops (0.151 g g^{-1}) and corn stover (0.144 g g^{-1}) treated with *P. chrysosporium* with *P. florida* in sugarcane trash (0.156 g g^{-1}) and corn husk (0.150 g g^{-1}) and also with UASD-LF1 in 30 days incubation period. The weight loss of 0.159 g per g observed with *P. chrysosporium* in corn husk was significantly superior over rest of the substrates inoculated with different lignolytic fungi at all the incubation periods (30, 20 and 10 days) except for sugarcane trash and corn husk. Both sugarcane trash and corn husk showed highest weight loss with lignolytic fungi, *P. florida* and UASD-LF1 inoculation at 30 days incubation. The significantly lowest weight loss was observed with uninoculated control at all the incubation periods with respect to weight loss.

The experimental set up of lignolytic pre-treatment and the growth of *P. chrysosporium* on different substrates are depicted in Plate 4a and 4b.

4.4.2 Cellulose content

The data on cellulose content of different substrates as influenced by lignolytic fungi pre-treatments is presented in Table 11. The lignolytic fungi inoculation, different substrates, particle sizes and the interaction effect between lignolytic fungi and particle size of substrates indicated significant differences in cellulose content of the substrates. However, the interaction between lignolytic fungi inoculation and substrates, substrates and particle size and combined interaction between lignolytic fungi, substrates and the particle size did not differ significantly with regards to cellulose content.

The inoculation of lignolytic fungi influenced the cellulose content. The mean maximum cellulose content (0.396 g g^{-1}) was observed with inoculation of *P. chrysosporium* at 30 days incubation, followed by both *P. florida* and UASD-LF1 (0.393 g g^{-1}) at 30 days incubation. All the three lignolytic fungi were found to be on par with each other at 30 days incubation and significantly superior over 20 days and 10 days incubation with regards to cellulose content. The significantly lowest mean cellulose content, 0.329 g per g was recorded with uninoculated control.

The cellulose content was found to vary differently with different substrates. The mean maximum cellulose content of 0.377 g per g was noticed in sugarcane bagasse. It was observed to be significantly superior over other substrates. The rest of the substrates with a range of maximum (0.367 g g^{-1}) and minimum (0.363 g g^{-1}) cellulose content found to be on par with each other.

The particle size of substrates indicated significant variation in cellulose content. The mean maximum cellulose content, 0.376 g per g was observed with 0.5 mm particle size and it was significantly superior over particle size of 1.0 mm (0.368 g g^{-1}) and 10.00 mm (0.356 g g^{-1}) particle size with regards to cellulose content. All the three particle sizes were found to be significantly different from each other.

Table 11: Effect of lignolytic fungi on cellulose content of substrates

Lignolytic fungi	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.380	0.360	0.367	0.373	0.360	**0.358**
	1.0	0.367	0.360	0.360	0.360	0.347	
	10.0	0.360	0.340	0.347	0.347	0.340	
Pleurotus florida (10 days)	0.5	0.380	0.367	0.367	0.367	0.360	**0.357**
	1.0	0.367	0.360	0.360	0.360	0.347	
	10.0	0.353	0.340	0.340	0.347	0.340	
UASD-LF1 (10 days)	0.5	0.380	0.357	0.360	0.367	0.360	**0.355**
	1.0	0.367	0.360	0.347	0.360	0.353	
	10.0	0.360	0.337	0.340	0.347	0.337	
Control (10 days)	0.5	0.353	0.333	0.340	0.330	0.323	**0.336**
	1.0	0.353	0.333	0.337	0.330	0.333	
	10.0	0.350	0.333	0.340	0.330	0.327	
Phanerochaete chrysosporium (20 days)	0.5	0.400	0.387	0.387	0.403	0.387	**0.381**
	1.0	0.393	0.377	0.380	0.380	0.380	
	10.0	0.377	0.373	0.363	0.370	0.363	
Pleurotus florida (20 days)	0.5	0.403	0.397	0.390	0.403	0.400	**0.384**
	1.0	0.397	0.390	0.377	0.387	0.387	
	10.0	0.377	0.357	0.357	0.370	0.363	
UASD-LF1 (20 days)	0.5	0.397	0.387	0.387	0.397	0.403	**0.383**
	1.0	0.390	0.383	0.377	0.390	0.387	
	10.0	0.377	0.357	0.363	0.377	0.373	
Control (20 days)	0.5	0.353	0.333	0.340	0.330	0.323	**0.336**
	1.0	0.353	0.333	0.337	0.330	0.333	
	10.0	0.350	0.333	0.340	0.330	0.327	
Phanerochaete chrysosporium (30 days)	0.5	0.427	0.407	0.400	0.413	0.413	**0.396**
	1.0	0.413	0.393	0.387	0.400	0.393	
	10.0	0.387	0.377	0.373	0.383	0.367	
Pleurotus florida (30 days)	0.5	0.413	0.413	0.410	0.413	0.407	**0.393**
	1.0	0.400	0.400	0.387	0.400	0.393	
	10.0	0.390	0.367	0.367	0.367	0.367	
UASD-LF1 (30 days)	0.5	0.413	0.400	0.400	0.407	0.410	**0.393**
	1.0	0.400	0.387	0.393	0.397	0.397	
	10.0	0.380	0.367	0.377	0.377	0.390	
Control (30 days)	0.5	0.343	0.323	0.330	0.323	0.317	**0.329**
	1.0	0.343	0.323	0.327	0.317	0.323	
	10.0	0.340	0.330	0.340	0.327	0.327	
Mean		**0.377**	**0.363**	**0.364**	**0.367**	**0.363**	
Mean	0.5	0.376					
	1.0	0.368					
	10.0	0.356					

Contd…..

Two way table for interaction (A × C)

Particle size / Lignolytic fungi	0.5 mm	1.0 mm	10 mm	Mean
Phanerochaete chrysosporium (10 days)	0.368	0.359	0.347	**0.358**
Pleurotus florida (10 days)	0.368	0.359	0.344	**0.357**
UASD-LF1 (10 days)	0.365	0.357	0.344	**0.355**
Control (10 days)	0.336	0.337	0.336	**0.336**
Phanerochaete chrysosporium (20 days)	0.393	0.382	0.369	**0.381**
Pleurotus florida (20 days)	0.399	0.387	0.365	**0.384**
UASD-LF1 (20 days)	0.394	0.385	0.369	**0.383**
Control (20 days)	0.336	0.337	0.336	**0.336**
Phanerochaete chrysosporium (30 days)	0.412	0.397	0.377	**0.396**
Pleurotus florida (30 days)	0.411	0.396	0.371	**0.393**
UASD-LF1 (30 days)	0.406	0.395	0.378	**0.393**
Control (30 days)	0.327	0.327	0.333	**0.329**
Mean	**0.376**	**0.368**	**0.356**	

	SE±	CD 1 %
Lignolytic fungi (A)	0.003	0.009
Substrates (B)	0.002	0.006
Particle size (C)	0.001	0.005
A x B	0.006	NS
A x C	0.004	0.016
B x C	0.003	NS
A x B x C	0.010	NS

Contd…..

Table 11a: Per cent increase in cellulose content over the initial value

Lignolytic fungi	Particle size (mm)	Substrates (%)				
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover
Phanerochaete chrysosporium (10 days)	0.5	7.65	8.11	7.00	12.01	10.43
	1.0	3.97	8.11	4.96	8.11	6.44
	10.0	1.98	2.10	1.17	4.20	4.29
Pleurotus florida (10 days)	0.5	7.65	10.21	7.00	10.21	10.43
	1.0	3.97	8.11	4.96	8.11	6.44
	10.0	0.00	2.10	-0.87	4.20	4.29
UASD-LF1 (10 days)	0.5	7.65	7.21	4.96	10.21	10.43
	1.0	3.97	8.11	1.17	8.11	8.28
	10.0	1.98	1.20	-0.87	4.20	3.37
Control (10 days)	0.5	0.00	0.00	-0.87	-0.90	-0.92
	1.0	0.00	0.00	-1.75	-0.90	2.15
	10.0	-0.85	0.00	-0.87	-0.90	0.31
Phanerochaete chrysosporium (20 days)	0.5	13.31	16.22	12.83	21.02	18.71
	1.0	11.33	13.21	10.79	14.11	16.56
	10.0	6.80	12.01	5.83	11.11	11.35
Pleurotus florida (20 days)	0.5	14.16	19.22	13.70	21.02	22.70
	1.0	12.46	17.12	9.91	16.22	18.71
	10.0	6.80	7.21	4.08	11.11	11.35
UASD-LF1 (20 days)	0.5	12.46	16.22	12.83	19.22	23.62
	1.0	10.48	15.02	9.91	17.12	18.71
	10.0	6.80	7.21	5.83	13.21	14.42
Control (20 days)	0.5	0.00	0.00	-0.87	-0.90	-0.92
	1.0	0.00	0.00	-1.75	-0.90	2.15
	10.0	-0.85	0.00	-0.87	-0.90	0.31
Phanerochaete chrysosporium (30 days)	0.5	20.96	22.22	16.62	24.02	26.69
	1.0	17.00	18.02	12.83	20.12	20.55
	10.0	9.63	13.21	8.75	15.02	12.58
Pleurotus florida (30 days)	0.5	17.00	24.02	19.53	24.02	24.85
	1.0	13.31	20.12	12.83	20.12	20.55
	10.0	10.48	10.21	7.00	10.21	12.58
UASD-LF1 (30 days)	0.5	17.00	20.12	16.62	22.22	25.77
	1.0	13.31	16.22	14.58	19.22	21.78
	10.0	7.65	10.21	9.91	13.21	19.63
Control (30 days)	0.5	-2.83	-3.00	-3.79	-3.00	-2.76
	1.0	-2.83	-3.00	-4.66	-4.80	-0.92
	10.0	-3.68	-0.90	-0.87	-1.80	0.31

Note : Initial cellulose content (g g^{-1}) Sugarcane bagasse (0.353), Sugarcane tops (0.333), Sugarcane trash (0.343), Corn husk (0.333) and Corn stover (0.327)

The interaction between lignolytic fungi and particle size of substrates showed significant variations in cellulose content. The significantly highest cellulose content of 0.412 g per g was recorded with inoculation of *P. chrysosporium* in 0.5 mm particle size substrates. However, it was on par with *P. florida* (0.411 g g^{-1}) and UASD-LF1 (0.406 g g^{-1}) in 0.50 mm particle size, with *P. chrysosporium* (0.397 g g^{-1}), *P. florida* (0.396 g g^{-1}) and UASD-LF1 (0.395 g g^{-1}) in 1.00 mm particle size at 30 days incubation as well as with *P. florida* (0.399 g g^{-1}) in 0.50 mm particle size at 20 days incubation with regards to cellulose content. It was observed that the cellulose content in the substrates of 0.5 mm and 1.0 mm were on par with each other and found to be superior over 10.0 mm particle size at 30 days incubation with lignolytic fungi.

The observations also revealed that the cellulose content of different particle sized substrates inoculated with different lignolytic fungi at 30 days incubation did not vary significantly when compared to 20 days incubation period in respective particle size and fungal inoculation with regards to cellulose content. However, 30 and 20 days incubation was observed to be significantly superior over 10 days incubation period for the respective particle size and lignlytic fungi inoculation. Significantly the lowest cellulose content was observed with uninoculated control with 0.329 g per g at 30 days incubation period.

4.4.3 Hemicellulose content

The hemicellulose content of the substrates as influenced by individual treatment factors such as inoculation of lignolytic fungi, different substrates, particle size of substrates was found to vary significantly. The results are presented in Table 12.

The pre-treatment of substrates with different lignolytic fungi indicated significant variations in hemicellulose content. The mean maximum and mean minimum hemicellulose content (0.274 and 0.250 g g^{-1}) was observed respectively with *P. florida* inoculated substrates and unioculated control treatments over a period of 30 days incubation.

All the lignolytic fungi cultures were found to be on par with respect to hemicellulose content at all incubation periods (30, 20 and 10 days) except in case of *P. chrysosporium*, which showed significantly less hemicellulose content (0.264 g g^{-1}) at 10 days incubation when compared to *P. florida* (0.272 g g^{-1}) at 30 days incubation.

The different substrates subjected for microbial pre-treatment showed significant variations in hemicellulose content. The significantly highest mean hemicellulose content (0.300 g g^{-1}) was observed with sugarcane bagasse. It was highly superior over corn stover (0.264 g g^{-1}), corn husk (0.263 g g^{-1}), sugarcane trash (0.254 g g^{-1}) and sugarcane tops (0.244 g g^{-1}). Corn stover and corn husk stood on par with each other with respect to hemicellulose content.

The particle size of the substrates also indicated significant variations in hemicellulose content. The mean maximum hemicellulose content, 0.269 g per g was observed with 0.50 mm particle size. It was found to be significantly superior over particle sizes of 1.0 (0.264 g g^{-1}) and 10.0 mm (0.261 g g^{-1}) with respect to hemicellulose content. The two and three way interactions between the factors, lignolytic fungi, different substrates and particle size of the substrates were found to be insignificant with regards to hemicellulose content.

4.4.4 Lignin content

The amount of lignin content present in the substrates due to pre-treatment by lignolytic fungi was found to vary differently. The data presented in Table 13 indicates that the individual factors such as lignolytic fungi inoculation, the substrates and their particle size differed significantly, while the combinations of factors did not differ with respect to lignin content in the substrates.

The pre-treatment of substrates with lignolytic fungal cultures had influenced lignin content significantly. The significant mean maximum lignin content, 0.150 g per g was observed with uninoculated substrates at all the incubation periods studied (10, 20 and 30 days). The significant mean minimum 0.115 g per g lignin content was observed with *P. florida* at 30 days of incubation.

Table 12: Effect of lignolytic fungi on hemicellulose content of substrates

Lignolytic fungi	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.303	0.243	0.257	0.267	0.273	**0.264**
	1.0	0.303	0.230	0.250	0.260	0.260	
	10.0	0.303	0.230	0.253	0.260	0.260	
Pleurotus florida (10 days)	0.5	0.300	0.253	0.260	0.267	0.273	**0.266**
	1.0	0.300	0.250	0.253	0.260	0.260	
	10.0	0.303	0.243	0.253	0.253	0.263	
UASD-LF1 (10 days)	0.5	0.307	0.260	0.273	0.273	0.273	**0.269**
	1.0	0.303	0.253	0.260	0.267	0.260	
	10.0	0.297	0.240	0.247	0.253	0.263	
Control (10 days)	0.5	0.293	0.223	0.240	0.260	0.250	**0.252**
	1.0	0.290	0.223	0.240	0.250	0.253	
	10.0	0.293	0.223	0.237	0.253	0.257	
Phanerochaete chrysosporium (20 days)	0.5	0.307	0.260	0.267	0.267	0.273	**0.269**
	1.0	0.303	0.253	0.260	0.260	0.267	
	10.0	0.303	0.237	0.253	0.263	0.267	
Pleurotus florida (20 days)	0.5	0.313	0.263	0.267	0.273	0.280	**0.270**
	1.0	0.300	0.247	0.253	0.267	0.267	
	10.0	0.297	0.250	0.247	0.260	0.260	
UASD-LF1 (20 days)	0.5	0.307	0.260	0.267	0.267	0.273	**0.270**
	1.0	0.300	0.263	0.260	0.270	0.267	
	10.0	0.303	0.247	0.253	0.257	0.260	
Control (20 days)	0.5	0.293	0.223	0.240	0.260	0.250	**0.252**
	1.0	0.290	0.223	0.240	0.250	0.253	
	10.0	0.293	0.223	0.237	0.253	0.257	
Phanerochaete chrysosporium (30 days)	0.5	0.307	0.260	0.267	0.267	0.283	**0.270**
	1.0	0.307	0.253	0.257	0.260	0.267	
	10.0	0.300	0.247	0.250	0.260	0.270	
Pleurotus florida (30 days)	0.5	0.310	0.260	0.273	0.267	0.283	**0.272**
	1.0	0.303	0.253	0.267	0.270	0.267	
	10.0	0.297	0.247	0.260	0.263	0.260	
UASD-LF1 (30 days)	0.5	0.310	0.260	0.273	0.280	0.280	**0.274**
	1.0	0.303	0.260	0.267	0.273	0.273	
	10.0	0.297	0.247	0.253	0.267	0.267	
Control (30 days)	0.5	0.283	0.217	0.230	0.253	0.240	**0.250**
	1.0	0.280	0.223	0.240	0.260	0.253	
	10.0	0.293	0.223	0.237	0.260	0.257	
Mean		**0.300**	**0.244**	**0.254**	**0.263**	**0.264**	
Mean	0.5	0.269					
	1.0	0.264					
	10.0	0.261					

	SE±	CD 1 %
Lignolytic fungi (A)	0.002	0.007
Substrates (B)	0.001	0.005
Particle size (C)	0.001	0.004
A x B	0.004	NS
A x C	0.003	NS
B x C	0.002	NS
A x B x C	0.008	NS

Contd…..

Table 12a: Per cent increase in hemicellulose over initial hemicellulose content

Lignolytic fungi	Particle size (mm)	Substrates (%)				
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover
Phanerochaete chrysosporium (10 days)	0.5	3.41	7.05	4.05	5.53	5.00
	1.0	3.41	1.32	1.21	2.77	0.00
	10.0	3.41	1.32	2.43	2.77	0.00
Pleurotus florida (10 days)	0.5	2.39	11.45	5.26	5.53	5.00
	1.0	2.39	10.13	2.43	2.77	0.00
	10.0	3.41	7.05	2.43	0.00	1.15
UASD-LF1 (10 days)	0.5	4.78	14.54	10.53	7.91	5.00
	1.0	3.41	11.45	5.26	5.53	0.00
	10.0	1.37	5.73	0.00	0.00	1.15
Control (10 days)	0.5	0.00	-1.76	-2.83	2.77	-3.85
	1.0	-1.02	-1.76	-2.83	-1.19	-2.69
	10.0	0.00	-1.76	-4.05	0.00	-1.15
Phanerochaete chrysosporium (20 days)	0.5	4.78	14.54	8.10	5.53	5.00
	1.0	3.41	11.45	5.26	2.77	2.69
	10.0	3.41	4.41	2.43	3.95	2.69
Pleurotus florida (20 days)	0.5	6.83	15.86	8.10	7.91	7.69
	1.0	2.39	8.81	2.43	5.53	2.69
	10.0	1.37	10.13	0.00	2.77	0.00
UASD-LF1 (20 days)	0.5	4.78	14.54	8.10	5.53	5.00
	1.0	2.39	15.86	5.26	6.72	2.69
	10.0	3.41	8.81	2.43	1.58	0.00
Control (20 days)	0.5	0.00	-1.76	-2.83	2.77	-3.85
	1.0	-1.02	-1.76	-2.83	-1.19	-2.69
	10.0	0.00	-1.76	-4.05	0.00	-1.15
Phanerochaete chrysosporium (30 days)	0.5	4.78	14.54	8.10	5.53	8.85
	1.0	4.78	11.45	4.05	2.77	2.69
	10.0	2.39	8.81	1.21	2.77	3.85
Pleurotus florida (30 days)	0.5	5.80	14.54	10.53	5.53	8.85
	1.0	3.41	11.45	8.10	6.72	2.69
	10.0	1.37	8.81	5.26	3.95	0.00
UASD-LF1 (30 days)	0.5	5.80	14.54	10.53	10.67	7.69
	1.0	3.41	14.54	8.10	7.91	5.00
	10.0	1.37	8.81	2.43	5.53	2.69
Control (30 days)	0.5	-3.41	-4.41	-6.88	0.00	-7.69
	1.0	-4.44	-1.76	-2.83	2.77	-2.69
	10.0	0.00	-1.76	-4.05	2.77	-1.15

Note : Initial hemicellulose content (g g^{-1}) Sugarcane bagasse (0.293), Sugarcane tops (0.227), Sugarcane trash (0.247), Corn husk (0.253) and Corn stover (0.260)

Table 13: Effect of lignolytic fungi on lignin content of substrates

Lignolytic fungi	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.117	0.137	0.133	0.120	0.133	**0.134**
	1.0	0.120	0.143	0.137	0.127	0.140	
	10.0	0.133	0.150	0.147	0.133	0.147	
Pleurotus florida (10 days)	0.5	0.120	0.133	0.133	0.120	0.133	**0.134**
	1.0	0.120	0.143	0.140	0.133	0.133	
	10.0	0.127	0.153	0.150	0.130	0.140	
UASD-LF1 (10 days)	0.5	0.120	0.140	0.140	0.120	0.133	**0.136**
	1.0	0.127	0.143	0.147	0.127	0.140	
	10.0	0.133	0.150	0.153	0.133	0.140	
Control (10 days)	0.5	0.140	0.157	0.160	0.140	0.153	**0.150**
	1.0	0.140	0.157	0.160	0.140	0.153	
	10.0	0.140	0.157	0.160	0.140	0.153	
Phanerochaete chrysosporium (20 days)	0.5	0.107	0.133	0.140	0.113	0.127	**0.130**
	1.0	0.120	0.140	0.140	0.120	0.133	
	10.0	0.120	0.140	0.147	0.127	0.140	
Pleurotus florida (20 days)	0.5	0.107	0.123	0.120	0.107	0.107	**0.123**
	1.0	0.113	0.127	0.133	0.113	0.120	
	10.0	0.127	0.140	0.147	0.127	0.133	
UASD-LF1 (20 days)	0.5	0.113	0.127	0.127	0.107	0.120	**0.127**
	1.0	0.120	0.133	0.140	0.113	0.120	
	10.0	0.127	0.140	0.147	0.133	0.140	
Control (20 days)	0.5	0.140	0.157	0.160	0.140	0.153	**0.150**
	1.0	0.140	0.157	0.160	0.140	0.153	
	10.0	0.140	0.157	0.160	0.140	0.153	
Phanerochaete chrysosporium (30 days)	0.5	0.100	0.113	0.120	0.100	0.107	**0.116**
	1.0	0.107	0.120	0.120	0.103	0.113	
	10.0	0.120	0.133	0.140	0.113	0.127	
Pleurotus florida (30 days)	0.5	0.100	0.113	0.113	0.100	0.100	**0.115**
	1.0	0.107	0.120	0.120	0.107	0.107	
	10.0	0.120	0.133	0.140	0.120	0.120	
UASD-LF1 (30 days)	0.5	0.100	0.113	0.123	0.100	0.107	**0.117**
	1.0	0.107	0.120	0.127	0.113	0.113	
	10.0	0.120	0.133	0.140	0.113	0.127	
Control (30 days)	0.5	0.140	0.157	0.160	0.140	0.153	**0.150**
	1.0	0.140	0.157	0.160	0.140	0.153	
	10.0	0.140	0.157	0.160	0.140	0.153	
Mean		**0.123**	**0.139**	**0.142**	**0.123**	**0.133**	
Mean	0.5	0.126					
	1.0	0.131					
	10.0	0.138					

	SE±	CD 1 %
Lignolytic fungi (A)	0.002	0.008
Substrates (B)	0.001	0.005
Particle size (C)	0.001	0.004
A x B	0.005	NS
A x C	0.004	NS
B x C	0.002	NS
A x B x C	0.008	NS

Table 13a: Per cent reduction in lignin content over initial lignin content

Lignolytic fungi	Particle size (mm)	Substrates (%)				
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover
Phanerochaete chrysosporium (10 days)	0.5	-16.43	-12.74	-16.88	-14.29	-13.07
	1.0	-14.29	-8.92	-14.38	-9.29	-8.50
	10.0	-5.00	-4.46	-8.13	-5.00	-3.92
Pleurotus florida (10 days)	0.5	-14.29	-15.29	-16.88	-14.29	-13.07
	1.0	-14.29	-8.92	-12.50	-5.00	-13.07
	10.0	-9.29	-2.55	-6.25	-7.14	-8.50
UASD-LF1 (10 days)	0.5	-14.29	-10.83	-12.50	-14.29	-13.07
	1.0	-9.29	-8.92	-8.13	-9.29	-8.50
	10.0	-5.00	-4.46	-4.38	-5.00	-8.50
Control (10 days)	0.5	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00
Phanerochaete chrysosporium (20 days)	0.5	-23.57	-15.29	-12.50	-19.29	-16.99
	1.0	-14.29	-10.83	-12.50	-14.29	-13.07
	10.0	-14.29	-10.83	-8.13	-9.29	-8.50
Pleurotus florida (20 days)	0.5	-23.57	-21.66	-25.00	-23.57	-30.07
	1.0	-19.29	-19.11	-16.88	-19.29	-21.57
	10.0	-9.29	-10.83	-8.13	-9.29	-13.07
UASD-LF1 (20 days)	0.5	-19.29	-19.11	-20.63	-23.57	-21.57
	1.0	-14.29	-15.29	-12.50	-19.29	-21.57
	10.0	-9.29	-10.83	-8.13	-5.00	-8.50
Control (20 days)	0.5	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00
Phanerochaete chrysosporium (30 days)	0.5	-28.57	-28.03	-25.00	-28.57	-30.07
	1.0	-23.57	-23.57	-25.00	-26.43	-26.14
	10.0	-14.29	-15.29	-12.50	-19.29	-16.99
Pleurotus florida (30 days)	0.5	-28.57	-28.03	-29.38	-28.57	-34.64
	1.0	-23.57	-23.57	-25.00	-23.57	-30.07
	10.0	-14.29	-15.29	-12.50	-14.29	-21.57
UASD-LF1 (30 days)	0.5	-28.57	-28.03	-23.13	-28.57	-30.07
	1.0	-23.57	-23.57	-20.63	-19.29	-26.14
	10.0	-14.29	-15.29	-12.50	-19.29	-16.99
Control (30 days)	0.5	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00

Note : Initial lignin content (g g^{-1}) Sugarcane bagasse (0.140), Sugarcane tops (0.157), Sugarcane trash (0.160), Corn husk (0.140) and Corn stover (0.153)

Among the fungal cultures, the significant mean maximum lignin content, 0.136 g per g was seen with UASD-LF1 at 10 days incubation period and it was found to be on par with both *P. chrysosporium* and *P. florida* (0.134 g g^{-1}) at 10 days incubation period. The lignin content in the substrates was found to decrease over a period of incubation with fungal cultures. Significantly the mean minimum 0.115 g per g lignin content was seen with *P. florida* at 30 days incubation and it was found to be on par with *P. chrysosporium* (0.116 g g^{-1}) and UASD-LF1 (0.117 g g^{-1}) with respect to lignin content at 30 days incubation period and it was significantly lower when compared to 20 days incubation with all the fungal cultures.

The lignin content due to substrates also varied significantly. The mean maximum of 0.142 g per g lignin content was observed with sugarcane trash and it was on par with sugarcane tops (0.139 g g^{-1}) and significantly superior over rest of the substrates. The lowest mean lignin content, 0.123 g per g was recorded in both sugarcane bagasse and corn husk.

The lignin content as influenced by particle size of the substrates indicated the mean maximum 0.138 g per g lignin content in 10.0 mm particle size. It was found to be significantly superior over 1.0 mm (0.131 g g^{-1}) and 0.5 mm (0.126 g g^{-1}) particle size. All the particle sizes differed significantly with each other with regards to lignin content.

All the two factor and three factor interactions were insignificant for lignin content. However, the highest lignin content was observed with uninoculated control, 0.160 g per g lignin in case of sugarcane trash in all the particle sizes. The lowest lignin content was observed with bagasse (0.100 g g^{-1}) with all the lignolytic fungal cultures for the particle size 0.5 mm.

4.5 Effect of crude enzymes of lignolytic fungi pre-treatment

4.5.1 Weight loss

The data concerned with loss in weight of substrates due to pre-treatment by crude enzymes of lignolytic fungi is furnished in Table 14. Significant variation in weight loss observed with individual factors (crude enzymes and particle size of substrates) and interaction of factors (between crude enzymes and particle size and between substrates and particle sizes). The individual factor, substrates and the interaction between crude enzymes and substrates as well as the combined interaction of all the three factors did not differ significantly with respect to weight loss.

The loss of weight as influenced by different crude enzymes of lignolytic fungi indicated significant variation. The mean weight loss in the substrates ranged from maximum 0.048 g per g with UASD-LF1 after 10 days of incubation to minimum 0.008 g per g with uninoculated control at all the incubation periods.

The weight loss observed with *P. chrysosporium* and *P. florida* crude enzyme (0.047 g g^{-1}) were found to be on par with UASD-LF1 at all the incubation periods with an exception of *P. chrysosporium* crude enzyme that showed significantly less weight loss (0.046 g g^{-1}) on 30th day after incubation.

With regards to weight loss as influenced by different substrates was found to be insignificant, where all the substrates showed weight loss of 0.037 g per g.

The different particle sizes evaluated for weight loss revealed the maximum mean weight loss of 0.038 g per g in 0.5 mm particle size as against 0.037 g per g recorded in both 1.0 mm and 10.0 mm particle sizes. They ere found to be significantly different from each other with respect to weight loss.

The interaction between crude enzymes of lignolytic fungi and particle size was found to be significant with regards to loss of weight. The highest weight loss (0.048 g g^{-1}) was observed in the UASD-LF1 crude enzyme inoculated substrates of particle sizes 1.0 mm and 10.0 mm at both 10 and 20 days of incubation. However, it was found to be on par with 0.5 mm particle size (0.047 g g^{-1}) at 10 and 20 days incubation periods. It was also noticed that all the crude enzymes of lignolytic fungi were found to be insignificant with respect to weight loss in all the particle sizes at all incubation periods with an exception of *P. chrysosporium* and *P. florida* crude enzymes which showed significantly the less weight loss of 0.046 g per g at the end of 30 days after incubation.

Table 14: Effect of crude enzymes of lignolytic fungi on weight loss of substrates

Lignolytic fungi	Particle size (mm)	Substrates (g g^{-1})					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.046	0.047	0.046	0.046	0.047	**0.047**
	1.0	0.047	0.047	0.047	0.047	0.047	
	10.0	0.048	0.048	0.048	0.048	0.048	
Pleurotus florida (10 days)	0.5	0.047	0.047	0.047	0.047	0.047	**0.047**
	1.0	0.047	0.047	0.047	0.047	0.047	
	10.0	0.048	0.048	0.048	0.048	0.048	
UASD-LF1 (10 days)	0.5	0.047	0.047	0.047	0.047	0.047	**0.048**
	1.0	0.047	0.047	0.048	0.048	0.048	
	10.0	0.048	0.049	0.049	0.048	0.048	
Control (10 days)	0.5	0.010	0.007	0.010	0.010	0.010	**0.008**
	1.0	0.010	0.010	0.010	0.010	0.010	
	10.0	0.002	0.007	0.003	0.003	0.001	
Phanerochaete chrysosporium (20 days)	0.5	0.046	0.046	0.046	0.046	0.046	**0.047**
	1.0	0.046	0.047	0.047	0.046	0.047	
	10.0	0.047	0.048	0.048	0.047	0.048	
Pleurotus florida (20 days)	0.5	0.046	0.046	0.046	0.046	0.046	**0.047**
	1.0	0.046	0.047	0.047	0.047	0.047	
	10.0	0.047	0.048	0.048	0.048	0.048	
UASD-LF1 (20 days)	0.5	0.046	0.047	0.047	0.047	0.047	**0.047**
	1.0	0.047	0.047	0.047	0.047	0.047	
	10.0	0.048	0.048	0.048	0.048	0.048	
Control (20 days)	0.5	0.010	0.007	0.010	0.010	0.010	**0.008**
	1.0	0.010	0.010	0.010	0.010	0.010	
	10.0	0.002	0.007	0.003	0.003	0.001	
Phanerochaete chrysosporium (30 days)	0.5	0.046	0.046	0.046	0.046	0.046	**0.046**
	1.0	0.046	0.047	0.046	0.046	0.046	
	10.0	0.047	0.048	0.048	0.047	0.048	
Pleurotus florida (30 days)	0.5	0.046	0.046	0.046	0.046	0.046	**0.047**
	1.0	0.046	0.047	0.047	0.046	0.046	
	10.0	0.047	0.047	0.047	0.048	0.048	
UASD-LF1 (30 days)	0.5	0.046	0.047	0.047	0.046	0.046	**0.047**
	1.0	0.047	0.047	0.047	0.047	0.046	
	10.0	0.048	0.048	0.048	0.048	0.048	
Control (30 days)	0.5	0.010	0.007	0.010	0.010	0.010	**0.008**
	1.0	0.010	0.010	0.010	0.010	0.010	
	10.0	0.002	0.007	0.003	0.003	0.001	
Mean		**0.037**	**0.037**	**0.037**	**0.037**	**0.037**	
Mean	0.5	0.037					
	1.0	0.038					
	10.0	0.037					

Contd…..

Two way table for interaction (A × C)

Particle size / Lignolytic fungi	0.5 mm	1.0 mm	10 mm	Mean
Phanerochaete chrysosporium (10 days)	0.047	0.047	0.047	0.047
Pleurotus florida (10 days)	0.047	0.047	0.047	0.047
UASD-LF1 (10 days)	0.047	0.048	0.048	0.048
Control (10 days)	0.007	0.008	0.008	0.008
Phanerochaete chrysosporium (20 days)	0.046	0.047	0.047	0.046
Pleurotus florida (20 days)	0.047	0.047	0.047	0.047
UASD-LF1 (20 days)	0.047	0.048	0.048	0.047
Control (20 days)	0.007	0.008	0.008	0.008
Phanerochaete chrysosporium (30 days)	0.046	0.047	0.047	0.046
Pleurotus florida (30 days)	0.046	0.047	0.047	0.047
UASD-LF1 (30 days)	0.047	0.047	0.047	0.047
Control (30 days)	0.007	0.008	0.008	0.008
Mean	**0.037**	**0.037**	**0.037**	**0.037**

Two way table for interaction (B × C)

Substrates / Particle size	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Mean
0.5 mm	0.037	0.037	0.037	0.037	0.037	**0.037**
1.0 mm	0.037	0.038	0.038	0.038	0.038	**0.038**
10 mm	0.036	0.038	0.037	0.037	0.036	**0.037**
Mean	**0.037**	**0.037**	**0.037**	**0.037**	**0.037**	

	SE±	CD 1 %
Lignolytic fungi (A)	0.0002	0.0008
Substrates (B)	0.0001	NS
Particle size (C)	0.0001	0.0004
A x B	0.0005	NS
A x C	0.0004	0.0013
B x C	0.0002	0.0008
A x B x C	0.0008	NS

The uninoculated control treatment showed significantly lowest weight loss between 0.007 and 0.008 g per g in all the particle sizes at all incubation periods studied.

The interaction between particle sizes and different substrates showed significant differences in weight loss. The maximum weight loss, 0.038 g per g was observed with 1.0 mm particle size in case of sugarcane tops, sugarcane trash, corn husk and corn stover and it was found to be at par with sugarcane bagasse (0.037 g g^{-1}) of 1.0 mm particle size and it was also on par with 0.5 mm and 10.0 mm particle sizes of all the substrates except sugarcane bagasse and corn stover of 10.0 mm particle size with respect to weight loss.

Although, the three way interaction was found to be insignificant for weight loss, the numerically highest weight loss was observed with UASD-LF1 followed by *P. florida* and *P. chrysosporium* for all the substrates.

4.5.2 Cellulose content

The cellulose content of different substrates as influenced by individual factors *viz.*, crude enzymes of lignolytic fungi, substrates and particle size and the interaction between crude enzymes and particle sizes were found to differ significantly. Whereas the other two way and three way interactions were found to be non-significant with respect to cellulose content. The results are furnished in Table 15.

The cellulose content of the substrates indicated marked variations due to inoculation of crude enzymes of lignolytic fungi. The mean maximum and minimum cellulose content of 0.396 and 0.329 g per g was observed with *P. florida* crude enzyme at 20 days after incubation and with uninoculated control at 30 days after incubation, respectively. The mean maximum cellulose content of 0.396 g per g was recorded in case of *P. florida* at 20 days after incubation as well as with *P. chrysosporium* and *P. florida* crude enzyme at 30 days after incubation. They were found to be on par with crude enzymes of UASD-LF1 (0.392 g g^{-1}) at both 20 and 30 days after incubation with regards to cellulose content and significantly superior over all the lignolytic crude enzymes at 10 days after incubation with regards to cellulose content .

The cellulose content as influenced by different substrates also differed significantly. The mean maximum cellulose content of 0.387 g per g was observed in sugarcane bagasse, which was found to be significantly superior over rest of the substrates. All other substrates with a range between 0.377 and 0.373 g per g cellulose content were found to be at par with each other.

The cellulose content showed significant variation between the particle sizes. The mean maximum, 0.387 g per g cellulose content was recorded with 0.50 mm particle size. It was found to be significantly superior over particle sizes of 1.0 mm (0.379 g g^{-1}) and 10.0 mm (0.365 g g^{-1}) with respect to cellulose content.

The interaction effect between crude enzymes and particle size showed significant differences in cellulose content. The significantly highest cellulose content, 0.411 g per g was observed with crude enzyme of *P. florida* at 20 days after incubation and with crude enzymes of *P. chrysosporium* and *P. florida* at 30 days after incubation in case of particle size of 0.5 mm. However, they were found to be on par with UASD-LF1 at all the incubation periods with respect to cellulose content in the particle size of 0.5 mm. The particle sizes of 0.5 mm and 1.0 mm were found to be on par with each other with regards to cellulose content due to different lignolytic crude enzymes at both 20 and 30 days incubation. Also, the *P. florida* and UASD-LF1 crude enzymes produced on par cellulose content in case of 0.5 mm particle size at 10 days incubation. All these treatment combinations were found to be significantly superior over 10.0 mm particle size at all incubation periods. The control treatment resulted in a significantly lowest cellulose content of 0.327 g per g both in 0.5 and 1.0 mm particle sizes at the end of 30 days incubation period.

4.5.3 Hemicellulose content

The data pertaining to hemicellulose content as influenced by different lignolytic crude enzymes is presented in Table 16. The hemicellulose content varied differently due to individual treatment factors such as crude enzymes, substrates and particle size. The combined interaction effects of the individual factors did not differ with regards to hemicellulose content.

Table 15: Effect of crude enzymes of lignolytic fungi on cellulose content of substrates

Lignolytic fungi	Particle size (mm)	Substrates (g g^{-1})					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.407	0.393	0.400	0.393	0.393	**0.385**
	1.0	0.393	0.387	0.393	0.387	0.387	
	10.0	0.380	0.367	0.373	0.367	0.353	
Pleurotus florida (10 days)	0.5	0.420	0.393	0.400	0.400	0.407	**0.389**
	1.0	0.400	0.393	0.380	0.393	0.393	
	10.0	0.380	0.373	0.373	0.373	0.353	
UASD-LF1 (10 days)	0.5	0.400	0.407	0.393	0.393	0.400	**0.386**
	1.0	0.393	0.387	0.380	0.380	0.387	
	10.0	0.380	0.373	0.373	0.367	0.373	
Control (10 days)	0.5	0.353	0.333	0.340	0.330	0.323	**0.336**
	1.0	0.353	0.333	0.337	0.330	0.333	
	10.0	0.350	0.333	0.340	0.330	0.327	
Phanerochaete chrysosporium (20 days)	0.5	0.413	0.400	0.407	0.400	0.400	**0.392**
	1.0	0.400	0.393	0.400	0.393	0.393	
	10.0	0.387	0.373	0.380	0.373	0.360	
Pleurotus florida (20 days)	0.5	0.427	0.400	0.407	0.407	0.413	**0.396**
	1.0	0.407	0.400	0.387	0.400	0.400	
	10.0	0.387	0.380	0.380	0.380	0.360	
UASD-LF1 (20 days)	0.5	0.407	0.400	0.400	0.400	0.407	**0.392**
	1.0	0.400	0.393	0.393	0.387	0.393	
	10.0	0.387	0.380	0.380	0.373	0.380	
Control (20 days)	0.5	0.353	0.333	0.340	0.330	0.323	**0.336**
	1.0	0.353	0.333	0.337	0.330	0.333	
	10.0	0.350	0.333	0.340	0.330	0.327	
Phanerochaete chrysosporium (30 days)	0.5	0.427	0.407	0.407	0.400	0.413	**0.396**
	1.0	0.407	0.393	0.400	0.393	0.400	
	10.0	0.387	0.380	0.380	0.373	0.367	
Pleurotus florida (30 days)	0.5	0.427	0.400	0.407	0.407	0.413	**0.396**
	1.0	0.407	0.400	0.387	0.400	0.400	
	10.0	0.387	0.380	0.380	0.380	0.360	
UASD-LF1 (30 days)	0.5	0.413	0.400	0.400	0.400	0.407	**0.392**
	1.0	0.400	0.393	0.393	0.387	0.393	
	10.0	0.387	0.380	0.380	0.373	0.380	
Control (30 days)	0.5	0.343	0.323	0.330	0.323	0.317	**0.329**
	1.0	0.343	0.323	0.327	0.317	0.323	
	10.0	0.340	0.330	0.340	0.327	0.327	
Mean		**0.387**	**0.375**	**0.377**	**0.373**	**0.373**	
Mean	0.5	0.387					
	1.0	0.379					
	10.0	0.365					

Contd…..

Two way table for interaction (A × C)

Lignolytic fungi \ Particle size	0.5 mm	1.0 mm	10 mm	Mean
Phanerochaete chrysosporium (10 days)	0.397	0.389	0.368	**0.385**
Pleurotus florida (10 days)	0.404	0.392	0.371	**0.389**
UASD-LF1 (10 days)	0.399	0.385	0.373	**0.386**
Control (10 days)	0.336	0.337	0.336	**0.336**
Phanerochaete chrysosporium (20 days)	0.404	0.396	0.375	**0.392**
Pleurotus florida (20 days)	0.411	0.399	0.377	**0.396**
UASD-LF1 (20 days)	0.403	0.393	0.380	**0.392**
Control (20 days)	0.336	0.337	0.336	**0.336**
Phanerochaete chrysosporium (30 days)	0.411	0.399	0.377	**0.396**
Pleurotus florida (30 days)	0.411	0.399	0.377	**0.396**
UASD-LF1 (30 days)	0.404	0.393	0.380	**0.392**
Control (30 days)	0.327	0.327	0.333	**0.329**
Mean	**0.387**	**0.379**	**0.365**	

	SE±	CD 1 %
Lignolytic fungi (A)	0.002	0.007
Substrates (B)	0.001	0.005
Particle size (C)	0.001	0.004
A x B	0.004	NS
A x C	0.003	0.013
B x C	0.002	NS
A x B x C	0.008	NS

Contd…..

Table 15a: Per cent increase in cellulose content over initial cellulose content

Lignolytic fungi	Particle size (mm)	Substrates (%)				
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover
Phanerochaete chrysosporium (10 days)	0.5 1.0 10.0	15.30 11.33 7.65	18.02 16.22 10.21	16.62 14.58 8.75	18.02 16.22 10.21	20.55 18.71 8.28
Pleurotus florida (10 days)	0.5 1.0 10.0	18.98 13.31 7.65	18.02 18.02 12.01	16.62 10.79 8.75	20.12 18.02 12.01	24.85 20.55 8.28
UASD-LF1 (10 days)	0.5 1.0 10.0	13.31 11.33 7.65	22.22 16.22 12.01	14.58 10.79 8.75	18.02 14.11 10.21	22.70 18.71 14.42
Control (10 days)	0.5 1.0 10.0	0.00 0.00 -0.85	0.00 0.00 0.00	-0.87 -1.75 -0.87	-0.90 -0.90 -0.90	-0.92 2.15 0.31
Phanerochaete chrysosporium (20 days)	0.5 1.0 10.0	17.00 13.31 9.63	20.12 18.02 12.01	18.66 16.62 10.79	20.12 18.02 12.01	22.70 20.55 10.43
Pleurotus florida (20 days)	0.5 1.0 10.0	20.96 15.30 9.63	20.12 20.12 14.11	18.66 12.83 10.79	22.22 20.12 14.11	26.69 22.70 10.43
UASD-LF1 (20 days)	0.5 1.0 10.0	15.30 13.31 9.63	20.12 18.02 14.11	16.62 14.58 10.79	20.12 16.22 12.01	24.85 20.55 16.56
Control (20 days)	0.5 1.0 10.0	0.00 0.00 -0.85	0.00 0.00 0.00	-0.87 -1.75 -0.87	-0.90 -0.90 -0.90	-0.92 2.15 0.31
Phanerochaete chrysosporium (30 days)	0.5 1.0 10.0	20.96 15.30 9.63	22.22 18.02 14.11	18.66 16.62 10.79	20.12 18.02 12.01	26.69 22.70 12.58
Pleurotus florida (30 days)	0.5 1.0 10.0	20.96 15.30 9.63	20.12 20.12 14.11	18.66 12.83 10.79	22.22 20.12 14.11	26.69 22.70 10.43
UASD-LF1 (30 days)	0.5 1.0 10.0	17.00 13.31 9.63	20.12 18.02 14.11	16.62 14.58 10.79	20.12 16.22 12.01	24.85 20.55 16.56
Control (30 days)	0.5 1.0 10.0	-2.83 -2.83 -3.68	-3.00 -3.00 -0.90	-3.79 -4.66 -0.87	-3.00 -4.80 -1.80	-2.76 -0.92 0.31

Note : Initial cellulose content ($g\ g^{-1}$) Sugarcane bagasse (0.353), Sugarcane tops (0.333), Sugarcane trash (0.343), Corn husk (0.333) and Corn stover (0.327)

Table 16: Effect of crude enzymes of lignolytic fungi on hemicellulose content of substrates

Lignolytic fungi	Particle size (mm)	Substrates (g g^{-1})					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.320	0.253	0.267	0.280	0.287	**0.272**
	1.0	0.313	0.247	0.260	0.267	0.273	
	10.0	0.300	0.240	0.247	0.267	0.267	
Pleurotus florida (10 days)	0.5	0.327	0.253	0.267	0.273	0.280	**0.271**
	1.0	0.313	0.247	0.260	0.267	0.273	
	10.0	0.287	0.240	0.247	0.267	0.267	
UASD-LF1 (10 days)	0.5	0.307	0.267	0.260	0.267	0.273	**0.268**
	1.0	0.300	0.253	0.253	0.260	0.267	
	10.0	0.300	0.247	0.247	0.253	0.260	
Control (10 days)	0.5	0.293	0.223	0.240	0.260	0.250	**0.252**
	1.0	0.290	0.223	0.240	0.250	0.253	
	10.0	0.293	0.223	0.237	0.253	0.257	
Phanerochaete chrysosporium (20 days)	0.5	0.320	0.260	0.273	0.287	0.287	**0.278**
	1.0	0.320	0.253	0.267	0.280	0.280	
	10.0	0.300	0.247	0.253	0.273	0.273	
Pleurotus florida (20 days)	0.5	0.327	0.260	0.267	0.280	0.287	**0.275**
	1.0	0.313	0.253	0.260	0.273	0.280	
	10.0	0.287	0.240	0.253	0.267	0.273	
UASD-LF1 (20 days)	0.5	0.313	0.273	0.267	0.273	0.280	**0.274**
	1.0	0.307	0.260	0.260	0.267	0.273	
	10.0	0.300	0.253	0.253	0.260	0.267	
Control (20 days)	0.5	0.293	0.223	0.240	0.260	0.250	**0.252**
	1.0	0.290	0.223	0.240	0.250	0.253	
	10.0	0.293	0.223	0.237	0.253	0.257	
Phanerochaete chrysosporium (30 days)	0.5	0.307	0.247	0.260	0.273	0.273	**0.265**
	1.0	0.307	0.240	0.253	0.267	0.267	
	10.0	0.287	0.233	0.240	0.260	0.260	
Pleurotus florida (30 days)	0.5	0.313	0.247	0.253	0.267	0.273	**0.261**
	1.0	0.300	0.240	0.247	0.260	0.267	
	10.0	0.273	0.227	0.240	0.253	0.260	
UASD-LF1 (30 days)	0.5	0.300	0.260	0.253	0.260	0.267	**0.260**
	1.0	0.293	0.247	0.247	0.253	0.260	
	10.0	0.287	0.240	0.240	0.247	0.253	
Control (30 days)	0.5	0.283	0.217	0.230	0.253	0.240	**0.250**
	1.0	0.280	0.223	0.240	0.260	0.253	
	10.0	0.293	0.223	0.237	0.260	0.257	
Mean		**0.301**	**0.243**	**0.251**	**0.264**	**0.267**	
Mean	0.5	0.271					
	1.0	0.265					
	10.0	0.259					

	SE±	CD 1 %
Lignolytic fungi (A)	0.002	0.008
Substrates (B)	0.001	0.005
Particle size (C)	0.001	0.004
A x B	0.005	NS
A x C	0.004	NS
B x C	0.002	NS
A x B x C	0.008	NS

Contd…..

Table 16a: Per cent increase in hemicellulose over initial hemicellulose content

Lignolytic fungi	Particle size (mm)	Substrates (%)				
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover
Phanerochaete chrysosporium (10 days)	0.5	9.22	11.45	8.10	10.67	10.38
	1.0	6.83	8.81	5.26	5.53	5.00
	10.0	2.39	5.73	0.00	5.53	2.69
Pleurotus florida (10 days)	0.5	11.60	11.45	8.10	7.91	7.69
	1.0	6.83	8.81	5.26	5.53	5.00
	10.0	-2.05	5.73	0.00	5.53	2.69
UASD-LF1 (10 days)	0.5	4.78	17.62	5.26	5.53	5.00
	1.0	2.39	11.45	2.43	2.77	2.69
	10.0	2.39	8.81	0.00	0.00	0.00
Control (10 days)	0.5	0.00	-1.76	-2.83	2.77	-3.85
	1.0	-1.02	-1.76	-2.83	-1.19	-2.69
	10.0	0.00	-1.76	-4.05	0.00	-1.15
Phanerochaete chrysosporium (20 days)	0.5	9.22	14.54	10.53	13.44	10.38
	1.0	9.22	11.45	8.10	10.67	7.69
	10.0	2.39	8.81	2.43	7.91	5.00
Pleurotus florida (20 days)	0.5	11.60	14.54	8.10	10.67	10.38
	1.0	6.83	11.45	5.26	7.91	7.69
	10.0	-2.05	5.73	2.43	5.53	5.00
UASD-LF1 (20 days)	0.5	6.83	20.26	8.10	7.91	7.69
	1.0	4.78	14.54	5.26	5.53	5.00
	10.0	2.39	11.45	2.43	2.77	2.69
Control (20 days)	0.5	0.00	-1.76	-2.83	2.77	-3.85
	1.0	-1.02	-1.76	-2.83	-1.19	-2.69
	10.0	0.00	-1.76	-4.05	0.00	-1.15
Phanerochaete chrysosporium (30 days)	0.5	4.78	8.81	5.26	7.91	5.00
	1.0	4.78	5.73	2.43	5.53	2.69
	10.0	-2.05	2.64	-2.83	2.77	0.00
Pleurotus florida (30 days)	0.5	6.83	8.81	2.43	5.53	5.00
	1.0	2.39	5.73	0.00	2.77	2.69
	10.0	-6.83	0.00	-2.83	0.00	0.00
UASD-LF1 (30 days)	0.5	2.39	14.54	2.43	2.77	2.69
	1.0	0.00	8.81	0.00	0.00	0.00
	10.0	-2.05	5.73	-2.83	-2.37	-2.69
Control (30 days)	0.5	-3.41	-4.41	-6.88	0.00	-7.69
	1.0	-4.44	-1.76	-2.83	2.77	-2.69
	10.0	0.00	-1.76	-4.05	2.77	-1.15

Note : Initial hemicellulose content ($g\ g^{-1}$) Sugarcane bagasse (0.293), Sugarcane tops (0.227), Sugarcane trash (0.247), Corn husk (0.253) and Corn stover (0.260)

The crude enzymes of lignolytic fungi showed differences in hemicellulose content. The significant mean maximum hemicellulose content, 0.278 g per g was recorded with *P. chrysosporium* at 20 days after incubation. It was observed to be on par with other crude enzymes of *P. florida* (0.275 g g^{-1}), UASD-LF1 at 20 days of incubation and with *P. chrysosporium* (0.272 g g^{-1}) and *P. florida* (0.271 g g^{-1}) at 10 days after incubation. It was observed that the hemicellulose content had increased due to crude enzyme inoculation from 10 days incubation upto 20 days incubation and thereafter decreased when analyzed at 30 days incubation period. The decrease in hemicellulose content after 20 days incubation was observed to be significantly low. The lowest mean hemicellulose content was observed in uninoculated control treatment, 0.250 g per g at 30 days incubation period.

The hemicellulose content with regards to different substrates also indicated significant differences. The mean maximum hemicellulose content, 0.301 g per g was recorded with sugarcane bagasse. It was significantly superior over rest of the substrate in hemicellulose content. The lowest mean hemicellulose content was observed with sugarcane tops (0.243 g g^{-1}).

Among the particle sizes, the significant mean maximum hemicellulose content (0.271 g g^{-1}) was noticed with 0.50 mm particle size which was found to be significantly superior over 1.0 mm (0.265 g g^{-1}) and 10.0 mm (0.259 g g^{-1}) particle size. These particle sizes differed significantly with each other.

The interactions of combinations of two and three factors did not differ with regards to hemicellulose content.

4.5.4 Lignin content

The data on influence of inoculation of lignolytic crude enzymes on lignin content is furnished in Table 17. The individual treatment factors *viz.*, lignolytic crude enzymes, substrates and particle sizes showed significant variations in lignin content. The interaction of any of these factors with the other as well as their combined intersections were found to be non-significant.

As regards the inoculation of lignolytic crude enzymes, all the crude enzymes were found to be significant in decreasing the lignin content at a given incubation period. The significantly mean maximum lignin content was observed with uninoculated control, 0.150 g per g lignin at all the incubation periods studied. Among the fungal crude enzymes, the significant mean maximum lignin content, 0.137 g per g was observed with *P. florida* at 10 days of incubation. It was found to be on par with *P. chrysosporium* and UASD-LF1 (0.136 g g^{-1}) crude enzymes at 10 days incubation period. The lignin content had decreased with extension of incubation period with all the lignolytic crude enzymes. The significant mean lowest lignin content of 0.126 g per g was observed with both *P. chrysosporium* and *P. florida* crude enzymes at the end of 30 days incubation and they were found to be on par (0.128 g g^{-1}) with UASD-LF1 crude enzyme after 30 days incubation with respect to lignin content.

Among the substrates subjected for crude lignolytic enzyme pre-treatment, sugarcane trash exhibited the mean maximum lignin content (0.143 g g^{-1}). It was found to be on par with sugarcane tops (0.142 g g^{-1}) and corn stover (0.139 g g^{-1}) and superior over both corn husk and sugarcane bagasse (0.128 g g^{-1}) substrates with respect to lignin content.

The particle size of the substrates also indicated significant influence on lignin content. The three particle sizes evaluated differed significantly with each other. The mean maximum of 0.142 g per g lignin was observed with 10.0 mm particle size, followed by 1.0 mm (0.136 g g^{-1}) and 0.50 mm (0.131 g g^{-1}) particle sizes with respect to lignin content.

The interaction between two and three factors was insignificant to lignin content. The highest lignin content was observed with uninoculated control (0.160 g g^{-1}) with sugarcane trash irrespective of particle size and incubation period. The lowest lignin content was recorded with bagasse (0.100 g g^{-1}) particle size 0.5 mm pre-treated with *P. chrysosporium* crude enzyme for 30 days incubation period.

Table 17: Effect of crude enzymes of lignolytic fungi on lignin content of substrates

Lignolytic fungi	Particle size (mm)	Substrates ($g\ g^{-1}$)					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
Phanerochaete chrysosporium (10 days)	0.5	0.120	0.133	0.133	0.127	0.133	**0.136**
	1.0	0.127	0.147	0.140	0.127	0.140	
	10.0	0.133	0.160	0.147	0.133	0.147	
Pleurotus florida (10 days)	0.5	0.120	0.133	0.140	0.127	0.133	**0.137**
	1.0	0.127	0.140	0.147	0.127	0.140	
	10.0	0.133	0.153	0.153	0.133	0.147	
UASD-LF1 (10 days)	0.5	0.127	0.133	0.133	0.120	0.133	**0.136**
	1.0	0.133	0.140	0.147	0.127	0.140	
	10.0	0.127	0.147	0.153	0.133	0.143	
Control (10 days)	0.5	0.140	0.157	0.160	0.140	0.153	**0.150**
	1.0	0.140	0.157	0.160	0.140	0.153	
	10.0	0.140	0.157	0.160	0.140	0.153	
Phanerochaete chrysosporium (20 days)	0.5	0.107	0.127	0.127	0.120	0.127	**0.130**
	1.0	0.113	0.140	0.133	0.120	0.133	
	10.0	0.133	0.147	0.140	0.140	0.140	
Pleurotus florida (20 days)	0.5	0.113	0.127	0.133	0.120	0.127	**0.131**
	1.0	0.113	0.133	0.140	0.127	0.133	
	10.0	0.133	0.147	0.147	0.127	0.140	
UASD-LF1 (20 days)	0.5	0.127	0.127	0.127	0.113	0.127	**0.130**
	1.0	0.133	0.133	0.133	0.120	0.133	
	10.0	0.133	0.140	0.140	0.127	0.140	
Control (20 days)	0.5	0.140	0.157	0.160	0.140	0.153	**0.150**
	1.0	0.140	0.157	0.160	0.140	0.153	
	10.0	0.140	0.157	0.160	0.140	0.153	
Phanerochaete chrysosporium (30 days)	0.5	0.100	0.123	0.127	0.117	0.123	**0.126**
	1.0	0.110	0.137	0.130	0.120	0.130	
	10.0	0.130	0.143	0.137	0.130	0.140	
Pleurotus florida (30 days)	0.5	0.110	0.123	0.130	0.117	0.120	**0.126**
	1.0	0.110	0.130	0.137	0.120	0.127	
	10.0	0.130	0.143	0.143	0.123	0.133	
UASD-LF1 (30 days)	0.5	0.123	0.127	0.123	0.107	0.123	**0.128**
	1.0	0.130	0.133	0.133	0.113	0.133	
	10.0	0.133	0.140	0.140	0.120	0.140	
Control (30 days)	0.5	0.140	0.157	0.160	0.140	0.153	**0.150**
	1.0	0.140	0.157	0.160	0.140	0.153	
	10.0	0.140	0.157	0.160	0.140	0.153	
Mean		**0.128**	**0.142**	**0.143**	**0.128**	**0.139**	
Mean	0.5	0.131					
	1.0	0.136					
	10.0	0.142					

	SE±	CD 1 %
Lignolytic fungi (A)	0.002	0.007
Substrates (B)	0.001	0.005
Particle size (C)	0.001	0.004
A x B	0.005	NS
A x C	0.004	NS
B x C	0.002	NS
A x B x C	0.008	NS

Contd…..

Table 17a: Per cent decrease in lignin content over initial lignin content

Lignolytic fungi	Particle size (mm)	Substrates (%)				
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover
Phanerochaete chrysosporium (10 days)	0.5	-14.29	-15.29	-16.88	-9.29	-13.07
	1.0	-9.29	-6.37	-12.50	-9.29	-8.50
	10.0	-5.00	1.91	-8.13	-5.00	-3.92
Pleurotus florida (10 days)	0.5	-14.29	-15.29	-12.50	-9.29	-13.07
	1.0	-9.29	-10.83	-8.13	-9.29	-8.50
	10.0	-5.00	-2.55	-4.38	-5.00	-3.92
UASD-LF1 (10 days)	0.5	-9.29	-15.29	-16.88	-14.29	-13.07
	1.0	-5.00	-10.83	-8.13	-9.29	-8.50
	10.0	-9.29	-6.37	-4.38	-5.00	-6.54
Control (10 days)	0.5	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00
Phanerochaete chrysosporium (20 days)	0.5	-23.57	-19.11	-20.63	-14.29	-16.99
	1.0	-19.29	-10.83	-16.88	-14.29	-13.07
	10.0	-5.00	-6.37	-12.50	0.00	-8.50
Pleurotus florida (20 days)	0.5	-19.29	-19.11	-16.88	-14.29	-16.99
	1.0	-19.29	-15.29	-12.50	-9.29	-13.07
	10.0	-5.00	-6.37	-8.13	-9.29	-8.50
UASD-LF1 (20 days)	0.5	-9.29	-19.11	-20.63	-19.29	-16.99
	1.0	-5.00	-15.29	-16.88	-14.29	-13.07
	10.0	-5.00	-10.83	-12.50	-9.29	-8.50
Control (20 days)	0.5	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00
Phanerochaete chrysosporium (30 days)	0.5	-28.57	-21.66	-20.63	-16.43	-19.61
	1.0	-21.43	-12.74	-18.75	-14.29	-15.03
	10.0	-7.14	-8.92	-14.38	-7.14	-8.50
Pleurotus florida (30 days)	0.5	-21.43	-21.66	-18.75	-16.43	-21.57
	1.0	-21.43	-17.20	-14.38	-14.29	-16.99
	10.0	-7.14	-8.92	-10.63	-12.14	-13.07
UASD-LF1 (30 days)	0.5	-12.14	-19.11	-23.13	-23.57	-19.61
	1.0	-7.14	-15.29	-16.88	-19.29	-13.07
	10.0	-5.00	-10.83	-12.50	-14.29	-8.50
Control (30 days)	0.5	0.00	0.00	0.00	0.00	0.00
	1.0	0.00	0.00	0.00	0.00	0.00
	10.0	0.00	0.00	0.00	0.00	0.00

Note : Initial lignin content ($g\ g^{-1}$) Sugarcane bagasse (0.140), Sugarcane tops (0.157), Sugarcane trash (0.160), Corn husk (0.140) and Corn stover (0.153)

4.6 Effect of different cellulolytic fungi and their inoculum levels on release of reducing sugars from different alkali pre-treated substrates

4.6.1 Sugarcane bagasse

Sugarcane bagasse which was treated with 3.0% NaOH for 8 hours at room temperature were then subjected to autoclaving at 121^0C for 1 hour and saccharified with cellulolytic fungi. The release of reducing sugars due to inoculation of cellulolytic fungi varied significantly with all the individual factors such as different cellulolytic fungi, their inoculum levels and different days of incubation. The two way interactions between cellulolytic fungi and inoculum levels, cellulolytic fungi and incubation days and incubation days and inoculum levels as well as the combined interaction of all the three factors also resulted in variations in release of reducing sugars. The data is presented in Table 18.

The cellulolytic fungi *Trichoderma reesei* produced the mean maximum release of reducing sugars (115.66 mg g^{-1}), which was significantly superior over other fungi. The lowest release of reducing sugars was observed in the control treatment which received no fungi inoculation.

The release of reducing sugars as influenced by inoculum levels indicated the maximum release of reducing sugars with 6.0% inoculum level (94.62 mg g^{-1}), which was on par with 4.0% inoculum level (91.16 mg g^{-1}) but significantly superior over 2.0% inoculum level across the cellulolytic fungi.

As regards incubation time, incubation upto 8 days resulted in significantly highest release of reducing sugars (122.19 mg g^{-1}) but found on par with 6 days incubation time (122.02 mg g^{-1}) with respect to release of reducing sugars. Incubation upto 6 days and 8 days were found to be significantly superior over 4 days (80.29 mg g^{-1}) and 2 days incubation (27.93 mg g^{-1}).

The interaction of factors, cellulolytic fungi and their inoculum levels indicated a maximum release of reducing sugars with *T. reesei* which yielded 122.27 mg per g reducing sugar at 6% inoculum level. It was found to be on par with inoculum level 4% (120.77 mg g^{-1}) of the same organism, with fungi *A. awamorii* (119.01 mg g^{-1}) and with *A. sydowii* (116.03 mg g^{-1}) at 6% inoculum level with regards to release of reducing sugar.

The least release of reducing sugars among the cellulolytic fungi was observed in case of *P. chrysosporium* at all levels of inoculum 88.23 (6%), 85.02 (4%) and 68.09 mg per g (2%). Significantly the lowest release of reducing sugars was found in the control treatment (15.12 mg g^{-1} reducing sugar).

The interaction between the cellulolytic fungi and incubation periods produced the significantly higher reducing sugar with *T. Reesei,* 167.82 mg per g reducing sugars in 8 days, however it was on par with 6 days incubation (162.52 mg g^{-1}) period by same organism and with *T. viridae* (157.98 mg g^{-1}) at 8 days incubation period. These combinations of cellulolytic fungi and incubation periods were found to be superior over other combinations. The control treatment resulted in significantly the lowest reducing sugar content of 17.27 mg per g at 8 days incubation period.

The release of reducing sugars due to combination of levels of inoculum and incubation period produced significantly the highest reducing sugars of 127.48 mg per g at 4% inoculum level in 6 days incubation period. However, it was on par with 8 days of incubation time (125.01 mg g^{-1}) of 4% inoculum level and with 6% inoculum level (123.40 mg g^{-1} at 6 days, 120.76 mg g^{-1}) at 8 days as well as with 2% inoculum level (120.80 mg g^{-1}) at 8 days incubation period.

The reducing sugars released in sugarcane bagasse due to effect of combinations of different cellulolytic cultures, their inoculum levels and incubation periods varied differently. *T. reesei* showed the highest release of reducing sugars with 2% inoculum level on 8th day of incubation, 174.12 mg per g with 24.76 per cent conversion of cellulose to glucose.

Table 18: Effect of different cellulolytic fungi and their various inoculum levels on release of reducing sugars (mg g^{-1}) from pre-treated bagasse

Cellulolytic fungi	Incubation period (days)	Inoculums			Mean
		2 %	4 %	6 %	
Trichoderma viridae	2	9.46 (01.35)	10.89 (01.55)	22.79 (03.24)	**101.34**
	4	63.55 (09.04)	74.03 (10.53)	98.33 (13.98)	
	6	149.81 (21.30)	162.73 (23.14)	150.59 (21.41)	
	8	152.58 (21.70)	164.87 (23.44)	156.49 (22.25)	
Trichoderma reesei	2	13.55 (01.93)	17.74 (02.52)	20.98 (02.98)	**115.66**
	4	83.27 (11.84)	124.00 (17.63)	137.33 (19.53)	
	6	144.79 (20.59)	173.33 (24.64)	169.44 (24.09)	
	8	174.12 (24.76)	168.00 (23.88)	161.33 (22.94)	
Aspergillus sydowiii	2	17.74 (02.52)	52.60 (07.48)	89.17 (12.68)	**104.89**
	4	84.41 (12.00)	110.79 (15.75)	114.31 (16.25)	
	6	136.70 (19.44)	135.74 (19.30)	134.98 (19.19)	
	8	129.46 (18.40)	127.17 (18.08)	125.65 (17.86)	
Aspergillus awamorii	2	32.12 (04.57)	63.74 (09.06)	78.60 (11.18)	**111.17**
	4	81.84 (11.64)	118.03 (16.78)	120.2 (217.09)	
	6	137.46 (19.55)	145.84 (20.73)	142.7 (92.30)	
	8	141.17 (20.07)	137.84 (19.60)	134.41 (19.11)	
Phanerochaete chrysosporium	2	9.46 (01.35)	15.46 (02.20)	20.89 (02.97)	**80.45**
	4	50.51 (07.18)	59.73 (08.49)	74.31 (10.56)	
	6	102.20 (14.53)	130.00 (18.48)	128.35 (18.25)	
	8	110.21 (15.67)	134.89 (19.18)	129.39 (18.39)	
Control	2	9.16 (01.30)	9.16 (01.30)	9.16 (01.30)	**15.12**
	4	16.83 (02.39)	16.83 (02.39)	16.83 (02.39)	
	6	17.22 (02.45)	17.22 (02.45)	17.22 (02.45)	
	8	17.27 (02.45)	17.27 (02.45)	17.27 (02.45)	
Mean		**78.54**	**91.16**	**94.62**	
Incubation period (days)	2	27.93			
	4	80.29			
	6	122.02			
	8	122.19			

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content (633 mg g^{-1})

Contd…..

Two way interaction A x B

Cellulolytic fungi \ Inoculums	2 %	4 %	6 %	Mean
Trichoderma viridae	93.85	103.13	107.05	**101.34**
Trichoderma reesei	103.94	120.77	122.27	**115.66**
Aspergillus sydowiii	92.08	106.58	116.03	**104.89**
Aspergillus awamorii	98.15	116.36	119.01	**111.17**
Phanerochaete chrysosporium	68.09	85.02	88.23	**80.45**
Control	15.12	15.12	15.12	**15.12**
Mean	**78.54**	**91.16**	**94.62**	**101.34**

Two way interaction A x C

Cellulolytic fungi \ Incubation period	2	4	6	8	Mean
Trichoderma viridae	14.38	78.64	154.38	157.98	**101.34**
Trichoderma reesei	17.43	114.87	162.52	167.82	**115.66**
Aspergillus sydowiii	53.17	103.17	135.81	127.42	**104.89**
Aspergillus awamorii	58.15	106.70	142.03	137.81	**111.17**
Phanerochaete chrysosporium	15.27	61.52	120.18	124.83	**80.45**
Control	9.16	16.83	17.22	17.27	**15.12**
Mean	**27.93**	**80.29**	**122.02**	**122.19**	

Two way interaction C x B

	2 %	4 %	6 %	Mean
2	15.25	28.26	40.27	**27.93**
4	63.40	83.90	93.56	**80.29**
6	114.70	127.48	123.90	**122.02**
8	120.80	125.01	120.76	**122.19**
Mean	**78.54**	**91.16**	**94.62**	

	SEm ±	CD at 1%
Treatments (A)	1.373	5.070
Inoculums (B)	0.971	3.585
Incubation period (C)	1.121	4.140
A x B	2.379	8.782
A x C	2.747	10.140
B x C	1.942	7.170
A x B x C	4.758	17.563

This combination was found to be at par with 4% inoculum (173.33 mg g^{-1} and 24.64%), 6% inoculum (169.44 mg g^{-1} and 24.09%) reducing sugars and per cent saccharification, respectively in 6 days of incubation period. The 4% (168.00 mg g^{-1}) and 6% (161.33 mg g^{-1}) inoculum levels did not result in release of higher reducing sugars on 8th day of incubation and were found to be at par with 6 days of incubation with *T. reesei*. Similarly, *T. viridae* with 4% inoculum level in 8 days (164.89 mg g^{-1}) and 6 days (162.73 mg g^{-1}) incubation was found to be on par with the above treatment combinations. These treatments were highly superior over other treatment combinations.

It was observed that the release of reducing sugars had increased with increase in inoculum levels from 2 to 6% from 2 days of incubation in all the cellulolytic fungi. Significant amount of reducing sugars release was produced by *A. sydowii* and *A. awamorii* with 89.17 and 78.60 mg per g reducing sugars and 12.68 and 11.18 per cent saccharification, respectively in 2 days incubation period, but the release of reducing sugars had reduced in these two organism from 4th day incubation onwards and reached a maximum of 135.74 and 145.84 mg per g reducing sugars in 6 days incubation with at 4% inoculum level when compared to *T. reesei* (173.33 mg g^{-1}) and *T. viridae* (162.73 mg g^{-1}) with the same inoculum level in 6 days incubation period.

4.6.2 Sugarcane tops

The substrate sugarcane tops pre-treated with 3.0% NaOH for 8 hours at room temperature and autoclaved at 121^{0}C for one hour was subjected to saccharification with cellulolytic fungi. The reducing sugars released due to inoculation of fungi varied differently in sugarcane tops. The individual factors as well as combination of two and three factors showed a significant variation for release of reducing sugars observed in Table 19.

The mean highest release of reducing sugar was observed with the cellulolytic fungi *T. reesei* (130.74 mg g^{-1} reducing sugars). It was significantly superior over all other fungi. All the fungi differed significantly over one another with respect to release of reducing sugars. *T. viridae* (122.58 mg g^{-1}), *A. awamorii* (116.43 mg g^{-1}), *A. sydowii* (113.06 mg g^{-1}) and *P. chrysosporium* (74.16 mg g^{-1}) exhibited the order of decreasing amounts of reducing sugar release was recorded in the control treatment (14.40 mg g^{-1} reducing sugar).

The inoculum level 6% resulted in significantly mean maximum release of reducing sugar (102.61 mg g^{-1}), followed by 4% (99.49 mg g^{-1}) and 2% (83.40 mg g^{-1}) inoculum levels. All these inoculum levels differed significantly with one another.

Incubation period of 8 days produced a statistically superior amounts of reducing sugar, 124.71 mg per g, when compared to 6 days (120.42 mg g^{-1}), 4 days (90.35 mg g^{-1}) and 2 days (45.19 mg g^{-1}) incubation periods with respect to release of reducing sugars all these incubation periods differed significantly with each other.

The interaction of cellulolytic fungi and inoculum levels indicated the significant highest release of reducing sugars for *T. reesei* at 6% (138.65 mg g^{-1}) and at 4% (137.16 mg g^{-1}) inoculum levels. These two levels were on par with each other and significantly superior over other combinatiuons of cellulolytic fungi and inoculum levels. *T. viridae* at 4% inoculum level was the second best interms of release of reducing sugars at 130.96 mg per g. P. ch ysosporiuym showed the least release of reducing sugars at all levels of inoculum and statistically lowest reducing sugar was observed with control, 14.04 mg per g reducing sugar.

Trichoderma reesei was found to release significantly the highest quantity of reducing sugars at 8 days of incubation period (169.69 mg g^{-1}) and at 6 days of incubation period (168.36 mg g^{-1}) due to interaction effects of cellulolytic fungi and incubation periods. These combinations were on par with *T. viridae* at 8 days incubation period (166.38 mg g^{-1}). *T. reesei* at 8 and 6 days incubation period was *T. viridae* at 8 days incubation were significantly superior over all other fungal inoculations at all incubation periods. *P. chysosporium* was observed to be the fungi to release least quantity of reducing sugars and the lowest release of reducing sugars was observed in control treatment with 15.93 mg per g reducing sugar at 8 days incubation period. The release of reducing sugars with respect to inoculum level and incubation period indicated the highest release of reducing sugar at 4% inoculum level in 8 days incubation period (127.81 mg g^{-1}). It was significantly superior over other combinations of inoculum levels and incubation periods.

Table 19: Effect of different fungi inoculation and their inoculum levels on release of reducing sugars (mg g^{-1}) from pre-treated sugarcane tops

Treatments	Incubation period (days)	Inoculums			Mean
		2 %	4 %	6 %	
Trichoderma viridae	2	17.46 (02.56)	55.94 (08.21)	62.13 (09.12)	122.58
	4	106.89 (15.69)	128.50 (18.87)	129.84 (19.06)	
	6	155.84 (22.88)	166.50 (24.45)	148.69 (21.83)	
	8	166.13 (24.39)	172.89 (25.38)	160.12 (23.51)	
Trichoderma reesei	2	23.46 (03.44)	74.03 (10.87)	83.65 (12.28)	130.74
	4	101.08 (14.84)	130.21 (19.12)	142.26 (20.88)	
	6	167.33 (24.57)	171.39 (25.16)	166.34 (24.42)	
	8	173.73 (25.51)	173.02 (25.40)	162.33 (23.83)	
Aspergillus sydowiii	2	25.08 (03.68)	75.46 (11.08)	89.36 (13.12)	113.06
	4	80.79 (11.86)	125.07 (18.36)	131.74 (19.34)	
	6	140.03 (20.56)	141.08 (20.71)	143.08 (21.01)	
	8	137.84 (20.24)	134.22 (19.71)	132.98 (19.52)	
Aspergillus awamorii	2	37.46 (05.50)	77.93 (11.44)	94.03 (13.81)	116.43
	4	87.27 (12.81)	128.31 (18.84)	134.22 (19.70)	
	6	142.89 (20.98)	144.98 (21.29)	141.08 (20.71)	
	8	139.07 (20.42)	137.08 (20.13)	132.89 (19.51)	
Phanerochaete chrysosporium	2	24.89 (03.66)	15.65 (02.30)	26.79 (03.94)	74.16
	4	35.08 (05.15)	55.36 (08.13)	66.69 (09.79)	
	6	78.88 (11.58)	90.12 (13.23)	121.65 (17.86)	
	8	104.31 (15.31)	133.74 (19.64)	136.69 (20.07)	
Control	2	10.03 (01.47)	10.03 (01.47)	10.03 (01.47)	14.04
	4	14.32 (02.10)	14.32 (02.10)	14.3202.10)	
	6	15.89 (02.33)	15.89 (02.33)	15.89 (02.33)	
	8	15.93 (02.34)	15.93 (02.34)	15.93 (02.34)	
Mean		**83.40**	**99.49**	**102.61**	
Incubation period (days)	2 %	45.19			
	4 %	90.35			
	6 %	120.42			
	8 %	124.71			

Figures in parentheses indicate per cent saccharification

te : Initial cellulose content (613 mg g^{-1})

Contd…..

Two way interaction A x B

	2 %	**4 %**	**6 %**	**Mean**
Trichoderma viridae	111.58	130.96	125.20	**122.58**
Trichoderma reesei	116.40	137.16	138.65	**130.74**
Aspergillus sydowiii	95.93	118.96	124.29	**113.06**
Aspergillus awamorii	101.67	122.08	125.55	**116.43**
Phanerochaete chrysosporium	60.79	73.72	87.96	**74.16**
Control	14.04	14.04	14.04	**14.04**
Mean	**83.40**	**99.49**	**102.61**	

Two way interaction A x C

	2	**4**	**6**	**8**	**Mean**
Trichoderma viridae	45.17	121.74	157.01	166.38	**122.58**
Trichoderma reesei	60.38	124.51	168.36	169.69	**130.74**
Aspergillus sydowiii	63.30	112.53	141.39	135.01	**113.06**
Aspergillus awamorii	69.81	116.60	142.98	136.35	**116.43**
Phanerochaete chrysosporium	22.44	52.38	96.88	124.92	**74.16**
Control	10.03	14.32	15.89	15.93	**14.04**
Mean	**45.19**	**90.35**	**120.42**	**124.71**	

Two way interaction C x B

	2 %	**4 %**	**6 %**	**Mean**
2	23.06	51.51	61.00	**45.19**
4	70.90	96.96	103.18	**90.35**
6	116.81	121.66	122.79	**120.42**
8	122.84	127.81	123.49	**124.71**
Mean	**83.40**	**99.49**	**102.61**	

	SEm ±	**CD at 1%**
Treatments (A)	0.607	2.241
Inoculums (B)	0.429	1.585
Incubation period (C)	0.496	1.830
A x B	1.052	3.882
A x C	1.214	4.483
B x C	0.859	3.170
A x B x C	2.103	7.764

The next best combination observed was 2% inoculum level in 8 days (122.84 mg g^{-1}), 6% inoculum level in 8 days (123.49 mg g^{-1}) and 6 days (122.79 mg g^{-1}) and 4% inoculum level in 6 days (121.66 mg g^{-1}). All these combinations are at par with each other for release of reducing sugars.

The combined interaction effect of cellulolytic cultures, levels of inoculum and incubation period showed a significant differences in release of reducing sugars and per cent Saccharification in sugarcane tops.

Trichoderma reesei in 8 days of incubation produced significantly the highest reducing sugar when 2% inoculum was used with a reducing sugar content of 173.73 mg per g at 25.51 per cent saccharification. It was found to be on par with 4% inoculum level in 8 days (173.02 mg g^{-1}) and in 6 days (171.39 mg g^{-1}) incubation in case of *T. reesei* and in 8 days (172.89 mg g^{-1}) and 6 days (166.50 mg g^{-1}) incubation with *T. viridae* of 4% inoculum level. Both the cellulolytic fungi fared well in releasing the reducing sugars from 4 days after incubation. The lowest release of reducing sugars was recorded in control with 15.93 mg per g reducing sugars in 8 days incubation period.

The maximum saccharification of sugars was achieved with *T. reesei* with 2% inoculum in 8 days incubation period (25.51%), which was significantly highest when compared to all other combinations of cellulolytic fungi inoculation levels and incubation periods.

4.6.3 Sugarcane trash

The data presented in Table 20 indicates reducing sugars released from 3.00% NaOH treated sugarcane trash for 3 hours at room temperature followed by autoclaving at 121^{0}C for one hour. A significant difference in release of reducing sugars was observed in sugarcane trash. All the individual factors and their two way and three way combinations varied significantly

Among the cellulolytic fungi, all the fungi differed statistically with each other interms of release of reducing sugars. A mean maximum reducing sugar of 135.17 mg per g was released by *T. reesei*. Followed by *T. reesei* was *T. viridae* (125.67 mg g^{-1}), *A. awamorii* (114.67 mg g^{-1}), *A. sydowii* (110.15 mg g^{-1}) and *P. chrysosporium* (74.79 mg g^{-1}) in that decreasing order of sugar released. The lowest reducing sugar release was observed in control at 13.41 mg per g reducing sugar.

The inoculum level 6% showed significantly highest average reducing sugar content of 103.22 mg per g, followed by 4% (94.96 mg g^{-1}) and 2% (83.74 mg g^{-1}) inoculums with respect to reducing sugar released. All these inoculum levels differed significantly over one another for inoculum levels.

The release of reducing sugars varied with incubation periods. A mean highest release was noticed in 8 days incubation at 125.04 mg per g, which was significantly superior over 6 days (122.32 mg g^{-1}), 4 days (89.53 mg g^{-1}) and 2 days (45.69 mg g^{-1}) incubation period. All these incubation periods differed statistically with one another.

The release of reducing sugars as influenced by different cellulolytic fungi and inoculum levels indicated a varied sugar content. A highest reducing sugar release was recorded by the fungi *T. reesei* at 6% inoculum level (142.70 mg g^{-1}) and at 4% inoculum level (141.81 mg g^{-1}). These two inoculum levels of *T. reesei* were on par with each other and significantly superior over the next best fungi *T. viridae* which recorded 131.41 mg per g reducing sugar at 6% inoculum level and 130.77 mg per g reducing sugar at 4% inoculum levels and over of the fungi and their inoculum levels interms of reducing sugars released. A least release of reducing sugar was observed in *P. chrysosporium*. The control treatment resulted in significantly lowest release of reducing sugars (13.41 mg g^{-1}).

As regards to different fungi inoculation and their incubation period, it was noticed that fungi *T. reesei* released significantly higher quantities of reducing sugar at 6 days incubation period with a release of 176.20 mg per g reducing sugar. The same organism at 8 days incubation released 175.74 mg per g, which was less compared to 6 days incubation but statistically at par with 6 days incubation. The next significantly higher reducing sugars was released by the fungi *T. viridae* at 6 days (167.46 mg g^{-1}) and 8 days (166.82 mg g^{-1}) incubation.

Table 20: Effect of different fungi inoculation and their inoculum levels on release of reducing sugars (mg g^{-1}) from pre-treated sugarcane trash

Treatments	Incubation period (days)	Inoculums			Mean
		2 %	4 %	6 %	
Trichoderma viridae	2	22.51 (03.31)	58.31 (08.56)	72.41 (10.63)	125.67
	4	108.31 (15.90)	120.41 (17.68)	123.27 (18.10)	
	6	161.27 (23.68)	171.46 (25.17)	169.65 (24.91)	
	8	167.27 (24.56)	172.89 (25.38)	160.31 (23.54)	
Trichoderma reesei	2	23.46 (03.44)	77.08 (11.32)	80.79 (11.86)	135.17
	4	111.45 (16.36)	130.60 (19.17)	142.89 (20.98)	
	6	174.07 (25.56)	180.50 (26.50)	174.03 (25.55)	
	8	175.08 (25.70)	179.08 (26.29)	173.08 (25.41)	
Aspergillus sydowiii	2	23.46 (03.44)	80.98 (11.89)	89.46 (13.13)	110.15
	4	82.03 (12.04)	120.50 (17.69)	128.50 (18.87)	
	6	137.74 (20.22)	135.08 (19.83)	129.08 (18.95)	
	8	132.70 (19.48)	132.89 (19.51)	129.36 (18.99)	
Aspergillus awamorii	2	36.69 (05.39)	79.17 (11.62)	95.17 (13.97)	114.67
	4	85.17 (12.50)	124.41 (18.27)	133.74 (19.63)	
	6	141.94 (20.84)	139.74 (20.52)	136.12 (19.99)	
	8	137.65 (20.21)	135.27 (19.86)	130.98 (19.23)	
Phanerochaete chrysosporium	2	8.31 (01.22)	16.31 (02.39)	25.84 (03.79)	74.79
	4	39.81 (05.85)	60.60 (08.90)	68.31 (10.03)	
	6	80.03 (11.75)	96.70 (14.20)	125.64 (18.44)	
	8	107.26 (15.75)	133.55 (19.61)	135.08 (19.83)	
Control	2	10.84 (01.59)	10.84 (01.59)	10.84 (01.59)	13.41
	4	10.50 (01.54)	10.50 (01.54)	10.50 (01.54)	
	6	16.22 (02.38)	16.22 (02.38)	16.22 (02.38)	
	8	16.08 (02.36)	16.08 (02.36)	16.08 (02.36)	
Mean		**83.74**	**99.96**	**103.22**	
Incubation period (days)	2 %	45.69			
	4 %	89.53			
	6 %	122.32			
	8 %	125.04			

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content (613 mg g^{-1})

Contd…..

Two way interaction A x B

	2 %	4 %	6 %	Mean
Trichoderma viridae	114.84	130.77	131.41	**125.67**
Trichoderma reesei	121.01	141.81	142.70	**135.17**
Aspergillus sydowiii	93.98	117.36	119.10	**110.15**
Aspergillus awamorii	100.36	119.65	124.00	**114.67**
Phanerochaete chrysosporium	58.85	76.79	88.72	**74.79**
Control	13.41	13.41	13.41	**13.41**
Mean	**83.74**	**99.96**	**103.22**	

Two way interaction A x C

	2	4	6	8	Mean
Trichoderma viridae	51.08	117.33	167.46	166.82	**125.67**
Trichoderma reesei	60.44	128.31	176.20	175.74	**135.17**
Aspergillus sydowiii	64.63	110.35	133.97	131.65	**110.15**
Aspergillus awamorii	70.34	114.44	139.27	134.63	**114.67**
Phanerochaete chrysosporium	16.82	56.24	100.79	125.30	**74.79**
Control	10.84	10.50	16.22	16.08	**13.41**
Mean	**45.69**	**89.53**	**122.32**	**125.04**	

Two way interaction C x B

	2 %	4 %	6 %	Mean
2	20.88	53.78	62.42	**45.69**
4	72.88	94.50	101.20	**89.53**
6	118.54	123.28	125.12	**122.32**
8	122.67	128.29	124.15	**125.04**
Mean	**83.74**	**99.96**	**103.22**	

	SEm ±	CD at 1%
Treatments (A)	0.320	1.182
Inoculums (B)	0.226	0.836
Incubation period (C)	0.261	0.965
A x B	0.555	2.047
A x C	0.640	2.364
B x C	0.453	1.672
A x B x C	1.109	4.095

The other fungi released significantly very less quantities of reducing sugar at all incubation periods, when compared to *T. reesei* at 6 days incubation. The lowest release of reducing sugar was noticed in control treatment without inoculation at 16.08 mg per g on 8th day of incubation.

The interaction between the inoculum levels of different fungi and their incubation periods indicated that significantly highest quantity of reducing sugar was released by *T. reesei* at 4% inoculum level (128.29 mg g^{-1}) in 8 days incubation. It was significantly superior over other combinations of inoculums and incubation periods. A second best combination of inoculum levels and incubation period observed was 6% inoculum with 6 days incubation period (125.12 mg g^{-1}) with respect to release of reducing sugars in sugarcane trash.

The per cent saccharification and release of reducing sugars due to combination of factors in sugarcane trash showed that significantly the highest saccharificaiton was done by *T. reesei* with 4% inoculum level in 6 days to be at 26.50 per cent (180.50 mg g^{-1} reducing sugar). It was found to be on par with 8 days incubation even though the quantity of reducing sugars did not increase (179.08 mg g^{-1}) on 8 days of incubation. These combinations were observed to be significantly superior over both 6% or 2% inoculum levels of 6 days or 8 days incubation with *T. reesei*, which released 172.89 mg per g reducing sugars in 8 days at 4% inoculum level.

A trend of increase in reducing sugars from 2% inoculum to 6% inoculum level was observed in the beginning stages in all the organisms upto six days of incubation and thereafter the reducing sugar content was stagnant.

4.6.4 Corn husk

The data concerning to the release of reducing sugars from 3.00% NaOH treated corn husk for 8 hours at room temperature followed by autoclaving for 1 hour at 121^{0}C temperature (Table 21) indicated significant variations due to individual effects such as cellulolytic fungi, their inoculum levels and different incubation periods. The interaction of these individual factors with one another as well as their combined interactions were found to be significant.

The different fungi used in the saccharification study differed significantly with one another as regards to release of reducing sugars. The mean maximum release of reducing sugar (135.70 mg per g was observed with *T. reesei* inoculation, followed by *T. viridae* (128.92 mg g^{-1}), *A. awamorii* (112.64 mg g^{-1}), *A. sydowii* (107.67 mg g^{-1}) and *P. chrysosporium* (78.39 mg g^{-1}) inoculations. The lowest release of reducing sugars content, 13.46 mg per g was recorded in control treatment (without fungal inoculation).

The inoculum levels of different fungi were found to differ significantly. The mean maximum reducing sugar content of 103.64 mg per g was observed with 6% inoculum level which was significantly superior to other inoculum levels of 4% (100.26 mg g^{-1}) and 2% (84.49 mg g^{-1}). All these inoculum levels differed significantly over one another.

The incubation periods analysed for release of reducing sugars indicated that the mean maximum 126.48 mg per g reducing sugars was released at 8 days incubation period followed by 6 days (122.89 mg g^{-1}), 4 days (89.20 mg g^{-1}) and 2 days (45.95 mg g^{-1}) incubation period with respect to release of reducing sugars. All these incubation days differed significantly with each other.

The combined effect of cellulolytic fungi and inoculum levels was found to differ for reducing sugars content. *T. reesei* showed significantly the highest release of reducing sugar with 6% inoculum level (143.08 mg g^{-1}) as well as at 4% inoculum (142.86 mg g^{-1}). These two levels were at par with each other and significantly superior over the next best combinations of *T. viridae* both with 4% (134.41 mg g^{-1}) and with 6% (133.31 mg g^{-1}) inoculation with respect to release of reducing sugars. The release of reducing sugars observed for *A. awamorii*, *A. sydowii* and *P. chrysosporium* were significantly lower than *T. reesei* and *T. viridae* at all levels of inoculums. The lowest reducing sugar content was observed in the control treatment, 13.46 mg per g reducing sugars.

The combined effect of cellulolytic fungi and incubation periods also differed significantly. *T. reesei* at 6 days incubation period showed significantly the higher reducing sugars release of 178.70 mg per g.

Table 21: Effect of different fungi inoculation and their inoculum levels on release of reducing sugars (mg g^{-1}) from pre-treated corn husk

Treatments	Incubation period (days)	Inoculums			Mean
		2 %	4 %	6 %	
Trichoderma viridae	2	24.12 (03.50)	66.31 (09.63)	72.51 (10.53)	128.92
	4	109.46 (15.89)	122.79 (17.83)	123.17 (17.88)	
	6	168.90 (24.52)	175.08 (25.41)	171.36 (24.87)	
	8	173.65 (25.21)	173.46 (25.18)	166.22 (24.13)	
Trichoderma reesei	2	24.31 (03.53)	73.08 (10.61)	83.84 (12.17)	135.70
	4	109.50 (15.89)	132.22 (19.20)	140.50 (20.39)	
	6	177.17 (25.72)	185.84 (26.98)	173.08 (25.13)	
	8	173.65 (25.21)	180.31 (26.17)	174.89 (25.39)	
Aspergillus sydowiii	2	27.74 (04.03)	75.08 (10.90)	87.27 (12.67)	107.67
	4	77.46 (11.24)	119.36 (17.33)	124.50 (18.07)	
	6	133.65 (19.40)	132.60 (19.25)	125.55 (18.23)	
	8	131.65 (19.11)	129.27 (18.77)	127.94 (18.57)	
Aspergillus awamorii	2	39.74 (05.77)	77.17 (11.20)	89.46 (12.99)	112.64
	4	80.41 (11.67)	120.85 (17.54)	128.41 (18.64)	
	6	138.60 (20.12)	138.60 (20.12)	135.46 (19.66)	
	8	138.03 (20.04)	133.84 (19.43)	131.17 (19.04)	
Phanerochaete chrysosporium	2	9.46 (01.37)	16.72 (02.43)	30.79 (04.47)	78.39
	4	44.70 (06.49)	56.89 (08.26)	74.98 (10.89)	
	6	79.36 (11.52)	103.65 (15.05)	129.08 (18.74)	
	8	112.31 (16.30)	139.36 (20.23)	143.36 (20.81)	
Control	2	9.84 (01.43)	9.84 (01.43)	9.84 (01.43)	13.46
	4	13.45 (01.95)	13.45 (01.95)	13.45 (01.95)	
	6	14.69 (02.13)	14.69 (02.13)	14.69 (02.13)	
	8	15.86 (02.30)	15.86 (02.30)	15.86 (02.30)	
Mean		**84.49**	**100.26**	**103.64**	
Mean	2 %	45.95			
	4 %	89.20			
	6 %	122.89			
	8 %	126.48			

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content (620 mg g^{-1})

Contd…..

Two way interaction A x B

	2 %	**4 %**	**6 %**	**Mean**
Trichoderma viridae	119.03	134.41	133.31	**128.92**
Trichoderma reesei	121.16	142.86	143.08	**135.70**
Aspergillus sydowiii	92.62	114.08	116.32	**107.67**
Aspergillus awamorii	99.20	117.61	121.12	**112.64**
Phanerochaete chrysosporium	61.46	79.16	94.55	**78.39**
Control	13.46	13.46	13.46	**13.46**
Mean	**84.49**	**100.26**	**103.64**	

Two way interaction A x C

	2	**4**	**6**	**8**	**Mean**
Trichoderma viridae	54.31	118.47	171.78	171.11	**128.92**
Trichoderma reesei	60.41	127.41	178.70	176.28	**135.70**
Aspergillus sydowiii	63.36	107.11	130.60	129.62	**107.67**
Aspergillus awamorii	68.79	109.89	137.55	134.35	**112.64**
Phanerochaete chrysosporium	18.99	58.86	104.03	131.68	**78.39**
Control	9.84	13.45	14.69	15.86	**13.46**
Mean	**45.95**	**89.20**	**122.89**	**126.48**	

Two way interaction C x B

	2 %	**4 %**	**6 %**	**Mean**
2	22.54	53.03	62.28	**45.95**
4	72.50	94.26	100.84	**89.20**
6	118.73	125.08	124.87	**122.89**
8	124.19	128.68	126.57	**126.48**
Mean	**84.49**	**100.26**	**103.64**	

	SEm ±	**CD at 1%**
Treatments (A)	0.257	0.950
Inoculums (B)	0.182	0.671
Incubation period (C)	0.210	0.775
A x B	0.446	1.645
A x C	0.514	1.899
B x C	0.364	1.343
A x B x C	0.891	3.290

The extension of incubation periods upto 8 days (176.28 mg g^{-1}) with this fungi did not increase the reducing sugar content. Followed by *T. reesei*, *T. viridae* recorded the release of 171.78 mg per g and 171.11 mg per g in 6 days and 8 days incubation, respectively. The rest of the fungi *A. awamorii*, *A. sydowii* and *P. chrysosporium* were found to be less efficient in releasing reducing sugars for all the tested incubation periods. The lowest quantity of reducing sugar release was noticed in the control treatment (15.86 mg g^{-1}) at 8 days incubation period.

Combinations of inoculum levels and incubation periods had indicated significantly the highest reducing sugar release, 128.68 mg per g with 4% inoculum level in 8 days incubation period. It was significantly superior over 6% inoculum for 8 days incubation period (126.57 mg g^{-1}) and over 4% and 6% inoculation levels for 6 days incubation period.

The interaction effects of cellulolytic fungi, inoculum levels and the incubation periods were found to vary significantly as regards to release of reducing sugars and per cent saccharification in alkali pre-treated corn husk.

T. reesei with a reducing sugar content of 185.84 mg per g and per cent saccharification of 26.50 with 4% inoculum, in 6 days incubation was found to be superior over rest of the combinations of cellulolytic fungi, inoculation levels and incubation periods with respect to reducing sugar release and per cent saccharification. The lowest reducing sugar content and per cent saccharification was noticed in case of *A. sydowii* which yielded 133.65 mg per g reducing sugar and 20.22 per cent saccharification in 6 days incubation among the cellulolytic fungi. All the cellulolytic fungi had recorded significantly the highest release of reducing sugars and per cent saccharification when compared to control treatment that received no fungal inoculation.

4.6.5 Corn stover

The substrate corn stover pre-treated with 3.00A% NaOH for 8 hours at room temperature and autoclaved at 121^0C for one hour indicated variations in reducing sugar content due to inoculation of cellulolytic fungi (Table 22). The individual effects like cellulolytic fungi, their inoculum levels and incubation periods as well as their interaction with one another and their combined interactions found to be varied significantly.

The different fungi behaved differently with respect to release of reducing sugars. The mean maximum, 134.86 mg per g reducing sugar was released by *T. reesei*. It was significantly higher than other cellulolytic fungi such as *T. viridae* (128.24 mg g^{-1}), *A. awamorii* (111.83 mg g^{-1}), *A. sydowii* (105.42 mg g^{-1}) and *P. chrysosporium* (78.23 mg g^{-1}) with respect to reducing sugars released. Significantly the lowest releasing reducing sugar was observed in the control treatment with 15.01 mg per g reducing sugars.

The inoculum levels as indicated in the Table 22 were also significant for release of reducing sugars. The mean maximum reducing sugar release of 103.49 mg per g was obtained with 6% inoculum level. It was significantly superior over 4% (99.07 mg g^{-1}) and 2% (82.78 mg g^{-1}) inoculum levels with respect to reducing sugars released.

Among the incubation periods, incubation upto 8 days was found to be significantly superior, which yielded 125.15 mg per g reducing sugar when compared to 6 days (121.53 mg g^{-1}), 4 days (89.50 mg g^{-1}) and 2 days (46.20 mg g^{-1}) incubation periods. All these incubation periods differed significantly with each other.

The interaction of cellulolytic fungi and inoculum levels produced the significantly higher release of reducing sugars for *T. reesei* with 6% inoculation (143.29 mg g^{-1}) as well as with 4% (140.80 mg g^{-1}) inoculum levels. These two levels of inoculum for *T. reesei* were found to be at par and significantly superior over *T. viridae* which released 133.55 and 132.08 mg per g reducing with 4% and 6% inoculum levels. The remaining fungi and their inoculum levels were found to be less efficient in releasing the reducing sugars from pre-treated corn stover. The lowest release of reducing sugars was observed with control treatment at 15.01 mg per g reducing sugars.

The combined effect of cellulolytic fungi and their incubation period also influenced greatly with respect to release of reducing sugars. Significantly the highest amount 176.47 mg per g reducing sugars was obtained with *T. reesei* in 6 days incubation time.

Table 22: Effect of different fungi inoculation and their inoculum levels on release of reducing sugars (mg g^{-1}) from pre-treated corn stover

Treatments	Incubation period (days)	Inoculums			Mean
		2 %	4 %	6 %	
Trichoderma viridae	2	23.35 (03.43)	65.46 (09.61)	69.70 (10.23)	128.24
	4	110.79 (16.27)	119.36 (17.52)	121.46 (17.83)	
	6	172.10 (25.27)	174.22 (25.58)	170.03 (24.96)	
	8	170.04 (24.97)	175.17 (25.72)	167.14 (24.54)	
Trichoderma reesei	2	25.25 (03.71)	74.22 (10.90)	79.17 (11.62)	134.86
	4	106.07 (15.57)	127.65 (18.74)	148.68 (21.83)	
	6	174.02 (25.55)	182.98 (26.87)	172.41 (25.31)	
	8	176.40 (25.90)	178.60 (26.22)	172.89 (25.38)	
Aspergillus sydowiii	2	25.70 (03.77)	74.03 (10.87)	88.12 (12.94)	105.42
	4	74.50 (10.94)	114.41 (16.80)	131.74 (19.34)	
	6	126.12 (18.52)	127.74 (18.75)	127.46 (18.71)	
	8	127.84 (18.77)	125.08 (18.36)	122.31 (17.96)	
Aspergillus awamorii	2	37.07 (05.44)	76.22 (11.19)	94.41 (13.86)	111.83
	4	79.70 (11.70)	121.65 (17.86)	131.93 (19.37)	
	6	138.22 (20.30)	133.93 (19.66)	132.51 (19.45)	
	8	135.36 (19.88)	132.32 (19.43)	128.60 (18.88)	
Phanerochaete chrysosporium	2	8.98 (01.32)	18.40 (02.70)	31.17 (04.58)	78.23
	4	48.03 (07.05)	55.55 (08.16)	77.17 (11.33)	
	6	81.46 (11.96)	100.70 (14.78)	126.69 (18.60)	
	8	109.84 (16.12)	140.03 (20.56)	140.69 (20.66)	
Control	2	13.46 (01.98)	13.46 (01.98)	13.46 (01.98)	15.01
	4	14.11 (02.07)	14.11 (02.07)	14.11 (02.07)	
	6	15.68 (02.30)	15.68 (02.30)	15.68 (02.30)	
	8	16.79 (02.47)	16.79 (02.47)	16.79 (02.47)	
Mean		**82.78**	**99.07**	**103.49**	
Mean	2 %	46.20			
	4 %	89.50			
	6 %	121.53			
	8 %	125.15			

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content (613 mg g^{-1})

Contd…..

Two way interaction A x B

	2 %	**4 %**	**6 %**	**Mean**
Trichoderma viridae	119.07	133.55	132.08	**128.24**
Trichoderma reesei	120.43	140.86	143.29	**134.86**
Aspergillus sydowiii	88.54	110.32	117.41	**105.42**
Aspergillus awamorii	97.59	116.03	121.86	**111.83**
Phanerochaete chrysosporium	62.08	78.67	93.93	**78.23**
Control	15.01	15.01	15.01	**15.01**
Mean	**83.79**	**99.07**	**103.93**	

Two way interaction A x C

	2	**4**	**6**	**8**	**Mean**
Trichoderma viridae	52.84	117.20	172.12	170.78	**128.24**
Trichoderma reesei	59.55	127.46	176.47	175.96	**134.86**
Aspergillus sydowiii	62.62	106.89	127.11	125.08	**105.42**
Aspergillus awamorii	69.23	111.09	134.89	132.09	**111.83**
Phanerochaete chrysosporium	19.52	60.25	102.95	130.19	**78.23**
Control	13.46	14.11	15.68	16.79	**15.01**
Mean	**46.20**	**89.50**	**121.53**	**125.15**	

Two way interaction C x B

	2 %	**4 %**	**6 %**	**Mean**
2	22.30	53.63	62.67	**46.20**
4	72.20	92.12	104.18	**89.50**
6	117.93	122.54	124.13	**121.53**
8	122.71	128.00	124.74	**125.15**
Mean	**83.79**	**99.07**	**103.93**	

	SEm ±	**CD at 1%**
Treatments (A)	0.443	1.634
Inoculums (B)	0.313	1.155
Incubation period (C)	0.361	1.334
A x B	0.767	2.830
A x C	0.885	3.267
B x C	0.626	2.310
A x B x C	1.533	5.659

The same organism in 8 days incubation did not significantly increase the reducing sugar content (175.96 mg g^{-1}). However, 6 days and 8 days incubation were found to be on par with *T. reesei* in releasing reducing sugars. The other organisms and their incubation periods were found to be less efficient with regard to release of reducing sugars. The lowest reducing sugar content (16.79 mg g^{-1}) was obtained with control treatment.

Reducing sugars with regards to inoculum levels and incubation period also varied differently. The highest reducing sugar release was recorded with the combination 4% inoculum and 8 days incubation, 128.00 mg per g reducing sugar. It was significantly superior over 6% (124.74 mg g^{-1}) and 2% (122.71 mg g^{-1}) inoculum levels at 8 days as well as at 6 days incubation period.

The per cent saccharification and release of reducing sugars due to the effect of combination of cellulolytic fungi, their inoculum levels and incubation periods significantly differed in corn stover.

Significantly the highest release of reducing sugars and per cent saccharification was recorded in *T. reesei* cellulolytic fungi with 4% inoculum level in 6 days of incubation, where the recorded 182.98 mg per g and 26.98 per cent, reducing sugars and saccharificaiton was found to be on par with 8 days of incubation (178.60 mg g^{-1}) with the same combinations and significantly superior over all other cellulolytic cultures, their inoculum levels and incubation periods. It was also observed that all these cellulolytic fungi almost reached saturation in 6 days incubation with respect to release of reducing sugar. The control treatment was observed to give significantly the lowest reducing sugar release with a maximum reducing sugar content of 16.99 mg per g.

4.7 Optimization of growth conditions for secretion of cellulase

4.7.1 Effect of different temperatures on growth of cellulolytic fungi

The effect of different temperatures *viz.*, 25, 30, 35 and 40^0C on growth of cellulolytic fungi to secrete cellulase enzyme was studied by using Mandel and Waber medium with 1% cellulose as substrate. The cellulase activity in terms of filter paper units indicated significant differences for different culture filtrates, the incubation temperatures of cellulolytic fungi and the combinations of cellulolytic culture filtrate and incubation temperatures. The data is presented in Table 23.

The filter paper activity of different cellulolytic culture filtrates differed significantly with respect to release of glucose from Whatman No. 1 filtrate paper per minute per ml. The mean FPU activity of different crude enzyme extracts ranged between 1.13 and 1.98 U. The significantly higher FPU activity of 1.98 U was observed with *T. reesei* crude enzyme filtrate which was significantly superior over all other crude enzyme extracts of different fungi. The significantly lowest enzyme activity was recorded in crude enzyme extract of *P. chrysosporium* (1.13 U).

The temperature for growth for cellulolytic fungi to secrete cellulase enzyme varied for different temperatures. The significant mean maximum FPU activity, 1.79 U was recorded in the culture filtrates of fungi grown at 30^0C temperature. It was found to be significantly superior over other temperatures studied and significantly lowest mean FPU activity was observed at 40^0C with an activity of 1.14 U.

The combination of crude enzyme extracts and different temperatures of incubation indicated that significantly higher FPU activity was observed at 30^0C in case of *T. reesei* crude enzyme extract. The recorded 2.31 U of FPU activity was found to be highly superior over rest of the crude enzyme extracts of different fungi grown at various temperatures. Also, all the crude enzyme extracts of different fungi were found to be significantly superior at 30^0C when compared to 25, 35 and 40^0C with respect to FPU activity.

4.7.2 Effect of different pH levels on growth of cellulolytic fungi

The five different cellulolytic fungi were grown at different pH such as 5.0, 5.5, 6.0 and 6.5 pH to determine the optimum pH conditions for maximum cellulase activity. The data presented in Table 24. The culture filtrates of different fungi, pH conditions as well as the combinations of fungi and pH conditions differed significantly with respect to cellulase activity as filter paper units (FPU).

Table 23: Effect of different temperatures on growth of cellulolytic fungi to secrete cellulase as FPU activity (U ml^{-1} min^{-1})

Cellulolytic fungi	Temperature ^{0}C (B)				Mean
	25	30	35	40	
Trichoderma viridae	2.03	2.18	2.02	1.36	**1.90**
Trichoderma reesei	2.11	2.31	2.15	1.37	**1.98**
Aspergillus sydowiii	1.36	1.63	1.41	1.04	**1.36**
Aspergillus awamorii	1.33	1.51	1.37	1.01	**1.30**
Phanerochaete chrysosporium	1.15	1.34	1.12	0.93	**1.13**
Mean	**1.60**	**1.79**	**1.61**	**1.14**	
	SE±		**CD (1%)**		
Cellulolytic fungi (A)	0.005		0.018		
Temperature (B)	0.004		0.017		
Interaction (A x B)	0.010		0.037		

Table 24: Effect of different pH levels on growth of cellulolytic fungi to secrete cellulose as FPU activity (U ml^{-1} min^{-1})

Cellulolytic fungi	pH				Mean
	5.0	5.5	6.0	6.5	
Trichoderma viridae	2.05	2.16	2.19	2.13	**2.13**
Trichoderma reesei	2.12	2.30	2.33	2.31	**2.26**
Aspergillus sydowiii	1.43	1.55	1.65	1.57	**1.55**
Aspergillus awamorii	1.40	1.38	1.54	1.45	**1.44**
Phanerochaete chrysosporium	1.28	1.30	1.31	1.30	**1.30**
Mean	**1.65**	**1.74**	**1.80**	**1.75**	
	SE±		**CD (1%)**		
Cellulolytic fungi (A)	0.005		0.017		
pH (B)	0.004		0.016		
Interaction (A x B)	0.009		0.035		

The crude enzyme extracts (filtrates) of different fungi showed significant variations in FPU activity. The mean maximum FPU activity of 2.26 U was observed with *T. reesei* crude enzyme extract, which was found to be significantly superior over *T. viridae* crude enzyme extract (2.13 U), *A. sydowii* (1.55 U), *A. awamorii* (1.44 U) and over *P. chrysosporium* (1.30 U) with respect to FPU activity.

The different pH variations on cellulase activity was also observed to be different. The mean maximum cellulase activity (1.80 U) was observed at pH 6.0. It was found to be significantly superior over 5.0 (1.65 U), 5.50 (1.74 U) and 6.50 (1.75 U) pH with regards to FPU activity.

The cellulase activity due to the effect of combination of crude enzyme extracts and different pH showed marked variations. The highest FPU activity was observed with crude enzyme extract obtained at pH 6.0 in case of *T. reesei* (2.33 U). It was found to be significantly superior over other fungal crude enzyme extracts at pH 6.0 as well as over different pH conditions.

All the crude enzyme extracts (different fungi) obtained at 6.0 pH growth conditions resulted in high FPU activity. *T. viridae* (2.19 U), *T. reesei* (2.33 U), *A. sydowii* (1.65 U), *A. awamorii* (1.54 U) and *P. chrysosporium* (1.31 U) were found to be superior over their respective crude enzyme extracts of 5.0, 5.5 and 6.5 pH growth conditions with respect to secretion of cellulase enzyme.

4.8 Optimization of conditions for crude enzyme activity (cellulase)

4.8.1 Effect of different temperatures on cellulase activity (FPU)

The different cellulolytic fungi crude enzymes extracted at 30^0C temperature and 6.0 pH were determined for FPU activity at different temperatures such as 30, 40 and 50^0C. The culture filtrates (crude enzymes), temperature of reaction as well as their combined effect differed significantly with regards to FPU activity. The data pertaining to FPU activity is presented in Table 25.

The different crude enzymes behaved differently with respect to FPU activity. The highest mean FPU activity was recorded in *T. reesei* crude enzyme extract, where the observed FPU activity of 2.24 U was found to be significantly superior over all other crude enzyme extracts of cellulolytic fungi. *P. crysosporium* recorded the significantly the lowest mean FPU activity, 1.33 U.

Among the temperatures of reaction to release glucose units from Whatman No. 1 filter paper by crude enzymes, it was observed that 50^0C temperature resulted in significantly highest mean FPU activity, 1.87 U. It was found to be significantly superior over 40^0C (1.80 U) and 30^0C (1.60 U) with regards to cellulase activity.

The interaction effect of crude enzyme extracts and different reaction temperatures on FPU activity was found to differ. The highest FPU activity (2.40 U) was observed at 50^0C temperature with *T. reesei* crude enzyme. It was found to be on par with *T. viridae* crude enzyme extract at 50^0C (2.33 U) and with *T. reesei* crude enzyme extract at 40^0C (2.33 U) and significantly superior over other crude enzyme extracts of different temperature combinations. The temperature 50^0C was found to be optimum for all the crude enzyme extracts of cellulolytic fungi for higher FPU activity. At this temperature, the observed FPU activities were 2.33 U (*T. viridae*), 2.40 U (*T. reesei*), 1.67 U (*A. sydowii*), 1.57 U (*A. awamorii*) and 1.40 U (*P. chrysosporium*) found to be significantly superior over 40 and 30^0C reaction temperatures with respect to FPU activity within the enzyme source.

4.8.2 Effect of different buffers on cellulase activity (FPU)

Two different buffers, sodium citrate (0.05 M) and sodium acetate (0.05 M) were used at different pH (4.6, 4.8 and 5.0) to determine the optimum buffer and pH conditions for the cellulase activity. The five different crude enzyme extracts (obtained from growth conditions of 30^0C temperature and pH 6.0) as well as the different pH of buffers and their combined interaction indicates significant difference in FPU activity (Table 26).

Table 25: Effect of different temperatures on cellulase activity (FPU activity) (U ml^{-1} min^{-1})

Cellulolytic fungi	Temperature ^{0}C (B)			Mean
	30	40	50	
Trichoderma viridae	1.97	2.20	2.33	**2.17**
Trichoderma reesei	2.00	2.33	2.40	**2.24**
Aspergillus sydowiii	1.42	1.64	1.67	**1.58**
Aspergillus awamorii	1.36	1.51	1.57	**1.48**
Phanerochaete chrysosporium	1.23	1.34	1.40	**1.33**
Mean	**1.60**	**1.80**	**1.87**	

	SE±	CD (1%)
Cellulolytic fungi (A)	0.01	0.06
Temperature (B)	0.01	0.04
Interaction (A x B)	0.02	0.10

Table 26: Effect of different pH levels on cellulase activity (FPU activity) (U ml^{-1} min^{-1})

Cellulolytic fungi	Buffers (B)						Mean
	Citrate 0.05 M			Acetate 0.05 M			
	4.60	4.80	5.00	4.60	4.80	5.00	
Trichoderma viridae	2.28	2.34	2.28	2.13	2.30	2.15	**2.24**
Trichoderma reesei	2.32	2.38	2.31	2.14	2.25	2.23	**2.27**
Aspergillus sydowiii	1.57	1.66	1.60	1.46	1.57	1.56	**1.57**
Aspergillus awamorii	1.51	1.57	1.52	1.40	1.50	1.46	**1.49**
Phanerochaete chrysosporium	1.37	1.43	1.39	1.34	1.39	1.35	**1.38**
Mean	**1.81**	**1.88**	**1.82**	**1.70**	**1.80**	**1.75**	

	SE±	CD (1%)
Cellulolytic fungi (A)	0.006	0.022
Buffers (B)	0.007	0.025
Interaction (A x B)	0.015	0.055

The different fungal enzyme extracts indicated significant variations in FPU activity. The mean maximum FPU activity of 2.27 U observed in *T. reesei* crude enzyme extract found to be significantly superior over *T. viridae* (2.24 U) and other crude enzyme extracts. The lowest FPU activity was measured in *P. chrysosporium* crude enzyme extract (1.38 U).

The two different buffers with varied pH levels were also differed significantly with regards to cellulase activity. The significant and highest mean FPU activity of 1.88 U was observed with citrate buffer (0.05 M) at pH 4.8. It was found to be significantly superior over other pH conditions of the same buffer as well as over acetate buffer (0.05 M) of 4.6, 4.8 and 5.0 pH conditions. The significantly lowest mean FPU activity 1.70 U was observed in 0.05 M solution of 4.6 pH acetate buffer.

The combined interaction of crude enzyme extracts and pH conditions of buffers showed significant variation with respect to cellulase activity. The significantly highest FPU activity of 2.38 U was observed in *T. reesei* crude enzyme extract with citrate buffer at pH 4.8. However, it was found to be on par with *T. viridae* crude enzyme extract at pH 4.8 (citrate buffer). The significantly lowest FPU activity was recorded with *P. chrysosporium* crude enzyme extract at pH 4.6 in acetate buffer (1.35 U). The results also indicated that citrate buffer of pH 4.8 was found to be optimum for higher FPU activities in all the crude enzyme extracts with measured FPU activities of 2.34, 2.38, 1.66, 1.57 and 1.47 U, respectively for *T. viridae*, *T. reesei*, *A. sydowii*, *A. awamorii* and *P. chrysosporium* crude enzyme extracts. It was found to be significantly superior over other pH conditions with citrate buffer as well as over all the three pH conditions of acetate buffer.

4.8.3 Effect of different alkali pre-treated substrates on FPU activity of crude enzymes

Six different substrates were used as carbon source and as inducer of cellulase enzyme at temperature 30^0C and pH 6.0. The FPU activity of crude enzyme extracts of different fungi differed significantly. Even the substrates as well as the interaction between crude enzyme extracts and substrates were found to be different.

The filter paper activity of five different crude enzymes differed with each other (Table 27). The mean maximum FPU activity, 1.90 U observed with *T. reesei* crude enzyme extract was found to be significantly highest over other crude enzyme extracts of cellulolytic fungi. The significantly lowest mean FPU activity of 1.13 U was recorded with *P. chrysosporium* crude enzyme extract.

The filter paper activity of crude enzymes also varied with the type of substrate used as carbon source. The substrate cellulose was found to be very encouraging to yield higher FPU activity. The mean maximum FPU activity of 1.81 U was recorded with cellulose as substrate and it was found to be significantly superior over all other substrates with respect to FPU activity of crude enzymes. All other substrates with a range between 1.27 (sugarcane bagasse) and 1.49 U (sugarcane tops) indicated lesser FPU activity.

The interaction between crude enzyme extracts and different substrates indicated a highly significant FPU activity. *T. reesei* crude enzyme extract that was produced on cellulose as carbon source resulted in FPU activity of 2.33 U. It was found to be highly superior over other carbon sources of the same cellulolytic fungi (crude enzyme extract) and also over other crude enzyme extracts of different fungi. Followed by *T. reesei*, *T. viridae* (2.21 U), *A. sydowii* (1.65 U), *A. awamorii* (1.52 U) and *P. chrysosporium* (1.33 U) showed maximum FPU activity with cellulose as substrate and it was significantly superior over other substrates as carbon source for the respective crude enzyme extracts of different fungi. Bagasse as a substrate resulted in lowest FPU activity for all the crude enzyme extracts with the determined highest FPU activity of 1.66 U in case of *T. reesei* crude enzyme extract and the lowest FPU activity of 0.95 U in case of *P. chrysosporium* crude enzyme extract.

4.8.4 Effect of different substrates on CMCase activity of crude enzymes

The data pertaining to CMCase activity of different crude enzyme extracts produced on different substrates at 30^0C temperature and 6.0 pH is presented in Table 28. The CMCase activity varied differently as influenced by different crude enzyme extracts, different substrates and their combined interaction.

Table 27: Effect of different substrates on FPU activity of crude enzymes (U ml^{-1} min^{-1})

Cultures	Substrates						
	Sugarcane bagasse	**Sugarcane tops**	**Sugarcane trash**	**Corn husk**	**Corn stover**	**Cellulose**	**Mean**
Trichoderma viridae	1.47	1.65	1.43	1.48	1.64	2.21	**1.65**
Trichoderma reesei	1.66	2.01	1.82	1.66	1.90	2.33	**1.90**
Aspergillus sydowiii	1.21	1.42	1.48	1.58	1.33	1.65	**1.45**
Aspergillus awamorii	1.08	1.25	1.29	1.11	1.25	1.52	**1.25**
Phanerochaete chrysosporium	0.95	1.11	1.18	1.08	1.11	1.33	**1.13**
Mean	**1.27**	**1.49**	**1.44**	**1.38**	**1.45**	**1.81**	

	SE±	CD (1%)
Cultures (A)	0.004	0.015
Substrates (B)	0.004	0.016
Interactions (A x B)	0.010	0.036

Table 28: Effect of different substrates on CMCase activity of crude enzymes (U ml^{-1} min^{-1})

Cultures	Substrates						
	Sugarcane bagasse	**Sugarcane tops**	**Sugarcane trash**	**Corn husk**	**Corn stover**	**Cellulose**	**Mean**
Trichoderma viridae	2.72	2.78	2.63	2.83	2.91	2.90	2.79
Trichoderma reesei	3.04	3.18	3.42	3.08	3.24	3.68	3.27
Aspergillus sydowiii	2.70	2.52	2.43	2.48	2.44	2.74	2.55
Aspergillus awamorii	2.43	2.48	2.45	2.29	2.34	2.47	2.41
Phanerochaete chrysosporium	2.18	2.32	2.33	2.38	2.29	2.80	2.38
Mean	**2.61**	**2.65**	**2.65**	**2.61**	**2.64**	**2.92**	

	SE±	CD (1%)
Cultures (A)	0.04	0.14
Substrates (B)	0.04	0.15
Interactions (A x B)	0.09	0.34

Among the crude enzyme extracts studied, the mean maximum CMCase activity of 3.27 U was recorded with *T. reesei* crude enzyme extract which was found to be significantly superior over all other crude enzyme extracts with respect to CMCase activity. The CMCase activity of other crude enzyme extracts were observed to be 2.79 U (*T. viridae*), 2.55 U (*A. sydowii*), 2.41 U (*A. awamorii*) and 2.38 U (*P. chrysosporium*).

Cellulose as a substrate was found to be very encouraging to yield higher CMCase activity at 2.92 U, which was found to be significantly superior over other substrates. All other substrates with a range between 2.61 and 2.65 U of CMCase activity were on par with each other.

The CMCase activity as influenced by combination of crude enzyme extracts and substrates indicated a highly significant and superior CMCase activity, 3.68 U for *T. reesei* with cellulose as substrate. Followed by this combination, sugarcane trash was found to be the second best substrate to yield higher CMCase activity (3.42 U) also in *T. reesei* crude enzyme extract. The lowest CMCase activity was observed in all the culture filtrates that were grown on sugarcane bagasse as substrate with highest and lowest CMCase activity of 3.04 U (*T. reesei*) and 2.18 U (*P. chrysosporium*).

4.8.5 Effect of different substrates on β-glucosidase activity of crude enzyme extracts

The β-glucosidase activity was found to vary significantly due to individual factors such as crude enzyme extracts and substrates and their combined interactions.

The data presented in Table 29 indicates that the mean maximum β-glucosidase activity, 1.34 IU was exhibited by *T. reesei* which was statistically superior over all other crude enzyme extracts of fungi. *T. viridae* showed an activity of 1.23 IU as the second best crude enzyme extract. Rest of the crude enzyme extracts was found to be less efficient with respect to β-glucosidase activity. The lowest mean β-glucosidase activity, 0.31 IU was recorded with *P. chrysosporium* crude enzyme extract.

The β-glucosidase activity differed with different substrates. Cellulose as a substrate produced mean maximum β-glucosidase activity of 0.94 IU. It was found to be significantly superior over corn husk (0.76 IU), corn stover (0.73 IU), sugarcane trash (0.69 IU), sugarcane tops (0.68 IU) and sugarcane bagasse (0.67 IU) with regards to β-glucosidase activity.

The combined interaction between crude enzyme extracts and different substrates indicated a significantly superior β-glucosidase activity for *T. reesei* crude enzyme extract with 1.82 IU when produced on cellulose as a carbon source. It was observed to be significantly superior over *T. viridae* crude enzyme extract (1.56 IU) and also over other crude enzyme extracts produced on cellulose with respect to β-glucosidase activity. The significantly lowest β-glucosidease activity was noticed with *P. chrysosporium* crude enzyme extract on all the substrates with maximum and minimum β-glucosidase activity of 0.34 IU (cellulose) and 0.28 IU (on both sugarcane bagasse and sugarcane tops).

4.8.6 Effect of different substrates on xylanase activity of crude enzymes

The crude enzyme extracts of different fungi produced on different substrates differed significantly with respect to xylanase activity as indicated in Table 30.

The different crude enzyme extracts exhibited different xylanase activities. *T. reesei* crude enzyme extract recorded mean maximum xylanase activity of 3.49 U which was significantly superior over all other crude enzyme extracts. The mean lowest xylanase activity was observed with *A. awamorii* with an activity of 0.01 U. The xylanase activity due to different substrates also differed significantly, where the mean maximum xylanase activity of 1.70 U was recorded with bagasse as a substrate. It was found to be significantly superior over rest of the substrates with regards to xylanase activity.

The interaction effect between different crude enzyme extracts and substrates showed varied xylanase activity. The highest, 3.65 U of xylanase activity was observed with *T. reesei* crude enzyme extract in sugarcane bagasse.

Table 29: Effect of different substrates on β-Glucosidase activity of crude enzymes (IU ml^{-1} min^{-1})

Cultures	Substrates						
	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Cellulose	Mean
Trichoderma viridae	1.06	1.09	1.14	1.28	1.26	1.56	1.23
Trichoderma reesei	1.16	1.25	1.22	1.32	1.28	1.82	1.34
Aspergillus sydowiii	0.44	0.38	0.41	0.48	0.42	0.55	0.45
Aspergillus awamorii	0.42	0.39	0.36	0.40	0.38	0.45	0.40
Phanerochaete chrysosporium	0.28	0.28	0.31	0.33	0.31	0.34	0.31
Mean	**0.67**	**0.68**	**0.69**	**0.76**	**0.73**	**0.94**	

	SE±	CD (1%)
Cultures (A)	0.01	0.02
Substrates (B)	0.01	0.03
Interactions (A x B)	0.02	0.06

Table 30: Effect of different substrates on Xylanase activity of crude enzymes (U ml^{-1} min^{-1})

Cultures	Substrates						
	Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	Cellulose	Mean
Trichoderma viridae	3.57	2.48	2.46	2.70	2.52	2.75	2.75
Trichoderma reesei	3.65	3.43	3.40	3.46	3.60	3.42	3.49
Aspergillus sydowiii	0.03	0.01	0.03	0.01	0.01	0.00	0.02
Aspergillus awamorii	0.00	0.01	0.00	0.00	0.00	0.00	0.01
Phanerochaete chrysosporium	1.24	1.25	1.14	1.13	1.06	1.03	1.14
Mean	**1.70**	**1.44**	**1.41**	**1.46**	**1.44**	**1.44**	

	SE±	CD (1%)
Cultures (A)	0.03	0.12
Substrates (B)	0.03	0.13
Interactions (A x B)	0.08	0.29

Table 31: Protein content (mg ml^{-1}) in the culture filtrate of cellulolytic fungi grown on different substrates after 8 days

Cultures	Substrates						
	Sugarcane bagasse	**Sugarcane tops**	**Sugarcane trash**	**Corn husk**	**Corn stover**	**Cellulose**	**Mean**
Trichoderma viridae	0.457	0.447	0.436	0.459	0.464	0.475	0.456
Trichoderma reesei	0.461	0.466	0.468	0.473	0.488	0.492	0.475
Aspergillus sydowiii	0.470	0.519	0.522	0.486	0.478	0.511	0.498
Aspergillus awamorii	0.519	0.496	0.533	0.541	0.524	0.565	0.530
Phanerochaete chrysosporium	0.395	0.434	0.450	0.429	0.387	0.399	0.416
Mean	**0.460**	**0.472**	**0.482**	**0.477**	**0.468**	**0.488**	

	SE±	**CD (1%)**
Cultures (A)	0.006	0.022
Substrates (B)	0.006	NS
Interactions (A x B)	0.014	NS

The 3.65 U of xylanase activity observed in *T. reesei* crude enzyme extract was found to be on par with all other substrates (in case of *T. reesei* crude enzyme extract) as well as with *T. viridae* crude enzyme extract (3.57 U) on bagasse as substrate with respect to xylanase activity. All the above combinations were found to be significantly superior over other combinations of crude enzyme extracts and substrates. The *A. sydowii* and *A. awamorii* crude enzyme extracts exhibited either zero or significantly negligible xylanase activities on all the substrates.

4.8.7 Effect of different substrates on protein content in the crude enzyme extracts of different fungi

The data on protein content of cellulolytic fungi crude enzyme extracts showed a significant variation. Different substrates and the interaction between cultures and substrates did not differ significantly as observed in Table 31.

As regards to protein content in the culture filtrates of cellulolytic fungi, culture filtrate of *A. awamorii* showed a significantly highest mean protein content of 0.530 mg per ml, which was significantly superior over other culture filtrates. *A. sydowii* had a protein content of 0.498 mg per ml and it was at par with culture filtrate of *T. reesei* (0.475 mg ml^{-1}). The lowest protein content was seen in culture filtrates of *P. chrysosporium* (0.416 mg ml^{-1}).

The protein content as influenced by different substrates was found to be insignificant, so also the interaction between crude enzyme extracts of fungi and substrates with respect to protein content. However, from the data, it could be revealed that all the cultures produced numerically highest protein content with cellulose as the substrate over other substrates.

4.9 Effect of different concentrations of crude enzyme on release of reducing sugars from different alkali pre-treated substrates

4.9.1 Sugarcane bagasse

The alkali pre-treated bagasse was subjected for conversion of cellulose to reducing sugars at 5% substrate concentration using different concentrations of crude cellulose enzyme extracted from various fungi. The individual factors such as incubation time, crude enzymes and their concentrations as well as their two and three way interactions differed significantly with respect to release of reducing sugars (Table 32).

The effect of incubation period influenced the release of reducing sugar significantly. A significantly higher mean reducing sugar release was recorded in 48 h incubation time (323.04 mg g^{-1}). It was found to be on par with 24 h incubation period (322.17 mg g^{-1}) and significantly superior over 12 h (258.45 mg g^{-1}) and 6 h (178.26 mg g^{-1}) incubation period in releasing reducing sugar.

Another factor, crude enzyme extracts of different fungi also influenced significantly the amount of reducing sugar released. Significantly higher mean reducing sugar release was observed in case of crude enzyme extracts of *T. reesei* (364.84 mg g^{-1}) which was statistically superior over rest of the enzyme extracts studied. Significantly the lowest reducing sugar release was recorded with *A. awamorii* (317.48 mg g^{-1}) crude enzyme extract.

Enzyme concentration also influenced significantly the release of reducing sugars in alkali pre-treated bagasse. The 30 U of cellulase crude enzyme showed significantly the higher mean reducing sugar release of 307.90 mg per g followed by 20 U (295.40 mg g^{-1}) and 10 U (208.13 mg g^{-1}). All the enzyme concentrations differed significantly with each other in releasing the reducing sugars.

Interaction effect of incubation time and crude cellulose enzyme extracts revealed significant variation in releasing the reducing sugars. The highest release of reducing sugar was observed at 48 h incubation period (435.79 mg g^{-1}) in *T. reesei*, cellulase enzyme extract which was on par with 24 h (432.11 mg g^{-1}) incubation time with the same enzyme extract. The above two combinations were significantly superior over all other combinations of incubation time and crude enzyme extracts of different fungi in releasing of reducing sugars. Significantly the lowest reducing sugar content was noted in control treatment (8.74 mg g^{-1}) without any crude enzyme extract.

Table 32: Effect of different concentrations of crude enzymes on release of reducing sugars (mg g^{-1}) in sugarcane bagasse

Incubation time (h)	Enzyme conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	
6	10 U	149.83 (21.30)	183.35 (26.07)	157.83 (22.44)	146.02 (20.76)	8.29 (1.15)	178.26
	20 U	243.54 (34.63)	266.40 (37.88)	204.49 (29.08)	231.73 (32.95)	8.33 (1.18)	
	30 U	260.30 (37.01)	293.06 (41.67)	267.92 (38.09)	243.93 (34.68)	8.81 (1.25)	
12	10 U	220.31 (31.32)	270.59 (38.47)	224.12 (31.87)	211.93 (30.13)	8.28 (1.15)	258.45
	20 U	347.16 (49.36)	357.45 (50.82)	344.11 (48.92)	352.49 (50.12)	8.71 (1.24)	
	30 U	370.40 (52.66)	403.54 (57.38)	368.88 (52.45)	379.92 (54.02)	8.85 (1.26)	
24	10 U	312.49 (44.43)	341.83 (48.60)	318.21 (45.24)	303.73 (43.19)	8.66 (1.23)	322.17
	20 U	445.16 (63.29)	477.45 (67.88)	438.59 (62.36)	394.40 (56.07)	8.90 (1.26)	
	30 U	428.60 (60.94)	477.07 (67.83)	427.16 (60.73)	441.30 (62.75)	8.93 (1.27)	
48	10 U	315.54 (44.86)	344.88 (49.03)	323.16 (45.95)	304.50 (43.29)	9.04 (1.29)	323.04
	20 U	451.93 (64.25)	480.85 (68.37)	440.87 (62.68)	396.30 (56.35)	9.09 (1.29)	
	30 U	432.46 (61.49)	481.64 (68.48)	442.78 (62.96)	403.54 (57.38)	8.98 (1.28)	
Mean		**331.48**	**364.84**	**329.84**	**317.48**	**8.74**	
Mean	10 U	208.13					
	20 U	295.40					
	30 U	307.90					

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content 633 mg g^{-1}

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Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	**Control**	**Mean**
6	217.89	247.61	210.08	207.23	8.48	**178.26**
12	312.62	343.86	312.37	314.78	8.61	**258.45**
24	395.42	432.11	394.65	379.81	8.83	**322.17**
48	399.98	435.79	402.27	368.11	9.04	**323.04**
Mean	**331.48**	**364.84**	**329.84**	**317.48**	**8.74**	

Two way interaction A x C

	10 U	**20 U**	**30 U**	**Mean**
6	129.06	190.90	214.81	**178.26**
12	187.04	281.99	306.32	**258.45**
24	256.98	352.90	356.61	**322.17**
48	259.42	355.81	353.88	**323.04**
Mean	**208.13**	**295.40**	**307.90**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	**Control**	**Mean**
10 U	249.54	285.16	255.83	241.54	8.57	**208.13**
20 U	371.95	395.54	357.02	343.73	8.76	**295.40**
30 U	372.94	413.83	376.69	367.17	8.89	**307.90**
Mean	**331.48**	**364.84**	**329.84**	**317.48**	**8.74**	

	SEm ±	**CD 1%**
Incubation hours (A)	1.399	5.177
Crude enzymes (B)	1.564	5.788
Enzyme concentration (C)	1.211	4.483
A x B	3.127	11.576
A x C	2.422	8.966
B x C	2.708	10.025
A x B x C	5.417	20.050

The combination of incubation period and crude enzyme concentrations were found to differ in release of reducing sugars. Significantly the highest reducing sugar release was observed at 24 h incubation period (356.61 mg g^{-1}) reducing sugar with 30 U enzyme concentrations. However, it was on par with 48 h incubation (353.88 mg g^{-1}) with 30 U enzyme concentrations as well as with 20 U enzyme concentrations both at 48 h (355.81 mg g^{-1}) and 24 h (352.90 mg g^{-1}) in release of reducing sugars. Incubation for 12 h and 6 h indicated a significantly lowest amount of reducing sugars with all the crude enzyme extracts and their concentrations.

The combined effect of enzyme concentrations and different cellulase crude enzyme extracts also differed significantly with respect to release of reducing sugars. Significantly the highest reducing sugar release was observed in case of *T. reesei* crude enzyme (413.83 mg g^{-1}). It was significantly superior over 20 U (395.94 mg g^{-1}) of the same crude enzyme extract (*T. reesei*) and over *T. viridae* (372.94 mg g^{-1}), *A. sydowii* (376.69 mg g^{-1}) and *A. awamorii* (367.17 mg g^{-1}) crude enzyme extracts of 30 U enzyme concentration.

Combined interaction effect of incubation periods, cellulase crude enzyme extracts and their concentrations differed significantly in releasing the reducing sugars. Significantly highest quantities of reducing sugars were released in 48 h incubation period with 30 U of *T. reesei* crude enzyme extract. The highest reducing sugar content of 481.64 mg per g with 68.48 per cent saccharification observed with this treatment combination was found to be on par with 20 U enzyme concentrations (480.85 mg g^{-1}) reducing sugar and 68.37 per cent saccharification in 48 h of incubation period. It was also found to be on par with 20 U (477.45 mg g^{-1}) and 30 U (477.07 mg g^{-1}) *T. reesei* crude enzyme at 24 h of incubation. The per cent saccharification achieved at 20 U and 30 U of enzyme concentration at 24 h of incubation was 67.88 and 67.83.

The lowest amount of reducing sugars release was observed with 10 U of enzyme concentration of 48 h of incubation in all the cellulase crude enzyme extracts. The highest amount of reducing sugars and per cent saccharificaiton at 48 h (344.88 mg g^{-1} and 49.03%) and at 24 h (341.00 mg g^{-1} and 48.60%) incubation was observed with 10 U of *T. reesei* crude enzyme extract.

4.9.2 Sugarcane tops

The substrate sugarcane tops, pre-treated with 3.0% NaOH for 8 h at room temperature and autoclaved at 121^{0}C temperature for one hour was saccharified with different crude enzyme extracts of cellulolytic fungi at different enzyme concentrations (10 U, 20 U and 30 U) at substrate concentrations of 5%. The data is presented in Table 33.

The release of reducing sugars in pre-treated sugarcane tops with all incubation periods varied significantly. A significantly higher amount of mean reducing sugars release was observed at 24 h of incubation 300.64 mg per g. This was found to be on par with 48 h (298.59 mg g^{-1}) and 6 h (174.19 mg g^{-1}) incubation.

The crude enzyme extracts of different fungi also differed with respect to release of reducing sugars. Crude enzyme extract of *T. reesei* showed significantly the highest release of mean reducing sugars (328.81 mg g^{-1}). It was found to be significantly superior over those of *T. viridae* (320.52 mg g^{-1}), *A. sydowii* (310.91 mg g^{-1}), *A. awamorii* (300.91 mg g^{-1}) and control (8.72 mg g^{-1}) with regards to release of reducing sugars.

The enzyme concentrations also differed significantly in releasing the reducing sugars. A mean highest 291.15 mg per g reducing sugar release was observed at 30 U enzyme concentration, which was found to be significantly superior over 20 U (271.58 mg g^{-1}) and 10 U (199.19 mg g^{-1}) of crude enzyme concentration with regards to release of reducing sugars. The combined interaction of incubation periods and crude enzyme extracts of different fungi revealed significant differences in releasing the reducing sugars. Significantly the highest release of reducing sugar was observed at 48 h incubation period, 396.18 mg per g, which was on par with 24 h of incubation period (394.27 mg g^{-1}) with the same enzyme extract but significantly superior over rest of the incubation periods and crude enzyme extracts of different fungi. Further, all the enzyme extracts of fungi showed a significant amount of release of reducing sugars from 6 h to 24 h of incubation period and the release of reducing sugars was found to be on par between 24 h and 48 h of incubation.

Table 33: Effect of different concentrations of crude enzymes on release of reducing sugars (mg g^{-1}) in sugarcane tops

Incubation hours	Enzyme conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	***Trichoderma reesei***	***Aspergillus sydowiii***	***Aspergillus awamorii***	**Control**	
6	10 U	144.88 (21.27)	162.40 (23.85)	211.54 (31.06)	134.21 (19.70)	8.29 (1.22)	174.19
	20 U	229.45 (33.69)	240.87 (35.36)	227.92 (33.46)	219.55 (32.23)	8.52 (1.25)	
	30 U	247.35 (36.31)	266.92 (39.19)	238.69 (35.04)	263.35 (38.66)	8.83 (1.30)	
12	10 U	245.83 (36.09)	211.54 (31.06)	211.54 (31.06)	203.16 (29.83)	8.28 (1.22)	242.48
	20 U	317.98 (46.69)	330.50 (48.52)	323.16 (47.45)	307.16 (45.10)	8.69 (1.28)	
	30 U	384.21 (56.41)	362.07 (53.16)	362.07 (53.16)	352.11 (51.70)	8.86 (1.30)	
24	10 U	302.97 (44.48)	319.35 (46.89)	293.27 (43.06)	290.74 (42.69)	8.28 (1.22)	300.64
	20 U	396.30 (58.18)	428.68 (62.94)	380.68 (55.89)	385.64 (56.62)	8.90 (1.31)	
	30 U	400.69 (59.10)	434.78 (63.83)	403.16 (59.19)	387.16 (56.84)	8.91 (1.31)	
48	10 U	304.49 (44.70)	321.64 (47.22)	296.96 (43.60)	295.35 (43.36)	9.04 (1.33)	298.59
	20 U	405.07 (59.47)	431.35 (63.33)	387.54 (56.90)	384.49 (56.45)	9.09 (1.33)	
	30 U	406.97 (59.75)	435.54 (63.94)	394.40 (57.91)	387.92 (56.95)	8.96 (1.32)	
Mean		**320.52**	**328.81**	**310.91**	**300.91**	**8.72**	
Mean	10 U	199.19					
	20 U	271.58					
	30 U	291.15					

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content 613 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	207.23	223.40	226.05	205.70	8.55	**174.19**
12	316.01	301.37	298.93	287.48	8.61	**242.48**
24	386.65	394.27	359.04	354.51	8.70	**300.64**
48	372.18	396.18	359.63	355.92	9.03	**298.59**
Mean	**320.52**	**328.81**	**310.91**	**300.91**	**8.72**	

Two way interaction A x C

	10 U	20 U	30 U	Mean
6	132.27	185.26	205.03	**174.19**
12	176.07	257.50	293.86	**242.48**
24	242.92	320.04	338.94	**300.64**
48	245.50	323.51	326.76	**298.59**
Mean	**199.19**	**271.58**	**291.15**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
10 U	249.54	253.73	253.33	230.87	8.47	**199.19**
20 U	337.20	357.85	329.83	324.21	8.80	**271.58**
30 U	374.81	374.83	349.58	347.64	8.89	**291.15**
Mean	**320.52**	**328.81**	**310.91**	**300.91**	**8.72**	

	SEm ±	CD 1%
Incubation hours (A)	0.667	2.468
Crude enzymes (B)	0.745	2.759
Enzyme concentration (C)	0.577	2.137
A x B	1.491	5.519
A x C	1.155	4.275
B x C	1.291	4.779
A x B x C	2.582	9.559

Significantly the lowest reducing sugar release (9.03 mg g^{-1}) was observed in control treatment over 48 h incubation period.

The interaction between incubation period and enzyme concentration indicated a significant variations, treatment with 24 h incubation period resulted in significantly the highest amount of release of reducing sugars with 30 U of enzyme concentration (338.94 mg g^{-1}) and was found to be on par with 48 h of incubation period (326.76 mg g^{-1}) while significantly superior over 12 h (293.86 mg g^{-1}) and 6 h (205.03 mg g^{-1}) of incubation in case for 30 U of enzyme concentration. Incubation period of 48 h showed a significantly higher release of reducing sugars for 20 U (323.51 mg g^{-1}) and 10 U (245.50 mg g^{-1}) however, they were found to be on par with 24 h of incubation for 20 U (320.04 mg g^{-1}) and 10 U (242.92 mg g^{-1}) of enzyme concentrations. These enzyme concentrations were significantly superior over 12 h and 6 h of incubation.

The effect of enzyme concentration and crude enzyme extracts of different fungi influenced differently the reducing sugar released. A maximum reducing sugar release was observed with *T. reesei* crude enzyme extract (374.83 mg g^{-1}) when 30 U of enzyme was used. This was found to be on par with *T. viridae* (374.81 mg g^{-1}) crude enzyme extract of 30 U. These two treatments were significantly higher over other crude enzyme extracts at 30 U as well as over 20 U and 10 U for all the enzyme extracts evaluated.

The combined influence of incubation periods, crude enzymes and different concentrations of crude enzyme showed variations in release of reducing sugars from alkali pre-treated sugarcane tops. At 48 h of incubation, *T. reesei* crude enzyme produced significantly the higher release of reducing sugars (435.54 mg) which corresponds 63.44 to per cent saccharification with 30 U of enzyme concentration. It was found to be on par with 20 U (431.35 mg g^{-1} reducing sugar and 63.33 per cent saccharification) at 48 h of incubation as well as with 24 h of incubation for both 30 U and 20 U of enzyme concentrations (434.78 mg g^{-1} and 63.83% and 428.68 and 62.94% reducing sugars and per cent saccharification, respectively). The crude enzyme from *T. reesei* at a concentration of 30 U and 20 U were found to be on par with each other at 48 h and 24 h of incubation but significantly superior over rest of the enzyme extracts across all concentrations and incubation periods.

A maximum of 321.64 mg per g reducing sugar and 47.22 per cent of saccharification was observed with 10 U enzyme concentration in case of *T. reesei* crude enzyme extract, which was significantly lower than that with 20 U either 30 U of enzyme concentrations in releasing reducing sugars from sugarcane tops.

4.9.3 Sugarcane trash

Sugarcane trash, pre-treated with 3.0% NaOH for 8 h at room temperature and autoclaved at 121^0C temperature for one hour was subjected for saccharification with different concentrations of crude extract of cellulase. All the individual factors *viz.*, incubation time, crude enzyme extracts and their enzyme concentrations as well as their combinations differed significantly in releasing the reducing sugars.

The release of reducing sugars in pre-treated sugarcane bagasse due to individual main factor, incubation h varied significantly as observed in Table 34. Significantly highest amount of mean reducing sugar release was observed at 48 h of incubation time (308.40 mg g^{-1}). It was found to be at par with 48 h of incubation time (308.09 mg g^{-1}) but significantly superior over 12 h (250.80 mg g^{-1}) and 6 h (177.04 mg g^{-1}) of incubations.

The crude cellulose enzyme extract of different fungi also revealed variations in release of reducing sugars. Crude enzyme extract of *T. viridae* showed significantly higher mean reducing sugar release (340.40 mg g^{-1}) which was found to be significantly superior over other cellulose crude enzyme extracts of *T. viridae* (325.88 mg g^{-1}), *A. sydowii* (320.05 mg g^{-1}) and *A. awamorii* (310.27 mg g^{-1}) and also over the uninoculated control (8.71 mg g^{-1}).

The crude enzyme concentrations also varied significantly with respect to release of reducing sugars. The mean highest (297.61 mg g^{-1}) of reducing sugar release was observed with 30 U enzyme concentration. This was significantly superior over 20 U (282.97 mg g^{-1}) and 10 U (202.61 mg/) of enzyme concentrations.

Table 34: Effect of different concentrations of crude enzymes on release of reducing sugars (mg g^{-1}) in sugarcane trash

Incubation hours	Enzyme conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	***Trichoderma reesei***	***Aspergillus sydowiii***	***Aspergillus awamorii***	**Control**	
6	10 U	150.21 (22.05)	172.69 (25.35)	161.25 (23.68)	138.40 (20.32)	8.28 (1.22)	177.04
	20 U	226.02 (33.18)	243.16 (35.70)	243.16 (35.70)	231.35 (33.97)	8.52 (1.25)	
	30 U	261.07 (38.33)	280.12 (41.13)	252.30 (37.04)	270.21 (39.67)	8.82 (1.30)	
12	10 U	217.64 (31.95)	254.97 (37.44)	222.97 (32.74)	208.87 (30.67)	8.28 (1.22)	250.86
	20 U	346.78 (50.91)	354.02 (51.98)	335.35 (49.24)	311.74 (45.77)	9.09 (1.33)	
	30 U	373.07 (54.77)	376.87 (55.33)	367.73 (53.99)	366.59 (53.82)	8.86 (1.30)	
24	10 U	307.16 (45.10)	327.73 (48.12)	314.78 (46.22)	297.25 (43.64)	8.28 (1.22)	308.09
	20 U	429.83 (63.11)	437.45 (64.22)	402.40 (59.08)	392.12 (57.57)	8.90 (1.31)	
	30 U	434.40 (63.78)	437.83 (64.28)	411.16 (60.37)	403.16 (59.19)	8.89 (1.31)	
48	10 U	310.87 (45.64)	326.21 (47.89)	310.86 (45.64)	296.88 (43.59)	8.66 (1.27)	308.27
	20 U	428.30 (62.88)	438.97 (64.45)	409.26 (60.09)	394.02 (57.85)	8.90 (1.31)	
	30 U	425.26 (62.43)	434.78 (63.83)	409.35 (60.10)	412.68 (60.59)	8.99 (1.32)	
Mean		**325.88**	**340.40**	**320.05**	**310.27**	**8.71**	
Mean	10 U	202.61					
	20 U	282.97					
	30 U	297.61					

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	212.43	231.99	218.91	213.32	8.54	**177.04**
12	312.50	328.62	308.69	295.73	8.74	**250.86**
24	390.46	401.00	376.12	364.18	8.69	**308.09**
48	388.14	399.99	376.49	367.86	8.85	**308.27**
Mean	**325.88**	**340.40**	**320.05**	**310.27**	**8.71**	

Two way interaction A x C

	10 U	20 U	30 U	Mean
6	126.17	190.44	214.50	**177.04**
12	182.55	271.39	298.63	**250.86**
24	251.04	334.14	339.09	**308.09**
48	250.70	335.89	338.21	**308.27**
Mean	**202.61**	**282.97**	**297.61**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
10 U	246.47	270.40	252.47	235.35	8.38	**202.61**
20 U	357.73	368.40	347.54	332.31	8.85	**282.97**
30 U	373.45	382.40	360.14	363.16	8.89	**297.61**
Mean	**325.88**	**340.40**	**320.05**	**310.27**	**8.71**	

	SEm $\pm$	CD 1%
Incubation hours (A)	0.517	1.914
Crude enzymes (B)	0.578	2.139
Enzyme concentration (C)	0.448	1.657
A x B	1.156	4.279
A x C	0.895	3.314
B x C	1.001	3.706
A x B x C	2.002	7.411

Variations in the amounts of reducing sugar released were also found to be due to the interaction of crude enzymes and incubation periods. Crude enzyme extract of *T. reesei* showed a significantly higher (401.00 mg g^{-1}) reducing sugar at 24 h incubation time and this was found to be at par when compared to 48 h incubation time (399.99 mg g^{-1}). These indicated that the reducing sugars released had reached saturation point in 24 h. Rest of the crude enzymes of different fungi and their incubation period found to be significantly less than the above combinations of *T. reesei* crude enzyme at 24 h and 48 h.

The interaction between incubation period and crude enzyme extract indicated significant variations with respect to release of reducing sugars. Significantly higher reducing sugars were released in case of 24 h incubation period with 30 U of enzyme concentration (339.09 mg g^{-1}) which was on par with 48 h incubation period (338.21 mg g^{-1}) but significantly superior over 20 U and 10 U of enzyme concentrations over all incubation periods. The lowest release of reducing sugars was observed in 10 U of enzyme (251.04 mg g^{-1}) over 24 h incubation period.

The effect of crude enzyme concentration and crude enzyme extract from different fungi influenced significantly the release of reducing sugars. The maximum amount of reducing sugars released was recorded with *T. reesei* crude enzyme extracts at 30 U enzyme concentration (382.40 mg g^{-1}), which was significantly superior over other crude enzyme extracts concentration of 30, 20 and 10 U of enzyme concentrations. Crude enzyme extract of *T. reesei* recorded a significantly higher release of reducing sugars over enzyme extracts from other fungi at concentrations of 30 U (382.40) 20 U (368.40) and 10 U (270.40 mg g^{-1}).

Effect of combinations of crude enzyme extracts, incubation periods and enzyme concentrations also showed significant variations for reducing sugar content. At 48 h of incubation, *T. reesei* recorded significantly higher reducing sugar and per cent saccharification with 20 U of enzyme concentration. The reducing sugar content of 438.97 mg per g and per cent saccharification of 64.45 observed with 20 U of enzyme concentration for 48 h incubation was at par with 20 U and 30 U of enzyme concentrations at 24 h incubation in case of *T. reesei* crude enzyme extract where it recorded 437.45 mg per g and 64.22 per cent and 437.83 mg per g and 64.28 per cent reducing sugars and per cent saccharification, respectively with 20 U and 30 U of enzyme concentration. All these combinations were found to be significantly superior over rest of the combinations of incubation time, crude cellulose enzymes and their enzyme concentrations with respect to release of reducing sugars.

4.9.4 Corn husk

Corn stover, pre-treated with 3.0% NaOH for 8 h under room temperature followed by autoclaving at 121^{0}C for one hour showed a significant variation in releasing the reducing sugars. The interaction between the factors and combination of all the factors also differed significantly with regards to release of reducing sugars (Table 35).

The incubation periods resulted in significant changes with respect to release of reducing sugars. Significantly higher release of mean reducing sugars was recorded at 48 h of incubation period (298.26 mg g^{-1}) which was at par with 24 h incubation period (296.33 mg g^{-1}), but significantly superior over 12 h (240.42 mg g^{-1}) and 6 h (166.18 mg g^{-1}) incubation period.

Significant variations in reducing sugar release were also observed due to different crude enzyme extracts. The highest mean reducing sugar release was recorded with *T. reesei* crude enzyme extract (329.73 mg g^{-1}) and was found to be significantly superior over all other enzyme extracts. This was followed by *T. viridae* (307.09 mg g^{-1}) and *A. sydowii* (305.97 mg g^{-1}) crude enzyme extracts, they were found to be on par with respect to release of reducing sugars and superior over *A. awamorii* (299.99 mg g^{-1}) crude enzyme extracts and also over control (8.70 mg g^{-1}).

All the enzyme concentrations also significantly the release of reducing sugar for substrate. At 30 U enzyme concentration influenced significantly the highest mean reducing sugar release (285.08 mg g^{-1}) followed by 20 U (266.09 mg g^{-1}) and 10 U (199.72 mg g^{-1}) enzyme concentrations was recorded.

Table 35: Effect of different concentrations of crude enzymes on release of reducing sugars (mg g^{-1}) in corn husk

Incubation hours	Enzyme conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	***Trichoderma reesei***	***Aspergillus sydowiii***	***Aspergillus awamorii***	**Control**	
6	10 U	152.88 (22.19)	163.54 (23.74)	147.55 (21.42)	138.78 (20.14)	8.28 (1.20)	166.18
	20 U	220.30 (31.98)	229.45 (33.31)	210.40 (30.54)	207.73 (30.15)	8.33 (1.21)	
	30 U	236.68 (34.36)	268.69 (39.00)	237.07 (34.41)	254.21 (36.90)	8.81 (1.28)	
12	10 U	211.54 (30.71)	266.40 (38.67)	207.35 (30.10)	209.64 (30.43)	8.28 (1.20)	240.42
	20 U	317.45 (46.08)	330.78 (48.02)	309.06 (44.86)	305.25 (44.31)	8.71 (1.27)	
	30 U	321.26 (46.64)	380.02 (55.17)	369.54 (53.64)	352.12 (51.11)	8.84 (1.28)	
24	10 U	301.83 (43.81)	323.92 (47.02)	307.54 (44.64)	297.25 (43.15)	8.28 (1.20)	296.33
	20 U	395.92 (57.47)	411.26 (59.70)	381.07 (55.32)	377.26 (54.76)	8.90 (1.29)	
	30 U	410.76 (59.63)	421.44 (61.18)	403.10 (58.52)	387.54 (56.25)	8.89 (1.29)	
48	10 U	303.49 (44.06)	322.78 (46.85)	307.92 (44.70)	298.02 (43.26)	9.04 (1.31)	298.26
	20 U	401.78 (58.32)	416.26 (60.42)	389.83 (56.59)	382.97 (55.59)	9.09 (1.32)	
	30 U	411.21 (59.69)	422.21 (61.29)	401.25 (58.25)	389.07 (56.48)	8.98 (1.30)	
Mean		**307.09**	**329.73**	**305.97**	**299.99**	**8.70**	
Mean	10 U	199.72					
	20 U	266.09					
	30 U	285.08					

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 620 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	***Trichoderma reesei***	***Aspergillus sydowiii***	***Aspergillus awamorii***	**Control**	**Mean**
6	203.29	220.56	198.34	200.24	8.47	**166.18**
12	283.42	325.73	295.32	289.00	8.61	**240.42**
24	369.50	385.54	363.90	354.02	8.69	**296.33**
48	372.16	387.08	366.33	356.69	9.04	**298.26**
Mean	**307.09**	**329.73**	**305.97**	**299.99**	**8.70**	

Two way interaction A x C

	10 U	**20 U**	**30 U**	**Mean**
6	122.21	175.24	201.09	**166.18**
12	180.64	254.25	286.35	**240.42**
24	247.77	314.88	326.35	**296.33**
48	248.25	319.98	326.54	**298.26**
Mean	**199.72**	**266.09**	**285.08**	

Two way interaction C x B

	Trichoderma viridae	***Trichoderma reesei***	***Aspergillus sydowiii***	***Aspergillus awamorii***	**Control**	**Mean**
10 U	242.43	269.16	242.59	235.92	8.47	**199.72**
20 U	333.86	346.94	322.59	318.30	8.76	**266.09**
30 U	344.98	373.09	352.74	345.73	8.88	**285.08**
Mean	**307.09**	**329.73**	**305.97**	**299.99**	**8.70**	

	SEm ±	**CD 1%**
Incubation hours (A)	0.578	2.141
Crude enzymes (B)	0.647	2.393
Enzyme concentration (C)	0.501	1.854
A x B	1.293	4.787
A x C	1.002	3.708
B x C	1.120	4.146
A x B x C	2.240	8.291

The combined effect of incubation period and crude enzyme extracts of showed significant variation in releasing the reducing sugars. Maximum reducing sugar release was observed at 48 h incubation period, 387.08 mg per g with *T. reesei*. This was found to be on par with 24 h of incubation period (385.54 mg g^{-1}) but significantly superior over 12 h (325.73 mg g^{-1}) and 6 h (220.56 mg g^{-1}) with the same enzyme extract. Similarly, *T. reesei* crude enzyme extract at either 48 h or 24 h incubation recorded a significantly higher reducing sugar release over other crude enzyme extracts in combination with all incubation periods. Significantly the lowest release of reducing sugars was recorded in *A. awamorii* crude enzyme extract at 48 h of incubation with maximum of 356.69 mg per g reducing sugars.

The combined effect of incubation periods and concentrations of crude enzyme also indicated significant differences with respect to release of reducing sugars. A 30 U enzyme concentration resulted in a significantly higher release of reducing sugars over 20 U and 10 U enzyme concentrations for all the incubation periods. Significantly higher release of reducing sugars was recorded at 48 h incubation period (326.54 mg g^{-1}) and was found to be at par with 24 h of incubation (326.35 mg g^{-1}) at 30 U of enzyme concentration but significantly superior over 20 U at 48 h (319.98 mg g^{-1}) and 20 U at 24 h (314.88 mg g^{-1}) incubation and also over 10 U at 48 h (248.25 mg g^{-1}) and 10 U at 24 h (247.77 mg g^{-1}) incubation period as well as over 12 h and 6 h incubation periods of incubation with respect to release of reducing sugars.

Crude enzyme concentrations and extracts of different fungi and their concentration also influenced the reducing sugar release from corn husk significantly highest release of reducing sugars was recorded in case of *T. reesei* crude enzyme extract at 30 U enzyme concentration. It was found to be significantly superior over other crude enzyme extracts at concentration of 30 U, 20 U and 10 U. Significantly higher release of reducing sugar was observed in *T. reesei* crude enzyme extract at 30 U (373.09 mg g^{-1}), 20 U (346.94 mg g^{-1}) and 10 U (269.16 mg g^{-1}) enzyme concentration and significantly lower reducing sugar release was recorded with *A. awamorii* at 30, 20 and 10 U of enzyme concentration, respectively of 345.73, 318.30 and 235.92 mg per h. The release of reducing sugar ranged between 8.47 and 8.88 mg per g in control treatment.

The release of reducing sugars due to combined effect of incubation time, crude enzyme extracts and enzyme concentration indicated a significant variation. Significantly higher release of reducing sugar 422.21 mg per g corresponding to 61.29 per cent saccharification was observed with *T. reesei* crude enzyme extract at 30 U enzyme concentration in 48 h incubation time. This was found to be at par with 24 h incubation period (421.44 mg g^{-1} reducing sugar and 61.18% saccharification), when 30 U of enzyme concentration was used and also with 48 h incubation period (416.26 mg g^{-1} and 60.42% saccharification) when 20 U of enzyme concentration was used. The combined interactions of treatments were highly superior over 20 U and 10 U of enzyme concentration for all the enzyme sources tried. Significantly higher release of reducing sugars with 10 U enzyme concentration was observed (322.78 mg g^{-1}) with a corresponding saccharification (46.85%) in case of *T. reesei* crude enzyme extract.

The control treatment recorded significantly lower release of reducing sugar with a range between 8.89 mg per g and 9.04 mg per g at 48 h of incubation period.

4.9.5 Corn stover

The alkali pre-treated corn stover was saccharified to obtain reducing sugars using crude enzyme extracts from fungi at different concentrations for various incubation periods at 5% solid concentration. All the individual factors *viz.*, incubation period, crude enzyme extract and their concentration, combination between the factors and their combinations showed significant variations with regard to release of reducing sugars. The data is presented in Table 36.

The incubation of crude enzyme extracts from different fungi for different periods of time indicated significant variations in releasing the reducing sugar from corn stover. The mean maximum release of reducing sugars was obtained at 24 h incubation period (295.14 mg g^{-1}) which was found to be on par at 48 h incubation period (294.63 mg g^{-1}) but significantly superior over 12 h (238.90 mg g^{-1}) and 6 h (163.42 mg g^{-1}) incubation period with regards to release of reducing sugar.

Table 36: Effect of different concentrations of crude enzymes on release of reducing sugars (mg g^{-1}) in corn stover

Incubation hours	Enzyme conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	***Trichoderma reesei***	***Aspergillus sydowiii***	***Aspergillus awamorii***	**Control**	
6	10 U	140.31 (20.60)	158.97 (23.34)	141.07 (20.71)	132.30 (19.43)	8.28 (1.22)	163.42
	20 U	210.78 (30.95)	222.97 (32.74)	208.12 (30.55)	209.29 (30.73)	8.52 (1.25)	
	30 U	228.68 (33.58)	259.16 (38.05)	256.11 (37.60)	257.99 (37.88)	8.80 (1.29)	
12	10 U	205.83 (30.22)	257.64 (37.83)	206.21 (30.28)	201.59 (29.60)	8.28 (1.22)	238.90
	20 U	321.64 (47.22)	331.93 (48.73)	310.21 (45.54)	300.31 (44.09)	8.71 (1.28)	
	30 U	335.06 (49.19)	362.40 (53.21)	361.64 (53.10)	363.16 (53.32)	8.86 (1.30)	
24	10 U	298.78 (43.87)	313.26 (45.99)	305.64 (44.87)	287.35 (42.19)	8.66 (1.27)	295.14
	20 U	400.45 (58.79)	421.83 (61.93)	374.95 (55.05)	380.68 (55.89)	8.90 (1.31)	
	30 U	408.50 (59.97)	424.50 (62.32)	393.26 (57.74)	391.40 (57.46)	8.92 (1.31)	
48	10 U	302.24 (44.37)	312.10 (45.82)	303.05 (44.49)	288.87 (42.41)	9.04 (1.33)	294.63
	20 U	403.64 (59.26)	419.24 (61.55)	378.40 (55.55)	385.25 (56.56)	8.90 (1.31)	
	30 U	410.45 (60.26)	421.11 (61.83)	382.21 (56.12)	386.02 (56.67)	8.98 (1.32)	
Mean		**305.53**	**325.43**	**301.74**	**298.69**	**8.74**	
Mean	10 U	194.47					
	20 U	265.74					
	30 U	283.86					

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	193.26	213.70	201.77	199.86	8.53	**163.42**
12	287.51	317.32	292.69	288.35	8.62	**238.90**
24	369.24	386.53	357.95	353.14	8.83	**295.14**
48	372.11	384.15	354.55	353.38	8.97	**294.63**
Mean	**305.53**	**325.43**	**301.74**	**298.69**	**8.74**	

Two way interaction A x C

	10 U	20 U	30 U	Mean
6	116.19	171.94	202.15	**163.42**
12	175.91	254.56	286.22	**238.90**
24	242.74	317.36	325.31	**295.14**
48	243.06	319.09	321.75	**294.63**
Mean	**194.47**	**265.74**	**283.86**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
10 U	236.79	260.49	238.99	227.53	8.57	**194.47**
20 U	334.13	348.99	317.92	318.88	8.76	**265.74**
30 U	345.67	366.79	348.30	349.64	8.89	**283.86**
Mean	**305.53**	**325.43**	**301.74**	**298.69**	**8.74**	

	SEm ±	CD 1%
Incubation hours (A)	0.453	1.678
Crude enzymes (B)	0.507	1.876
Enzyme concentration (C)	0.393	1.453
A x B	1.014	3.752
A x C	0.785	2.906
B x C	0.878	3.249
A x B x C	1.756	6.498

The different crude cellulase enzyme extracts indicated significant differences with each other in releasing the reducing sugars. Significantly higher mean reducing sugar release was observed with *T. reesei* crude enzyme extract. This was found to be superior over *T. viridae* (305.53 mg g^{-1}), *A. sydowii* (301.74 mg g^{-1}) and *A. awamorii* (298.69 mg g^{-1}) crude cellulase enzyme extracts as well as over control (8.74 mg g^{-1}) in releasing the reducing sugar.

The enzyme concentrations also influenced significantly the release of reducing sugar. Significantly higher mean reducing sugar release (283.86 mg g^{-1}) was observed at 30 U enzyme concentration, which was highly superior over 20 U (265.74 mg g^{-1}) and 10 U (194.47 mg g^{-1}) of enzyme concentrations.

The reducing sugars released due to incubation periods and source of crude enzyme extracts showed varied differences. A 24 h incubation had indicated release of significantly higher reducing sugars (386.53 mg g^{-1}) with *T. reesei* crude cellulase enzyme extract, which was found to be on par with 48 h incubation (384.15 mg g^{-1}) also with *T. reesei* crude enzyme extract. Similarly, 24 h and 48 h incubation periods with *T. reesei* crude enzyme extracts were found to be significantly superior over 12 h (317.32 mg g^{-1}) and 6 h (213.70 mg g^{-1}) with the same fungal enzyme extract and also over all other fungal crude enzyme extracts for all the incubation periods. Among the fungal crude enzymes extracts, the lowest release of reducing sugars was recorded in *A. awamorii* crude enzyme extract at 48 h (353.38 mg g^{-1}) and 24 h (353.14 mg g^{-1}) incubation periods. Significantly lower release of reducing sugars was observed in control treatment with a range between 8.53 and 8.97 mg per g over 6 h to 48 h of incubation period.

The combination of incubation periods and different enzyme concentrations indicated that a significantly higher release of reducing sugars was obtained at the end of 24 h incubation with 30 U enzyme concentration resulting in a reducing sugar content of 325.31 mg per g. This was found to be significantly superior over 48 h (321.75 mg g^{-1}), 12 h (286.22 mg g^{-1}) and over 6 h of incubation periods (202.15 mg g^{-1}) at 30 U enzyme concentration. Similarly, 20 U enzyme concentration at the end of 48 h incubation produced a maximum reducing sugar (319.09 mg g^{-1}). This was on par with 24 h incubation (317.36 mg g^{-1}) but significantly superior over 12 h and 6 h of incubation periods. Contrarily, 10 U enzyme concentration could only release a maximum of 243.06 mg per g reducing sugar at the end of 48 h incubation period, which was on par with 24 h incubation period (242.74 mg g^{-1}) but significantly superior over 12 h and 6 h incubation periods with respect to release of reducing sugars.

The different crude enzyme extracts and their enzyme concentrations were found to significantly influence the release of reducing sugars. A significantly higher release of reducing sugars was recorded with 30 U enzyme concentration in case of *T. reesei* crude enzyme extract (366.79 mg g^{-1}). This was found to be significantly superior over all other enzyme extracts with 30 U concentrations. Also the reducing sugars released with 30 U enzyme concentrations for all the enzyme extracts were highly significant over enzyme concentrations of 20 U and 10 U within the same enzyme source.

The combination of all the factors also differed significantly with regards to release of reducing sugars in alkali pre-treated corn stover. Significantly the highest quantities of reducing sugars (424.50 mg g^{-1}) with 62.32 per cent saccharification were observed in case of *T. reesei* enzyme extract with 30 U concentration over 24 h incubation period. This was found to be on par with 20 U enzyme concentration (421.83 mg g^{-1} reducing sugar and 61.93% saccharification) at 24 h and also with 30 U and 20 U enzyme concentration at the end of 48 h incubation period (421.11 mg per g and 61.83 per cent and 419.24 mg per g and 61.55 per cent reducing sugars released and per cent saccharification respectively) with the same enzyme extract. All the above combinations were found to be significantly superior over rest of the combinations of sources of enzyme extracts, concentrations of enzyme and incubation periods. The maximum reducing sugar released by the respective fungal crude cellulase enzymes at 24 h incubation with 30 U enzyme concentration *viz.*, *T. viridae* (408.50 mg g^{-1}), *T. reesei* (424.50 mg g^{-1}), *A. sydowii* (393.26 mg g^{-1}) and *A. awamorii* (391.40 mg g^{-1}) were found to be on par with 20 U enzyme concentration at 48 h incubation period *viz.*, *T. viridae* (403.64 mg g^{-1}), *T. reesei* (419.24 mg g^{-1}), *A. sydowii* (378.40 mg g^{-1}) and *A. awamorii* (385.25 mg g^{-1}) with respect to release of reducing sugars.

A significantly lowest reducing sugar release was recorded in 10 U enzyme concentration for all the crude enzyme sources with a maximum reducing sugar release of 313.26 mg per g at the end of 24 h incubation period in *T. reesei* crude enzyme extract saccharified corn stover.

4.10 Effect of different substrate concentrations on release of reducing sugars from different alkali pre-treated substrates by crude enzymes (20 FPU g^{-1})

4.10.1 Sugarcane bagasse

The substrate sugarcane bagasse pre-treated with 3.00% NaOH for 8 hours at room temperature and autoclaved at 121^{0}C temperature for one hour was subjected for saccharification with crude enzyme extracts of cellulolytic fungi at different substrate concentrations of 2.5%, 5.0% and 7.50% using 20 FPU per g substrate (Table 37).

The release of reducing sugar in pre-treated sugarcane bagasse with incubation hours varied significantly. Significantly the highest amount of mean reducing sugar was released at 48 hours incubation time (326.40 mg g^{-1}). It was found to be significantly superior over incubation time of 24 h (318.42 mg g^{-1}), 12 h (262.84 mg g^{-1}) and 6 h (203.10 mg g^{-1}) reducing sugars released.

The crude enzyme extracts of different fungi also yielded varying amounts of reducing sugars from different substrate differing in concentrations. Crude enzymes extract of *T. reesei* yielded the highest reducing sugar content at 368 mg per g. It was significantly superior over *T. viridae* (353.79 mg g^{-1}), *A. sydowii* (342.02 mg g^{-1}) and *A. awamorii* (315.90 mg g^{-1}) in releasing the reducing sugar from substrate. Control treatment recorded significantly the lowest (8.73 mg g^{-1}) reducing sugar.

The substrate concentrations influenced the release of reducing sugar. A substrate concentration of 25% yielded the mean highest reducing sugar (329.53 mg g^{-1}). This was significantly superior over substrate concentration of 5.0% (297.69 mg g^{-1}) and 7.5% (205.84 mg g^{-1}) with respect to release of reducing sugars.

Variation in the amounts of reducing sugar released was found due to combination of crude fungal enzymes and incubation periods. Enzyme extract of *T. viridae* showed significantly the maximum 429.82 mg per g reducing sugar at 48 h incubation period, however, the reducing sugar released was found to be on par at 24 h incubation time (422.85 mg g^{-1}) by *T. reesei*. Similarly, reducing sugars released by *T. viridae* (415.69 mg g^{-1}), *A. sydowii* (400 mg g^{-1}) at 48 h incubation were found to be higher as compared to those of 407.69 mg per g and 396.01 mg per g at 24 h incubation, respectively. Both these incubation periods were non-significant. *A. awamorii* at 48 h incubation released a maximum of 376.55 mg per g reducing sugar at 48 h incubation these values were significantly superior over other incubation periods.

The interaction between incubation period and crude enzymes concentrations also indicated significant variations. Incubation for 48 h recorded significantly the highest amount of reducing sugars at 379.59 mg per g in 2.5% substrate concentration. This was significantly superior over those incubated for 24 h (364.35 mg g^{-1}) 12 h (316.51 mg g^{-1}) and 6 h (257.69 mg g^{-1}) at similar substrate concentration. Also, substrate concentration of 5.0% and 7.5% resulted in the maximum release of reducing sugars on 48 h (359.18 and 240.43 mg g^{-1}) this was found to be on par with 24 h incubation (355.07 and 335.83 mg g^{-1}) at 5.0% and 7.5% substrate concentration, respectively.

The effect of substrate concentration and crude enzyme extract of different fungi influenced the quantum of reducing sugar released. Maximum reducing sugar release was recorded in *T. reesei* at 2.5% substrate concentration (433.55 mg g^{-1}), which was significantly superior over 5.0% (393.43 mg g^{-1}) and 7.5% (277.01 mg g^{-1}) substrate concentrations. Among the crude enzymes extracted from different fungi *A. awamorii* resulted in the lowest amount of reducing sugars irrespective of the substrate concentrations as compared to other fungi.

Table 37: Effect of inoculation of crude enzymes (20 FPU g^{-1}) on release of reducing sugars (mg g^{-1}) from sugarcane bagasse at different substrate concentrations

Incubation time (h)	Substrate conc. (%)	Fungal crude enzymes					Mean
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	
6	2.5%	323.28 (45.96)	340.04 (48.34)	325.56 (46.29)	291.28 (41.41)	8.29 (1.18)	203.10
	5.0%	242.40 (34.46)	267.92 (38.09)	245.07 (34.84)	233.64 (33.21)	8.33 (1.18)	
	7.5%	197.54 (28.09)	209.21 (29.75)	178.25 (25.34)	166.83 (23.72)	8.81 (1.25)	
12	2.5%	397.18 (56.47)	423.85 (60.26)	391.09 (55.61)	362.13 (51.49)	8.28 (1.18)	262.84
	5.0%	355.92 (50.61)	346.83 (49.31)	346.02 (49.20)	327.73 (46.60)	8.71 (1.24)	
	7.5%	258.96 (36.82)	270.13 (38.40)	227.49 (32.35)	209.47 (29.78)	8.85 (1.26)	
24	2.5%	481.00 (68.39)	478.67 (68.06)	459.27 (65.30)	394.13 (56.04)	8.66 (1.23)	318.42
	5.0%	443.52 (63.06)	477.67 (67.91)	434.38 (61.76)	410.90 (58.42)	8.90 (1.26)	
	7.5%	298.56 (42.45)	312.20 (44.39)	294.38 (41.86)	265.10 (37.69)	8.93 (1.27)	
48	2.5%	485.56 (69.03)	491.66 (69.90)	464.99 (66.11)	446.70 (63.51)	9.04 (1.29)	326.40
	5.0%	450.78 (64.09)	481.32 (68.43)	440.11 (62.57)	414.59 (58.95)	9.09 (1.29)	
	7.5%	310.74 (44.18)	316.49 (45.00)	297.59 (42.31)	268.35 (38.15)	8.98 (1.27)	
Mean		**353.79**	**368.00**	**342.02**	**315.90**	**8.74**	
Mean	2.5%	329.53					
	5.0%	297.69					
	7.5%	205.84					

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content 633 mg g^{-1}

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Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	254.41	272.39	249.63	230.58	8.48	**203.10**
12	337.35	346.93	321.53	299.78	8.61	**262.84**
24	407.69	422.85	396.01	356.71	8.83	**318.42**
48	415.69	429.82	400.90	376.55	9.04	**326.40**
Mean	**353.79**	**368.00**	**342.02**	**315.90**	**8.74**	

Two way interaction A x C

	2.5%	5.0%	7.5%	Mean
6	257.69	199.47	152.13	**203.10**
12	316.51	277.04	194.98	**262.84**
24	364.35	355.07	235.83	**318.42**
48	379.59	359.18	240.43	**326.40**
Mean	**329.53**	**297.69**	**205.84**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
2.5%	421.75	433.55	410.23	373.56	8.57	**329.53**
5.0%	373.16	393.43	366.40	346.72	8.76	**297.69**
7.5%	266.45	277.01	249.43	227.44	8.89	**205.84**
Mean	**353.79**	**368.00**	**342.02**	**315.90**	**8.74**	

	SEm $\pm$	CD 1%
Incubation time (A)	1.762	6.523
Crude enzymes (B)	1.970	7.293
Enzyme concentration (C)	1.526	5.649
A x B	3.940	14.586
A x C	3.052	11.298
B x C	3.413	12.632
A x B x C	6.825	25.263

The combined effect of crude enzyme from cellulolytic fungi, incubation period and substrate concentrations showed a significant variation for reducing sugar content. At 48 h of incubation period, *T. reesei* produced a maximum of 491.66 mg per g reducing sugar with 69.90 per cent saccharificaiton at 2.5% substrate concentration and was found to be on par with *T. viridae* (485.56 mg g^{-1}) resulting in 69.03 per cent saccharification. However, incubation of 48 h was on par with 24 h in both the crude enzyme extracts at 478.67 mg per g and 481.00 mg per g reducing sugars yielding 68.06 and 68.39 per cent saccharification, respectively for *T. reesei* and *T. viridae* under 2.5 per cent substrate concentration.

Incubation for a period of 48 h with 30 U *T. reesei* crude enzyme showed significantly the highest release of reducing sugars for 5.0% substrate concentration with a maximum of 481.32 mg per g reducing sugar and 68.43 per cent saccharification. It was found to be on par with 24 h incubation with 477.67 mg per g reducing sugar and 67.91 per cent saccharification and significantly superior over other combinations of incubation periods and fungal crude enzyme.

Maximum of 316.49 mg per g reducing sugars and 45.00 per cent saccharificaiton was observed at 48 h incubation period for 7.5 per cent substrate concentration with 30 U of *T. reesei* crude enzyme. It was found to be at par with 24 h incubation period (312.20 mg g^{-1}) reducing sugar with 30 U of *T. reesei* crude enzyme extract with *T. viridae* 310.74 mg per g and 298.56 mg per g reducing sugars, respectively at 48 and 24 h incubation period. *A. sydowii* recorded 297.59 mg per g and 294.38 mg per g reducing sugars, respectively at 48 and 24 h incubation period but significantly superior over *A. awamorii* (268.35 mg g^{-1} reducing sugar and 38.15% saccharification at 48 h of incubation).

4.10.2 Sugarcane tops

Sugarcane tops which were treated with 3.00% NaOH for 8 h at room temperature and then autoclaved at 121^{0}C temperature for one hour were further subjected to saccharification using different crude enzyme extracts and substrate concentrations at 20 FPU per g cellulose activity. The release of reducing sugars in pre-treated sugarcane tops varied differently due to individual factors such as incubation periods, crude enzyme extracts and substrate levels as well as all their (two way and three way) interactions with respect to release of reducing sugars (Table 38).

The incubation period differed significantly for release of reducing sugars in sugarcane tops. Significantly mean highest reducing sugar (313.67 mg g^{-1}) release was observed at 48 h incubation period, which was significantly superior over the values recorded at 24 h (302.94 mg g^{-1}), 12 h (254.88 mg g^{-1}) and 6 h (195.25 mg g^{-1}).

Among the fungal crude extracts, *T. viridae*, 349.76 mg per g showed significantly higher amount of mean reducing sugars released which was significantly superior over all other fungal crude enzyme extracts, followed by the crude enzyme extracts of *T. viridae* (340.27 mg g^{-1}), *A. sydowii* (326.65 mg g^{-1}) and *A. awamorii* (308.02 mg g^{-1}) in that order of releasing reducing sugars. Significantly lower reducing sugar was released in control treatment (8.72 mg g^{-1}). All these treatments were found to be significantly different with each other.

The substrate concentration as a factor also influenced significantly the reducing sugars released. Substrate concentration of 2.5% resulted in significantly higher mean reducing sugar release (315 mg g^{-1}), followed by 5.0% (283.52 mg g^{-1}) and 7.5% (201.15 mg g^{-1}) substrate concentration.

The quantity of reducing sugars released as a measure of incubation periods and crude enzyme extracts were found to differ significantly. A maximum reducing sugar release was recorded at 48 h incubation period with crude enzymes of *T. reesei* (406.30 mg g^{-1}), which was on par with those of *T. viridae* at 48 h incubation as well as *T. reesei* (397.54 mg g^{-1}) at 24 h incubation period. These were significantly superior over other combinations of incubation periods and sources of crude enzyme extracts. Crude enzyme extract of *A. awamorii* (370.99 mg g^{-1}) at 48 h incubation period was observed to be the least efficient among the crude extracts of different fungi in releasing the reducing sugars.

Table 38: Effect of inoculation of crude enzymes (20 FPU g^{-1}) on release of reducing sugars (mg g^{-1}) from sugarcane tops at different substrate concentrations

Incubation time (h)	Substrate conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	
6	2.5%	312.47 (45.88)	327.85 (48.13)	318.71 (46.79)	282.13 (41.42)	8.29 (1.22)	195.25
	5.0%	227.54 (33.41)	241.64 (35.48)	229.07 (33.63)	231.35 (33.97)	8.52 (1.25)	
	7.5%	190.94 (28.03)	195.25 (28.67)	178.25 (26.17)	167.84 (24.64)	8.83 (1.30)	
12	2.5%	394.13 (57.86)	405.47 (59.53)	375.09 (55.07)	340.80 (50.04)	8.28 (1.22)	254.88
	5.0%	350.21 (51.42)	353.64 (51.92)	330.78 (48.57)	310.21 (45.54)	8.69 (1.27)	
	7.5%	256.42 (37.65)	261.75 (38.43)	220.64 (32.39)	198.30 (29.12)	8.86 (1.30)	
24	2.5%	428.42 (62.90)	442.13 (64.91)	432.23 (63.46)	388.04 (56.97)	8.28 (1.22)	302.94
	5.0%	430.27 (63.17)	439.20 (64.48)	408.11 (59.92)	399.16 (58.61)	8.90 (1.30)	
	7.5%	292.46 (42.94)	311.27 (45.70)	281.22 (41.29)	265.45 (38.97)	8.91 (1.31)	
48	2.5%	461.94 (67.82)	465.00 (68.27)	460.42 (67.60)	439.09 (64.47)	8.96 (1.32)	313.67
	5.0%	434.50 (63.79)	442.33 (64.94)	403.54 (59.25)	403.54 (59.25)	9.04 (1.33)	
	7.5%	303.89 (44.62)	311.56 (45.74)	281.80 (41.37)	270.34 (39.69)	9.09 (1.33)	
Mean		**340.27**	**349.76**	**326.65**	**308.02**	**8.72**	
Mean	2.5%	315.39					
	5.0%	283.52					
	7.5%	201.15					

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}

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Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	243.65	254.91	242.01	227.11	8.55	**195.25**
12	333.59	340.29	308.83	283.10	8.61	**254.88**
24	383.72	397.54	373.85	350.88	8.70	**302.94**
48	400.11	406.30	381.92	370.99	9.03	**313.67**
Mean	**340.27**	**349.76**	**326.65**	**308.02**	**8.72**	

Two way interaction A x C

	2.5%	5.0%	7.5%	Mean
6	249.89	187.63	148.22	**195.25**
12	304.75	270.71	189.20	**254.88**
24	339.82	337.13	231.86	**302.94**
48	367.10	338.60	235.31	**313.67**
Mean	**315.39**	**283.52**	**201.15**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
2.5%	399.24	410.11	396.61	362.51	8.47	**315.39**
5.0%	360.63	369.20	342.88	336.07	8.80	**283.52**
7.5%	260.93	269.96	240.48	225.49	8.89	**201.15**
Mean	**340.27**	**349.76**	**326.65**	**308.02**	**8.72**	

	SEm ±	CD 1%
Incubation time (A)	1.099	4.068
Crude enzymes (B)	1.229	4.548
Enzyme concentration (C)	0.952	3.523
A x B	2.457	9.095
A x C	1.903	7.045
B x C	2.128	7.877
A x B x C	4.256	15.754

The reducing sugars released due to combination of incubation period and substrate concentrations were also varied significantly. Significantly the highest amount of reducing sugars was released at 48 h incubation period (367.10 mg per g reducing sugar) at 2.5% substrate concentration. This was significantly higher over the incubation periods of 24, 12 and 6 h for substrate concentration of 2.5%.

Substrate concentration of 5% along with 48 h incubation time resulted in maximum release of 338.6 mg per g reducing sugars and was found to be superior over 24 h incubation time (337.13 mg g^{-1} reducing sugar). Substrate concentration of 7.5% resulted in a maximum reducing sugar content of 235.31 mg per g over 48 h incubation period but was found to be on par with these recorded with 24 h incubation (231.86 mg g^{-1}).

With respect to substrate concentrations and crude enzyme extract of different fungi, significantly the highest amount of reducing sugar was noticed at a substrate concentration of 2.5% for all the fungal crude enzymes extract. However, in case of *T. reesei* significantly higher amount of 410.11, 369.20 and 269.00 mg per g of reducing sugar at 2.5, 5.0 and 7.5% substrate concentrations found to be superior.

The combined influence of incubation time, crude enzyme extracts and substrate concentration differed greatly with respect to release of reducing sugars. Significantly the highest amount of reducing sugar was released for 48 h incubation with *T. reesei* crude enzyme at 2.5% substrate concentration (465.00 mg g^{-1}) with 68.27 per cent saccharification. The release of reducing sugars by *T. reesei* was found to be on par with *T. viridae* (461.44 mg g^{-1}) and *A. sydowii* (460.42 mg g^{-1}) at 48 h incubation and was found to be superior over *A. awamorii* (439.09 mg g^{-1}) and control (9.04 mg g^{-1}) for 2.5% substrate concentration. Also, 48 h of incubation with crude enzyme extract significantly superior reducing sugar release over other incubation periods.

A maximum reducing sugar yield of 442.33 mg per g with 64.94 per cent saccharification was noticed from *T. reesei* over 48 h incubation period at 5% substrate concentration. It was statistically on par with *T. viridae* (434.50 mg g^{-1}) at 48 h incubation as well as with *T. reesei* (439.20 mg g^{-1}) and *T. viridae* (430.27 mg g^{-1}) at 24 h incubation period but significantly superior over other crude enzyme extracts of *A. sydowii* and *A. awamorii* at all incubation periods studied for 5.0% substrate concentration.

With respect to release of reducing sugars at 7.5% substrate concentration, it was noticed that *T. reesei* crude enzyme extract with 311.56 mg per g reducing sugar and 45.74 per cent saccharificaiton yielded better over 48 h incubation. However, it was found to be at par with those of 24 h incubation (311.27 mg g^{-1}) in case of *T. reesei* crude enzyme extract and significantly superior over other fungal crude enzyme extracts at 7.5% substrate concentration.

4.10.3 Sugarcane trash

Saccharification of alkali pre-treated sugarcane tops was done by using different crude enzyme extracts with varied substrate concentrations. The effect of individual factors such as incubation period, different enzyme extracts and substrate concentrations as well as the interactions among the factors showed significant variations in releasing reducing sugars (Table 39).

The incubation of crude enzyme extracts for different periods influenced significantly the release of reducing sugars. A mean maximum reducing sugar release of 305.27 mg per g was observed over 48 h of incubation. It was significantly superior over those recorded for 24 h, 12 h and 6 h of incubation periods with a release of 290.73, 251.68 and 191.84 mg per g reducing sugars, respectively for 24, 92 and 6 h of incubation.

The different fungal crude enzyme extracts yielded significant variations in release of reducing sugars. A mean maximum 343.39 mg per g reducing sugar release was shown by *T. reesei* crude enzyme extract which was significantly superior over other fungal crude enzyme extracts. Significantly the lowest reducing sugar release was observed in control treatment (8.71 mg g^{-1}).

The substrate concentrations also showed a significant difference with respect to release of reducing sugars. A mean maximum of 307.65 mg per g reducing sugar was released when 2.5% substrate was used.

Table 39: Effect of inoculation of crude enzymes (20 FPU g^{-1}) on release of reducing sugars (mg g^{-1}) from sugarcane trash at different substrate concentrations

Incubation time (h)	Substrate conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	
6	2.5%	291.28 (42.77)	318.70 (46.79)	314.13 (46.12)	277.56 (40.75)	8.28 (1.22)	191.84
	5.0%	230.21 (33.80)	242.78 (35.65)	227.92 (33.47)	226.02 (33.18)	8.52 (1.25)	
	7.5%	187.13 (27.47)	193.48 (28.41)	175.97 (25.84)	166.83 (24.49)	8.82 (1.30)	
12	2.5%	387.27 (56.86)	395.66 (58.09)	364.42 (53.50)	333.18 (48.92)	8.28 (1.22)	251.68
	5.0%	335.35 (49.24)	349.07 (51.25)	324.69 (47.67)	305.64 (44.87)	9.09 (1.33)	
	7.5%	281.35 (41.31)	257.44 (37.80)	220.64 (32.39)	194.24 (28.52)	8.86 (1.30)	
24	2.5%	416.99 (61.22)	437.56 (64.24)	419.27 (61.56)	386.51 (56.75)	8.28 (1.22)	290.73
	5.0%	392.11 (57.57)	426.40 (62.60)	382.97 (56.23)	384.49 (56.45)	8.90 (1.30)	
	7.5%	286.88 (42.12)	298.30 (43.80)	258.96 (38.02)	244.49 (35.90)	8.89 (1.30)	
48	2.5%	437.56 (64.24)	460.42 (67.60)	446.70 (65.58)	432.23 (63.46)	8.66 (1.27)	305.27
	5.0%	416.88 (61.20)	432.88 (63.55)	403.93 (59.31)	395.16 (58.02)	8.90 (1.30)	
	7.5%	291.20 (42.75)	307.95 (45.21)	275.21 (40.41)	252.36 (37.05)	8.99 (1.32)	
Mean		**329.52**	**343.39**	**317.90**	**299.89**	**8.71**	
Mean	2.5%	307.65					
	5.0%	275.60					
	7.5%	196.40					

Figures in parentheses indicate per cent saccharification

Note : Initial cellulose content 613 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	236.21	251.65	239.34	223.47	8.54	**191.84**
12	334.66	334.05	303.25	277.69	8.74	**251.68**
24	365.33	387.42	353.73	338.50	8.69	**290.73**
48	381.88	400.41	375.28	359.92	8.85	**305.27**
Mean	**329.52**	**343.39**	**317.90**	**299.89**	**8.71**	

Two way interaction A x C

	2.5%	5.0%	7.5%	Mean
6	241.99	187.09	146.45	**191.84**
12	297.76	264.77	192.51	**251.68**
24	333.72	318.98	219.51	**290.73**
48	357.12	331.55	227.14	**305.27**
Mean	**307.65**	**275.60**	**196.40**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
2.5%	383.28	403.09	386.13	357.37	8.38	**307.65**
5.0%	343.64	362.78	334.88	327.83	8.85	**275.60**
7.5%	261.64	264.29	232.69	214.48	8.89	**196.40**
Mean	**329.52**	**343.39**	**317.90**	**299.89**	**8.71**	

	SEm ±	CD 1%
Incubation time (A)	1.370	5.070
Crude enzymes (B)	1.531	5.668
Enzyme concentration (C)	1.186	4.391
A x B	3.063	11.336
A x C	2.372	8.781
B x C	2.652	9.818
A x B x C	5.305	19.635

The 2.5% substrate concentration was observed to be significantly superior over 5.0% (275.60 mg g^{-1}) and over 7.5% (196.40 mg g^{-1}) substrate concentrations for achieving higher release of reducing sugars.

The reducing sugars released due to effect of incubation periods and crude enzyme extracts indicated varied differences. Values recorded for 48 h incubation showed significantly high reducing sugars in case of *T. reesei* (400.41 mg g^{-1}). This combination showed significantly superior reducing sugars content over other fungi both at 48 h and at 24 h incubation. The lowest reducing sugar released among the fungal crude enzyme extract was noticed in case of *A. awamorii* at all incubation time periods such as 6, 12, 24 and 48 h and with a maximum reducing sugar content (359.92 mg g^{-1}) at 48 h after incubation. Significantly the lowest reducing sugar release was recorded with control treatment (8.71 mg g^{-1}) over 48 h incubation period.

The combination of incubation periods and substrate concentrations also differed with respect to release of reducing sugars. Significantly the higher reducing sugar release was recorded with 2.5% substrate concentration at all incubation periods *viz.*, 6 h (241.99 mg g^{-1}), 12 h (297.76 mg g^{-1}), 24 h (333.73 mg g^{-1}) and 48 h (357.12 mg g^{-1}) when compared to 5.0% and 7.5% substrate concentrations for the respective incubation periods. However, 48 h incubation period with a reducing sugar release of 357.12, 331.55 and 227.14 mg per g was significantly superior over 24 h 333.72, 318.98 and 219.51 mg per g at 2.5%, 5.0% and 7.5% substrate concentrations, respectively.

The substrate concentration and crude enzyme extracts of different fungi varied differently in releasing the reducing sugars. Crude enzyme extract of *T. reesei* recorded significantly higher reducing sugar content at all the concentrations of substrates *viz.*, 2.5% (403.09 mg g^{-1}), 5.0% (362.78 mg g^{-1}) and 7.5% (264.29 mg g^{-1}). These were significantly superior over all other crude enzyme extracts at respective substrate concentrations except in case of 7.5% substrate concentrations where crude enzyme extracts of *T. reesei* (261.64 mg g^{-1}) and *T. reesei* (264.29 mg g^{-1}) was found to be at par.

The release of reducing sugars due to combined interactions of all the three factors such as incubation period, crude enzyme extract and substrate concentration varied significantly. Significantly higher reducing sugar content was recorded at 48 h incubation period in case of *T. reesei* (460.42 mg g^{-1}) at 2.5% substrate concentration with a 67.60 per cent saccharification. It was found to be significantly superior over other fungi at 48 h and at 24 h incubation period.

The substrate concentration of 5.0% recorded the significantly highest reducing sugar (432.88 mg g^{-1}) released with 63.55 per cent saccharification in case of *T. reesei* at 48 h incubation and this was found to be on par with 24 h of incubation (426.40 mg g^{-1} reducing sugar) crude enzyme extract of same fungus and with fungal crude enzyme extract of *T. viridae* at 48 h incubation (416.88 mg g^{-1} reducing sugar).

Substrate concentration of 7.5% resulted in a release of the highest reducing sugar content of 307.95 mg per g with 45.21 per cent saccharification in case of *T. reesei* at 48 h incubation period. However, this treatment was at par with 24 h of incubation (298.30 mg g^{-1}) using the same as well as the crude enzyme extracts of *T. viridae* at 48 h (291.20 mg g^{-1}) of incubation *A. awamorii* recorded the lowest release of reducing sugars for all concentrations of substrate *viz.*, 2.5% (433.23 mg g^{-1}), 5.0% (395.16 mg g^{-1}) and 7.5% (252.36 mg g^{-1}) over 48 h of incubation.

4.10.4 Corn husk

Corn stover was pre-treated with 3.0% NaOH for 8 h under room temperature followed by autoclaving at 121^0C for one hour was subjected for saccharification using different crude enzyme extracts at different substrate concentrations using 20 FPU per g substrate. Each of the main factors such as incubation period, extracts of crude enzyme and substrate concentrations and then along their combinations differed significantly with respect to release of reducing sugars (Table 40).

The period of incubation was found to be significantly influence the amount of reducing sugars. The different incubation differed with each other with respect to release of reducing sugars.

Table 40: Effect of inoculation of crude enzymes (20 FPU g^{-1}) on release of reducing sugars (mg g^{-1}) from corn husk at different substrate concentrations

Incubation time (h)	Substrate conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	
6	2.5%	277.56 (40.29)	317.37 (46.07)	305.00 (44.27)	273.66 (39.72)	8.28 (1.20)	187.26
	5.0%	219.54 (31.87)	227.92 (33.08)	210.78 (30.60)	249.83 (36.27)	8.33 (1.21)	
	7.5%	176.47 (25.62)	188.15 (27.31)	172.92 (25.10)	164.29 (23.85)	8.81 (1.28)	
12	2.5%	368.23 (53.45)	382.70 (55.55)	363.66 (52.79)	323.27 (46.93)	8.28 (1.20)	241.69
	5.0%	319.35 (46.36)	331.92 (48.18)	308.69 (44.81)	303.35 (44.03)	8.71 (1.27)	
	7.5%	245.76 (35.67)	253.63 (36.82)	213.28 (30.96)	185.61 (26.94)	8.84 (1.28)	
24	2.5%	408.61 (59.31)	420.80 (61.08)	410.13 (59.53)	381.94 (55.44)	8.28 (1.20)	287.66
	5.0%	398.65 (57.87)	410.36 (59.57)	386.02 (56.04)	374.96 (54.43)	8.90 (1.29)	
	7.5%	292.66 (42.48)	293.96 (42.67)	264.46 (38.39)	246.31 (35.76)	8.89 (1.29)	
48	2.5%	432.99 (62.85)	432.23 (62.74)	428.42 (62.19)	405.56 (58.87)	9.04 (1.31)	293.79
	5.0%	402.40 (58.41)	410.78 (59.63)	383.35 (55.65)	378.78 (54.99)	9.09 (1.32)	
	7.5%	291.20 (42.27)	299.32 (43.45)	266.07 (38.62)	248.71 (36.10)	8.98 (1.30)	
Mean		**319.45**	**330.76**	**309.40**	**294.69**	**8.70**	
Mean	2.5%	298.30					
	5.0%	267.59					
	7.5%	191.92					

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 620 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	224.53	244.48	229.57	229.26	8.47	**187.26**
12	311.12	322.75	295.21	270.74	8.61	**241.69**
24	366.64	375.04	353.54	334.41	8.69	**287.66**
48	375.53	380.78	359.28	344.35	9.04	**293.79**
Mean	**319.45**	**330.76**	**309.40**	**294.69**	**8.70**	

Two way interaction A x C

	2.5%	5.0%	7.5%	Mean
6	236.37	183.28	142.13	**187.26**
12	289.23	254.40	181.42	**241.69**
24	325.95	315.78	221.26	**287.66**
48	341.65	316.88	222.85	**293.79**
Mean	**298.30**	**267.59**	**191.92**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
2.5%	371.85	388.28	376.80	346.11	8.47	**298.30**
5.0%	334.99	345.25	322.21	326.73	8.76	**267.59**
7.5%	251.52	258.77	229.18	211.23	8.88	**191.92**
Mean	**319.45**	**330.76**	**309.40**	**294.69**	**8.70**	

	SEm $\pm$	CD 1%
Incubation time (A)	1.409	5.217
Crude enzymes (B)	1.576	5.832
Enzyme concentration (C)	1.220	4.518
A x B	3.151	11.665
A x C	2.441	9.035
B x C	2.729	10.102
A x B x C	5.458	20.204

After 48 h incubation a maximum release of reducing sugars at 293.79 mg per g was recorded. It was highly significant over 24 h (289.66 mg g^{-1}), 12 h (241.69 mg g^{-1}) and 6 h (187.26 mg g^{-1}) incubation period with regards to release of reducing sugars.

Crude enzyme extracts of different fungi also differed significantly with one another. *T. reesei* with a mean maximum amount of reducing sugars released, 330.76 mg per g was found to be highly superior over *T. viridae* (319.45 mg g^{-1}), *A. sydowii* (309.40 mg g^{-1}) and *A. awamorii* (294.69 mg g^{-1}) and also over control (8.70 mg g^{-1}).

All the substrate concentrations differed significantly with each other in releasing the reducing sugar 2.5% substrate concentration and resulted in significantly higher amount of reducing sugars (298.30 mg g^{-1}) at 5.0% (267.59 mg g^{-1}) and 7.5% (191.92 mg g^{-1}).

The combined effect of incubation period and crude extract of fungal enzymes were found to vary significant in releasing of reducing sugars. Maximum amount of reducing sugars was observed at 48 h incubation period with *T. reesei* crude enzyme extract (380.78 mg g^{-1}). This was found to be at par with crude enzyme extracts of *T. viridae* (375.53 mg g^{-1}) and *T. reesei* extracts (375.04 mg g^{-1}) at 48 and 24 h of incubation period, respectively and statistically superior over rest of the combinations of crude enzymes and incubation periods. Significantly lower reducing sugar was recorded in *A. awamorii* at all incubation periods as compared to other fungal crude enzyme extracts.

The combining effect of incubation period and substrate concentration indicated significant differences for release of reducing sugars, substrate concentration of 2.5% resulted in significantly higher release of reducing sugar at all the incubation periods, at 6 h (236.37 mg g^{-1}) and at 48 h (341.65 mg g^{-1}) of incubation, substrate concentration of 5.0% recorded a maximum of 316.88 mg per g reducing sugar at 48 h incubation, which was found to be on par with those at 24 h incubation (315.78 mg g^{-1}) and significantly superior over 12 and 6 h of incubation. Substrate concentration of 7.5% had recorded a maximum reducing sugar content of 222.85 mg per g for 48 h of incubation and 221.26 mg per g for 24 h of incubation. These two incubation periods were found to be significantly superior to 12 and 6 h of incubation.

Substrate concentration and crude enzyme extracts of different fungi also influenced the release of reducing sugar from corn husk. Significantly higher reducing sugar release (388.28 mg g^{-1}) was by the crude enzyme extracts of *T. reesei* at 2.5% substrate concentration, which was significantly superior over other crude enzyme extracts at 2.5%, 5.0% and 7.5% substrate concentrations. Further, *T. reesei* recorded significantly higher (345.25 mg g^{-1}) reducing sugar at 5.0% substrate concentration, when compared to other crude enzyme extracts. At 7.5% substrate concentration, the activity of crude enzyme extract from *T. reesei* (258.77 mg g^{-1}) and *T. viridae* (251.50 mg g^{-1}) were found to be on par with each other but significantly superior over other two fungi crude enzyme extracts and control treatment.

The release of reducing sugars due to combined effect of incubation, crude enzyme extracts and substrate concentration differed significantly. Significantly higher release of reducing sugar and per cent saccharification was noticed at 2.5% substrate concentration over 48 h of incubation with enzyme extract of *T. viridae* (432.99 mg g^{-1}) resulting in and 62.85 per cent saccharificaiton. It was found to be on par with crude enzyme extracts of *T. viridae* (432.23 mg g^{-1}) and *A. sydowii* (428.42 mg g^{-1}) over 48 h incubation as well as with *T. reesei* (420.80 mg g^{-1}) at 24 h incubation but significantly superior over all other combined interaction.

At 5.0% substrate concentrations, *T. reesei* recorded a maximum relese of reducing sugar (410.79 mg g^{-1}) and (59.63 per cent saccharification) over 48 h incubation. However, this was found to be on par with *T. reesei* crude enzyme at 24 h (410.36 mg g^{-1}) incubation and with *T. viridae* crude enzyme extract both at 48 h (402.40 mg g^{-1}) and 24 h (398.65 mg g^{-1}) and significantly superior over the activity of other crude fungal enzymes at either 5% or 7.5% substrate concentration.

Significantly higher reducing sugar release was observed at 7.5% substrate concentration with *T. reesei* over 48 h incubation (399.32 mg g^{-1}) and 43.45 per cent saccharification.

This was found to be on par with 24 h incubation (293.96 mg g^{-1}) of the same enzyme extract as well as with extract of *T. viridae* at 48 and 24 h incubation and significantly superior over control and other crude enzyme extracts for all incubation periods at 7.5% substrate concentration.

4.10.5 Corn stover

Saccharification of alkali pre-treated sugarcane tops was done by using different crude enzyme extracts with varied substrate concentrations. The effect of individual factors as well as their interaction varied significantly in releasing reducing sugars.

The incubation of crude enzyme extracts with substrate influenced the release of reducing sugars. A mean maximum reducing sugar release of 299.18 mg per g was recorded at 48 h of incubation (Table 41). It was significantly superior over 24, 12 and 6 h incubation periods with a release of 292.85, 245.86 and 186.31 mg per g reducing sugars at 24, 12 and 6 h of incubation, respectively.

The various crude cellulases from fungi showed a significant differences with regard to release of reducing sugars. A mean maximum, 336.24 mg per g reducing sugar release was shown by crude enzyme extract of *T. reesei*. This was significantly superior over the treatments other fungal crude enzyme extracts. Significantly lower reducing sugar release was observed in the control treatment (8.74 mg g^{-1}).

The substrate concentration also showed significant variation with respect to release of reducing sugars. Mean maximum of 303.76 mg per g reducing sugar was released when 2.5% substrate was used. Substrate concentration of 2.5% was shown to be significantly superior over 5.0% (270.03 mg g^{-1}) and over 7.5% (194.36 mg g^{-1}).

The reducing sugars released due to incubation periods and crude enzyme extracts indicated varied differences. At 48 h incubation had given significantly high reducing sugars in *T. reesei* with 390.17 mg per g reducing sugars. This combination showed significantly higher reducing sugars over other fungi at both 48 and 24 h of incubation. The lowest reducing sugar release was noticed in case of *A. awamorii* over all the incubation periods with a maximum reducing sugar release for 48 h of incubation reducing sugar (352.59 mg g^{-1}).

The combination of incubation periods and substrate concentrations also differed significantly with respect to release of reducing sugars. Significantly the highest reducing sugar release was recorded for 2.50% substrate concentration at all incubation periods *viz.*, 6 h (239.55 mg/h), 12 h (295.63 mg g^{-1}) 24 h (330.14 mg g^{-1}) and 48 h (349.72 mg g^{-1}). This compared to 5.0% and 7.5% substrate concentrations at respective incubation periods. The reducing sugar released at 5% substrate concentration in 48 h (323.78 mg g^{-1}) was found to be on par with 24 h (321.77 mg g^{-1}) incubation.

The substrate concentration and crude enzyme extracts of different fungi varied differently in release of reducing sugars, crude enzyme extracts of *T. reesei* recorded significantly higher reducing sugar content at all concentrations of substrates *viz.*, 2.5% (394.32 mg g^{-1}), 5.0% (353.07 mg g^{-1}) and 7.5% (461.34 mg g^{-1}). These were found to be significantly superior over all other crude enzyme extracts at respective substrate concentrations in releasing the reducing sugars.

The release of reducing sugars as a measure of combined interactions of all the three factors varied differentially. Significantly higher reducing sugar content was recorded for 48 h incubation in case of *T. reesei* (45.94 mg g^{-1}) with 2.5% substrate concentration 49.79 per cent saccharification. It was found to be significantly superior over other fungi crude enzyme extracts at 48 h both at 24 h of incubation.

Substrate concentration of 5.0 per cent recorded significantly higher (422.97 mg g^{-1} reducing sugar) resulting in 47.23 per cent saccharification in case of *T. reesei* at 48 h of incubation. This was found to be on par with 24 h incubation (422.97 mg g^{-1}) in case of *T. reesei* crude enzyme extract.

Substrate concentration of 7.5% resulted in a release of highest reducing sugar content (301.60 mg g^{-1}) and the per cent saccharification of 33.68 with *T. reesei* at 48 h incubation.

Table 41: Effect of inoculation of crude enzymes (20 FPU g^{-1}) on release of reducing sugars (mg g^{-1}) from corn stover at different substrate concentrations

Incubation time (h)	Substrate conc.	Fungal crude enzymes					Mean
		Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	
6	2.5%	285.94 (41.98)	316.42 (46.46)	309.56 (45.45)	277.56 (40.75)	8.28 (0.92)	186.31
	5.0%	216.35 (31.76)	227.92 (33.46)	216.49 (31.78)	213.70 (31.38)	8.52 (0.95)	
	7.5%	178.25 (26.17)	191.19 (28.07)	172.92 (25.39)	162.77 (23.90)	8.80 (0.98)	
12	2.5%	378.13 (55.52)	386.51 (56.76)	378.13 (55.52)	327.09 (48.02)	8.28 (0.92)	245.86
	5.0%	325.07 (47.73)	338.40 (49.68)	313.26 (45.99)	304.49 (44.70)	8.71 (0.97)	
	7.5%	248.55 (36.49)	254.65 (37.39)	217.59 (31.95)	190.18 (27.92)	8.86 (0.99)	
24	2.5%	414.70 (60.89)	428.42 (62.90)	414.70 (60.89)	384.23 (56.41)	8.66 (0.97)	292.85
	5.0%	409.49 (60.12)	422.97 (62.10)	381.07 (55.95)	386.39 (56.73)	8.90 (1.00)	
	7.5%	291.47 (42.79)	297.93 (43.74)	286.43 (42.05)	248.41 (36.47)	8.92 (0.99)	
48	2.5%	435.28 (63.91)	445.94 (65.47)	439.85 (64.58)	418.51 (61.45)	9.04 (1.01)	299.18
	5.0%	410.02 (60.20)	422.97 (62.10)	389.07 (57.12)	387.92 (56.95)	8.90 (1.00)	
	7.5%	292.72 (42.98)	301.60 (44.28)	265.56 (38.99)	251.35 (36.90)	8.98 (1.00)	
Mean		**323.83**	**336.24**	**315.39**	**296.05**	**8.74**	
Mean	2.5%	303.76					
	5.0%	270.03					
	7.5%	194.36					

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}

Contd…..

Two way interaction A x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
6	226.85	245.18	232.99	218.01	8.53	**186.31**
12	317.25	326.52	302.99	273.92	8.62	**245.86**
24	371.89	383.11	360.73	339.68	8.83	**292.85**
48	379.34	390.17	364.82	352.59	8.97	**299.18**
Mean	**323.83**	**336.24**	**315.39**	**296.05**	**8.74**	

Two way interaction A x C

	2.5%	5.0%	7.5%	Mean
6	239.55	176.60	142.79	**186.31**
12	295.63	257.99	183.97	**245.86**
24	330.14	321.77	226.63	**292.85**
48	349.72	323.78	224.04	**299.18**
Mean	**303.76**	**270.03**	**194.36**	

Two way interaction C x B

	Trichoderma viridae	*Trichoderma reesei*	*Aspergillus sydowiii*	*Aspergillus awamorii*	Control	Mean
2.5%	378.51	394.32	385.56	351.85	8.57	**303.76**
5.0%	340.23	353.07	324.97	323.13	8.76	**270.03**
7.5%	252.75	261.34	235.63	213.18	8.89	**194.36**
Mean	**323.83**	**336.24**	**315.39**	**296.05**	**8.74**	

	SEm $\pm$	CD 1%
Incubation time (A)	0.694	2.569
Crude enzymes (B)	0.776	2.873
Enzyme concentration (C)	0.601	2.225
A x B	1.552	5.745
A x C	1.202	4.450
B x C	1.344	4.976
A x B x C	2.688	9.951

However, this was at par with 24 h incubation (297.93 mg g^{-1}) with same enzyme extract as well as with *T. vinidae* crude enzyme extract at 48 h (292.72 mg g^{-1}) incubation. Crude enzyme extract of *A. awamorii* recorded the lowest release of reducing sugars at substrate concentration of 2.50 per cent (418.51 mg g^{-1}), 5.0 per cent (387.92 mg g^{-1}) and 7.5 per cent (251.35 mg g^{-1}) at the end of 48 h incubation.

4.11 Effect of different concentrations of commercial cellulase on release of reducing sugars from different alkali pre-treated substrates

4.11.1 Sugarcane bagasse

The substrate sugarcane bagasse, pre-treated with 3.0% NaOH for 8 h at room temperature and autoclaved at 121^{0}C temperature for one hour was subjected for saccharification at 5% solids using commercial cellulase of different concentrations (10, 15 and 20 FPU) along with 10 IU per g β-glucosidase and 5 U per g xylanase. The data presented in Table 42 and Fig. 1 indicates significant variations in release of reducing sugars due to factors, incubation period, concentrations of enzyme and combination of incubation period and enzyme concentrations.

The release of reducing sugars over a different period of incubation varied significantly. The mean higher release of reducing sugars, 422.30 mg per g was recorded at the end of 24 h incubation period. It was found to be on par with 12 h incubation period (419.87 mg g^{-1}) and significantly superior over 8 h (376.67 mg g^{-1}), 4 h (308.50 mg g^{-1}) and over 2 h (223.58 mg g^{-1}) incubation periods with respect to release of reducing sugars.

The different concentrations of commercial cellulase indicated marked variations with regard to release of reducing sugars. Significantly the mean maximum reducing sugar release, 565.58 mg per g was recorded for 20 U of enzyme concentration, which was significantly superior over 15 U (516.21 mg g^{-1}) and over 10 U (310.285 mg g^{-1}) enzyme concentrations.

The combined effect of different concentrations and incubation period also differed significantly with regards to release of reducing sugars in pre-treated bagasse. Significantly the highest reducing sugars release was observed at 24 h incubation period, 658.41 mg per g, when 15 U of enzyme concentration was used. However, it was found to be at par with 12 h of incubation with the same enzyme concentration (655.32 mg g^{-1}) as well as with 20 U of enzyme concentration at both 24 h (658.01 mg g^{-1}) and 12 h (654.24 mg g^{-1}) incubation period. The 15 U and 20 U enzyme concentrations either at 12 h or at 24 h incubation were found to be significantly superior over other incubation periods (8, 4 and 2 h) and also over 10 U enzyme concentration for all the incubation periods. It was also observed that the per cent saccharification achieved with 15 and 20 U enzyme concentration either at 12 h or at 24 h incubation ranged between 93.02 and 93.61 per cent and found to be very high for pre-treated bagasse. The control treatment recorded a significantly the lowest release of reducing sugar with a range between 1.18 and 1.94 mg per g between all the incubation periods.

4.11.2 Sugarcane tops

The saccharification of alkali pre-treated sugarcane tops at 5% solids was done by using different concentrations of commercial cellulase enzymes at various incubation periods. The saccharification results indicated a significant variation due to concentrations of enzymes, incubation periods as well as their combined interaction. The data is presented in Table 43 and Fig. 1.

Significant differences in release of reducing sugars were recorded for different incubation periods. A mean maximum of reducing sugar release was observed at 24 h incubation, 398.40 mg per g. It was found to be on par with 12 h incubation period (397.02 mg g^{-1}) and significantly superior over 8, 4 and 2 h incubation periods.

The concentrations of cellulase enzyme showed significant variations in reducing sugar release. The higher reducing sugar release of 533.14 mg per g was recorded for 20 U enzyme concentration, which was significantly superior over both 15 U (488.36 mg g^{-1}) and 10 U (296.73 mg g^{-1}) enzyme concentrations.

Table 42: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from sugarcane bagasse

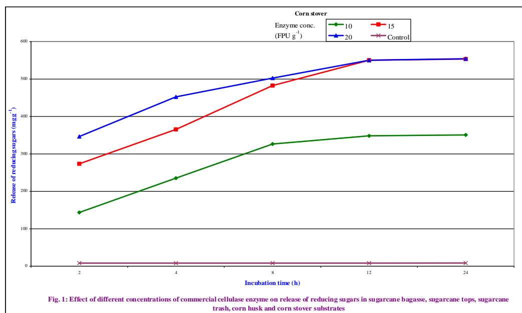
Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	184.38 (26.21)	305.29 (43.41)	396.35 (56.35)	8.29 (1.18)	**223.58**
4	296.23 (42.12)	420.44 (59.78)	509.00 (72.37)	8.32 (1.18)	**308.50**
8	346.48 (49.26)	541.59 (77.00)	610.25 (86.77)	8.36 (1.19)	**376.67**
12	361.51 (51.39)	655.32 (93.17)	654.24 (93.02)	8.38 (1.19)	**419.86**
24	364.33 (51.80)	658.41 (93.61)	658.05 (93.56)	8.39 (1.19)	**422.30**
Mean	**310.59**	**516.21**	**565.58**	**8.35**	
	SE±		**CD (1%)**		
Incubation time (A)	1.271		4.863		
Concentrations of enzyme (B)	1.137		4.350		
Interaction (A x B)	2.543		9.726		

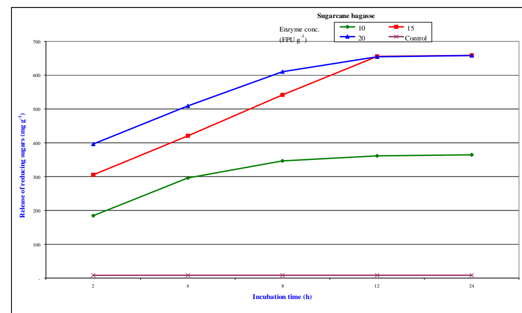
Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 633 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

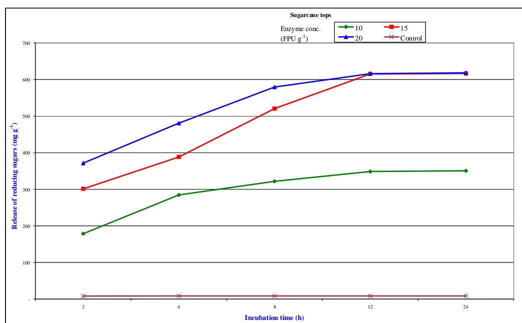
Table 43: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from sugarcane tops

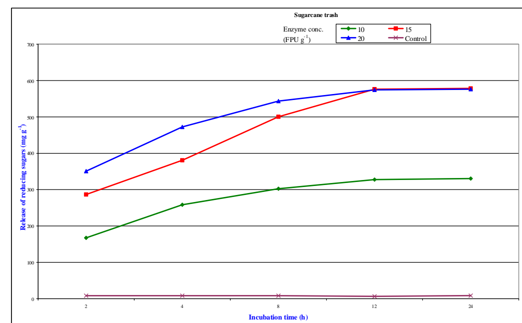
Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	178.48 (26.20)	301.17 (44.22)	371.38 (54.52)	8.26 (1.21)	**214.83**
4	284.52 (41.77)	388.49 (57.04)	480.64 (70.56)	8.40 (1.24)	**290.51**
8	321.68 (47.23)	520.53 (76.43)	579.41 (85.10)	8.40 (1.23)	**357.51**
12	348.54 (51.17)	615.23 (90.33)	615.89 (90.42)	8.41 (1.24)	**397.02**
24	350.44 (51.45)	616.37 (90.49)	618.35 (90.79)	8.45 (1.24)	**398.40**
Mean	**296.73**	**488.36**	**533.14**	**8.39**	
	SE±		**CD (1%)**		
Incubation time (A)	1.474		5.636		
Concentrations of enzyme (B)	1.318		5.041		
Interaction (A x B)	2.947		11.272		

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly









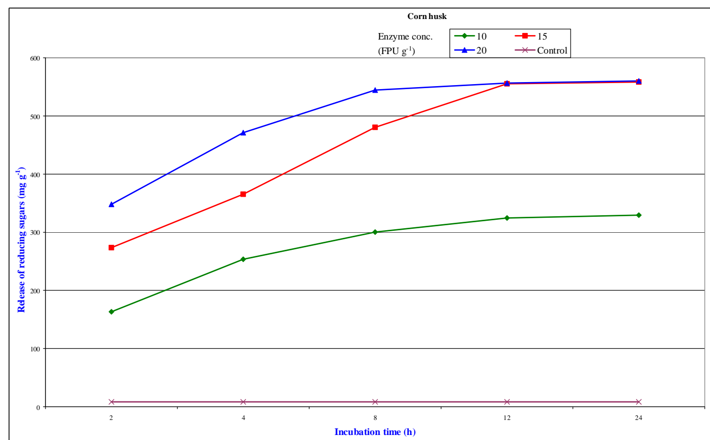


Fig . Effect of different concentrations of commercial cellulase enzyme on release of reducing sugars in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover substrates

The combination of enzyme concentrations and incubation periods also differed significantly with respect to release of reducing sugars in sugarcane tops. Significantly the highest release of reducing sugars was observed at 20 U enzyme concentrations on 24 h incubation, 618.35 mg per g with a per cent saccharification of 90.79 per cent. It was observed that the 20 U enzyme concentration was significantly at par with 12 h incubation which recorded 615.89 mg per g and 90.42 per cent, reducing sugars release and per cent saccharification and also with 15 U of enzyme concentration both at 24 h and 12 h incubation periods (616 mg g^{-1} and 90.49% and 615.23 mg g^{-1} and 90.33% reducing sugar release and per cent saccharification, respectively at 24 and 12 h incubation) and found to be significantly superior over 15 and 20 U enzyme concentrations at 8, 4 and 2 h incubation periods and over 10 U enzyme concentration at all incubation periods with regards to release of reducing sugars.

4.11.3 Sugarcane trash

The alkali pre-treated sugarcane trash at 5% solids was saccharified by using different concentrations of commercial cellulase enzyme at various incubation periods. The release of reducing sugars indicated marked variations as an effect of concentrations of enzymes, incubation periods as well as their combined interactions.

The Table 44 and Fig. 1 indicates significant differences with respect to release of reducing sugars due to incubation periods. The mean maximum of reducing sugar release was observed at 24 h incubation period, 398.40 mg per g which was significantly on par with 12 h incubation period (397.02 mg g^{-1}) and significantly superior over 8, 4 and 2 h of incubation periods.

The concentration of cellulase enzyme recorded a significant variation with regard to release of reducing sugars in sugarcane trash. The mean higher release of reducing sugar, 503.51 mg per g was observed with 20 U enzyme concentration and it was found to be significantly superior over both 15 U (464.45 mg g^{-1}) and 10 U (277.26 mg g^{-1}) enzyme concentrations.

The combination of enzyme concentrations and incubation periods showed significant differences with regard to release of reducing sugars in sugarcane trash. Significantly the highest release of reducing sugars was observed at 15 U enzyme concentration at the end of 24 h incubation period, 578.34 mg per g with a per cent saccharification of 84.91 per cent. It was observed that the 15 U enzyme concentration was significantly at par with 12 h incubation time which recorded 576.34 mg per g and 84.61 per cent, reducing sugar release and per cent saccharification and also with 20 U enzyme concentration both at 24 and 12 h incubation periods (576.34 mg g^{-1} and 84.62% and 574.34 mg g^{-1} and 84.32%, reducing sugars and per cent saccharification, respectively at 24 and 12 h incubation periods) and found to be significantly superior over 15 U and 20 U enzyme concentrations at 8, 4 and 2 h incubation periods and also over 10 U enzyme concentrations at all the incubation periods (24, 12, 8, 4 and 2 h) with respect to release of reducing sugars. The control treatment recorded a significantly the lowest reducing sugar release with a range between 1.21 and 1.24 mg per g between all the incubation periods.

4.11.4 Corn husk

The saccharification of alkali pre-treated corn husk at 5.0% solids was done by using different concentrations of commercial cellulase enzyme. The saccharification results indicated a significant variation due to concentrations of enzymes, incubation periods and their combined interactions.

Significant difference in release of reducing sugars was observed for different incubation periods. The mean higher reducing sugar release was observed at the end of 24 h incubation period, 364.12 mg per g. It was significantly on par with 12 h incubation period (361.17 mg g^{-1}) and superior over 8, 4 and 2 h incubation periods (Table 45 and Fig. 1).

The reducing sugars released to different concentrations of enzymes was also significant. The mean maximum reducing sugar release of 496.13 mg per g was recorded for 20 U enzyme concentrations and it was significantly superior over 15 U (446.61 mg g^{-1}) and 10 U (274.22 mg g^{-1}) incubation periods.

Table 44: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from sugarcane trash

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	167.45 (24.59)	286.56 (42.07)	350.90 (51.52)	8.24 (1.21)	**203.29**
4	258.38 (37.94)	380.58 (55.87)	472.43 (69.36)	8.26 (1.21)	**279.91**
8	302.51 (44.41)	500.49 (73.48)	543.54 (79.80)	8.35 (1.23)	**338.72**
12	327.50 (48.08)	576.28 (84.61)	574.34 (84.32)	6.40 (0.94)	**371.13**
24	330.44 (48.51)	578.34 (84.91)	576.34 (84.62)	8.42 (1.24)	**373.38**
Mean	**277.26**	**464.45**	**503.51**	**7.94**	
	SE±		**CD (1%)**		
Incubation time (A)	1.316		5.034		
Concentrations of enzyme (B)	1.177		4.503		
Interaction (A x B)	2.633		10.069		

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 45: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from corn husk

Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	163.33 (23.71)	273.49 (39.70)	348.04 (50.52)	8.25 (1.20)	**198.28**
4	253.51 (36.80)	365.43 (53.05)	471.37 (68.42)	8.27 (1.20)	**274.65**
8	300.37 (43.60)	480.47 (69.75)	544.45 (79.03)	8.33 (1.21)	**333.41**
12	324.47 (47.09)	555.30 (80.61)	556.55 (80.79)	8.36 (1.21)	**361.17**
24	329.43 (47.82)	558.38 (81.10)	560.25 (81.33)	8.43 (1.22)	**364.12**
Mean	**274.22**	**446.61**	**496.13**	**8.33**	
	SE±		**CD (1%)**		
Incubation time (A)	1.074		4.108		
Concentrations of enzyme (B)	0.961		3.674		
Interaction (A x B)	2.148		8.215		

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 620 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

A significant variation in release of reducing sugar was also noticed for the interactions between enzyme concentrations and incubation periods. Significantly the highest release of reducing sugars in corn husk was recorded with 20 U enzyme concentration at the end of 24 h incubation period, 560.25 mg per g with a per cent saccharification of 81.33 per cent. It was revealed that the 20 U enzyme concentration was significantly at par with 12 h incubation time which recorded 556.55 mg per g reducing sugars and 80.79 per cent saccharification and also with 15 U enzyme concentrations both at 24 and 12 h incubation periods (558.38 mg g^{-1} and 81.06% and 55.30 mg g^{-1} and 80.61%, reducing sugars and per cent saccharification respectively at 24 h and 12 h incubation) and found to be significantly superior over 15 U and 20 U enzyme concentrations at 8, 4 and 2 h incubation periods and also over 10 U enzyme concentration at all the incubation periods *viz.*, 24, 12, 8, 4 and 2 h with regard to release of reducing sugars.

4.11.5 Corn stover

The substrate corn stover which was pre-treated with alkali was subjected for commercial cellulase enzyme saccharification at 5.0% solids concentration. The saccharification involved three concentrations of enzyme (10, 15 and 15 U) along with 10 IU per g β-glucosidase and 5 U per g xylanase and four incubation periods (2, 4, 8, 12 and 24 h). The data depicted in Table 46 and Fig. 1 indicates significant variations in reducing sugar release in response to concentrations of enzymes, incubation periods and their combined interactions.

Significant variations in release of reducing sugar were recorded due to incubation periods. The mean maximum reducing sugar release was observed at the end of 24 h incubation period, 366.66 mg per g, where the treatment did not differ significantly when compared to 12 h incubation period, which recorded 364.32 mg per g reducing sugar. Both the above incubation periods were found to be significantly superior over 8, 4 and 2 h incubation periods with respect to release of reducing sugars.

The different concentrations of enzyme also indicated significant variations with regard to release of reducing sugars. Significantly mean maximum reducing sugar (481.18 mg g^{-1}) was recorded when 20 U of enzyme concentration was used and it was found to be significantly superior over 15 U (445.04 mg g^{-1}) and 10 U (280.82 mg g^{-1}) incubation periods with respect to release of reducing sugars.

The interactions between concentrations of enzymes and incubation periods also varied significantly in releasing the reducing sugars. Significantly the highest release of reducing sugars (554.23 mg g^{-1}) was recorded with 20 U enzyme concentration at the end of 24 h incubation period, where the per cent saccharification achieved was 81.37 per cent. It was found to be on par with 20 U enzyme concentration at the end of 12 h incubation period with a reducing sugar release and per cent saccharification of 550.24 mg per g and 80.79 per cent, respectively and with 15 U enzyme concentrations both at the end of 24 and 12 h incubation periods (553.41 mg g^{-1} and 81.06% and 550.37 mg g^{-1} and 80.61%, respectively reducing sugars and per cent sacharification at 24 and 12 h incubation periods). Both 20 and 15 U enzyme concentrations after 12 and 24 h incubation were found to be significantly superior over their 2, 4 and 8 h incubation as well as over 10 U enzyme concentrations at all the incubation periods studied with regard to release of reducing sugars. Significantly the lowest reducing sugars release was observed in the control treatment with a range between 8.24 and 8.38 mg per reducing sugar for all the incubation periods.

4.12 Effect of different substrate concentrations on release of reducing sugar from different alkali pre-treated substrates by commercial cellulase enzyme (15 FPU g^{-1})

4.12.1 Sugarcane bagasse

The alkali pre-treated sugarcane bagasse was saccharified by using commercial cellulose at 15 FPU per g with different substrate concentrations (2.5%, 5.0% and 7.5%). The results obtained indicated significant differences with respect to release of reducing sugars due to individual factors incubation period, substrate concentration and their combined interaction effects as shown in Table 47 and Fig. 2.

Table 46: Effect of different concentrations of commercial cellulase on release of reducing sugars (mg g^{-1}) from corn stover

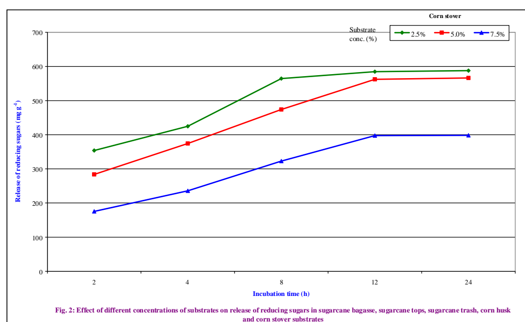
Incubation time (h)	Concentrations of enzyme FPU				Mean
	10	15	20	Control	
2	143.56 (21.07)	273.49 (40.15)	346.56 (50.88)	8.24 (1.21)	**192.96**
4	235.20 (34.53)	365.47 (53.66)	452.36 (66.41)	8.29 (1.23)	**265.33**
8	326.40 (47.92)	482.44 (70.83)	502.52 (73.78)	8.33 (1.22)	**329.93**
12	348.32 (51.14)	550.37 (80.80)	550.24 (80.79)	8.36 (1.23)	**364.32**
24	350.63 (51.48)	553.41 (81.25)	554.23 (81.37)	8.38 (1.23)	**366.66**
Mean	**280.82**	**445.04**	**481.18**	**8.32**	
	SE±		**CD (1%)**		
Incubation time (A)	1.310		5.010		
Concentrations of enzyme (B)	1.172		4.481		
Interaction (A x B)	2.620		10.020		

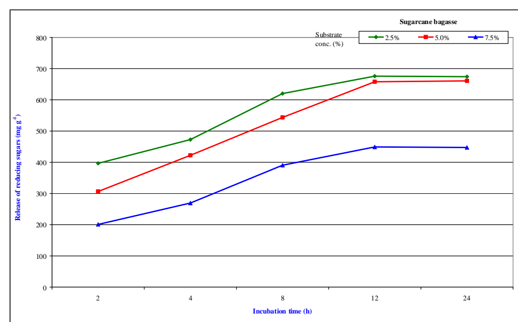
Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

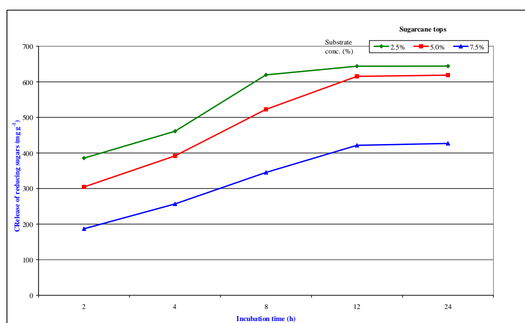
Table 47: Effect of different substrate concentrations on release of reducing sugars (mg g^{-1}) from sugarcane bagasse 15 FPU per g

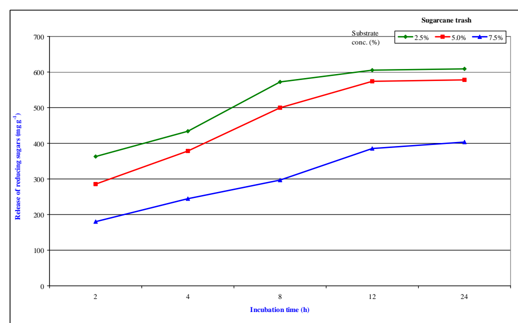
Incubation time (h)	Concentrations of Substrates			Mean
	2.5%	5.0%	7.5%	
2	396.42 (56.36)	306.40 (43.56)	200.84 (28.56)	**301.22**
4	472.61 (67.20)	422.21 (60.03)	269.11 (38.26)	**387.98**
8	620.18 (88.18)	543.71 (77.30)	390.61 (55.54)	**518.17**
12	675.73 (96.08)	657.84 (93.53)	449.10 (63.85)	**594.22**
24	674.30 (95.87)	660.61 (93.92)	447.46 (63.62)	**594.12**
Mean	**567.85**	**518.15**	**351.42**	
	SE±	**CD (1%)**		
Incubation time (A)	3.45	13.40		
Concentrations of substrate (B)	2.67	10.38		
Interaction (A x B)	5.97	23.21		

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 633 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly









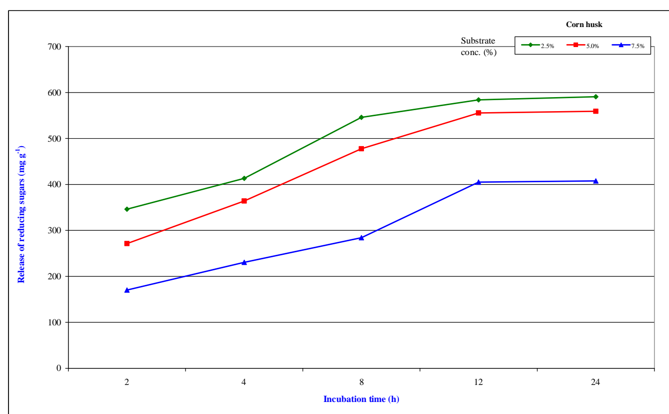


Fig 2 . Effect of different concentrations of substrates on release of reducing sugars in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover substrates

The individual factor incubation period showed significant variations with regard to release of reducing sugars in bagasse. Significant mean maximum release of reducing sugars was observed at the end of 12 h incubation period, 594.22 mg per g, which was significantly on par with 24 h incubation period (594.12 mg g^{-1}) and superior over 8, 4 and 2 h incubation periods.

The factor substrate concentration also differed significantly with regard to release of reducing sugar. Significantly the mean maximum reducing sugar release was obtained at 2.5% substrate concentration, 567.85 mg per g. It was significantly superior over 5.0% (518.15 mg g^{-1}) and over 7.5% (351.42 mg g^{-1}).

The combined interactions of incubation period and substrate concentration were significant with regard to release of reducing sugars. Significantly the highest release of reducing sugars was recorded in 2.5% substrate concentration at 12 h incubation period, 675.73 mg which corresponds to 96.08 per cent saccharification. It was found to be significantly at par with 24 h incubation (674.30 mg g^{-1} reducing sugars and 95.87% saccharification) at 2.5% substrate concentration and as well as with 5.0% substrate concentration (660.61 mg g^{-1} and 93.92% reducing sugars and per cent saccharification). They ere found to be significantly superior over rest of the combinations of substrate concentrations and incubation periods.

The 5.0% concentration of substrate with 660.61 mg per g reducing sugar at the end of 24 h incubation was found to be on par with 12 h incubation period (657.84 mg g^{-1}) reducing sugar and significantly superior over other incubation periods with respect to release of reducing sugars, whereas 7.5% substrate concentration recorded a maximum saccharification of 63.85% per cent at 12 h incubation period with 449.10 mg per g reducing sugars. It was found to be on par with 24 h incubation (447.46 mg g^{-1} and 63.85% reducing sugars and per cent saccharification) and significantly superior over 8, 4 and 2 h incubation periods.

4.12.2 Sugarcane tops

The sugarcane tops which was pre-treated with alkali was further subjected for saccharification by using commercial cellulose enzyme at 15 FPU per g substrate by varying the substrate concentrations. The data obtained revealed significant variations in release of reducing sugars for incubation periods, substrate concentrations and their combinations (substrate concentrations and incubation periods).

Significant differences in release of reducing sugars were recorded with regard to different incubation periods. Significant mean maximum reducing sugar release was observed at the end of 24 h incubation period, 424.50 mg per g. It was found to be on par with 12 h (422.27 mg g^{-1}) and significantly superior over rest of the incubation periods (8, 4 and 2 h) with respect to release of reducing sugars (Table 48 and Fig. 2).

The different substrate concentrations also indicated variations in release of reducing sugars due to 15 FPU per g cellulose activity. Significant mean higher reducing sugar release of 550.89 mg per g was observed in 2.5% substrate concentration. It was found to be significantly superior over 5.0% (490.60 mg g^{-1}) and 7.5% (327.40 mg g^{-1}) substrate concentrations.

The interaction between substrate concentration and incubation period showed significant differences with regard to release of reducing sugars. The 2.5% substrate concentration recorded significantly the higher reducing sugar release at the end of 24 h incubation period, 644.40 mg per g with 94.56 per cent saccharification however it was found to be on par with 12 h incubation (643.94 mg g^{-1} and 94.54%, reducing sugars at per cent saccharification, respectively) and superior over 8, 4 and 2 h incubation periods when 2.5% substrate concentration was used with regard to 5.0% substrate concentration. A significantly highest reducing sugar content of 618.78 mg per g was recorded at the end of 24 h incubation period, which corresponds 90.85 per cent saccharification. It was found to be on par with 12 h incubation period, 615.37 mg per g reducing sugar with 90.35 per cent saccharification and significantly superior over rest of the incubation periods with respect to release of reducing sugars.

Table 48: Effect of different substrate concentrations on release of reducing sugars (mg g^{-1}) from sugarcane tops 15 FPU per g

Incubation time (h)	Concentrations of Substrates			Mean
	2.5%	5.0%	7.5%	
2	385.70 (56.63)	304.50 (44.71)	186.88 (27.44)	**292.36**
4	461.18 (67.71)	391.92 (57.54)	256.68 (37.69)	**369.93**
8	619.59 (90.97)	522.41 (76.70)	345.35 (50.70)	**495.78**
12	643.94 (94.54)	615.37 (90.35)	421.38 (61.87)	**560.23**
24	644.04 (94.56)	618.78 (90.85)	426.73 (62.65)	**563.18**
Mean	**550.89**	**490.60**	**327.40**	
	SE±	**CD (1%)**		
Incubation time (A)	4.08	15.87		
Concentrations of substrate (B)	3.16	12.29		
Interaction (A x B)	7.07	27.49		

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 49: Effect of different substrate concentrations on release of reducing sugars (mg g^{-1}) from sugarcane trash 15 FPU per g

Incubation time (h)	Concentrations of Substrates			Mean
	2.5%	5.0%	7.5%	
2	362.90 (53.28)	285.45 (41.91)	180.02 (26.43)	**276.12**
4	433.80 (63.69)	378.40 (55.56)	244.50 (35.90)	**352.23**
8	574.32 (84.32)	499.94 (73.40)	296.78 (43.57)	**457.01**
12	605.28 (88.87)	573.91 (84.26)	385.29 (56.57)	**521.49**
24	608.99 (89.41)	578.02 (84.86)	403.38 (59.22)	**530.13**
Mean	**517.06**	**463.14**	**301.99**	
	SE±	**CD (1%)**		
Incubation time (A)	2.85	11.09		
Concentrations of substrate (B)	2.21	8.59		
Interaction (A x B)	4.94	19.21		

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

A maximum of 426.73 mg per g reducing sugar release was recorded when 7.5% substrate concentration was used at the end of 24 h incubation period, where it showed a per cent saccharification of 62.65 per cent. It was found to be on par with 12 h incubation period and significantly superior over 8, 4 and 2 h incubation period in releasing the reducing sugars.

4.12.3 Sugarcane trash

The alkali pre-treated sugarcane trash was saccharified by using commercial cellulose enzyme at 15 FPU per g substrate by varying the substrate concentration. Table indicates significant differences with regards to release of reducing sugars for individual factors substrate concentrations and incubation periods and for their combined interactions. The data is furnished in Table 49 and Fig. 2.

The incubation period as a factor influenced significantly in releasing the reducing sugars. The mean maximum reducing sugar release of 530.13 mg per g was recorded at the end of 24 h incubation period, which was found to be at par with 12 h incubation period (521.13 mg g^{-1}) and superior over 8, 4 and 2 h incubation periods with respect to release of reducing sugars.

The substrate concentrations as a factor also influenced significantly in releasing the reducing sugars. The mean higher reducing sugar release of 517.06 mg per g was recorded in 2.5% substrate concentration. It was found to be significantly superior over 5.0% (463.14 mg g^{-1}) and 7.50% (301.99 mg g^{-1}) substrate concentration with regards to release of reducing sugars.

The substrate concentrations and incubation periods as a combined factor revealed significant variation in releasing the reducing sugars. 2.5% substrate concentration recorded significantly the highest reducing sugar release at the end of 24 h incubation period. The recorded reducing sugar content 608.99 mg per h showed per cent saccharification of 89.41 per cent. It was found to be on par with 12 h incubation (605.28 mg g^{-1} and 88.81% reducing sugar released and per cent saccharification) and superior over rest of the incubation periods.

5% substrate concentration recorded a maximum of 578.02 mg per g reducing sugars and 84.86 per cent saccharification at 24 h incubation period, it was found to be on par with 12 h incubation with 573.91 mg per g reducing sugar and 84.26 per cent saccharification and significantly superior over rest of the incubation periods with respect to release of reducing sugars released.

7.5% substrate concentration resulted in a maximum of 403.38 mg per g reducing sugar and 59.22 per cent saccharification and it was on par with 12 h incubation but significantly superior over 12, 8, 4 and 2 h incubation periods in releasing the reducing sugars.

4.12.4 Corn husk

The alkali pre-treated corn husk was saccharified by using commercial cellulose at 15 FPU per g substrate on various concentrations of substrate for different incubation periods. The release of reducing sugars indicates a significant difference with regards substrate concentration, incubation periods and the combined effect of substrate concentration and incubation periods. The data is presented in Table 50 and Fig. 2.

The different incubation periods influenced differently about releasing the reducing sugars due to cellulose enzyme activity. The mean maximum reducing sugar release of 519.20 mg per g was recorded at the end of 24 h incubation period, which was on par with 12 h incubation (514.88 mg g^{-1}) and significantly superior over rest of the incubation periods.

The different substrate concentrations resulted in significant variation with regards to release of reducing sugars. The mean higher reducing sugar release of 495.95 mg per g reducing sugars was observed when 2.5% substrate concentration was used. It was found to be significantly superior over 5.0% (445.53 mg g^{-1}) and 7.5% (299.50 mg g^{-1}) substrate concentrations.

The combined effect of substrate concentration and incubation periods also differed significantly with respect to release of reducing sugars. Significantly the higher release of reducing sugars was noticed in 2.5% substrate concentration at the end of 24 h incubation period with 590.67 mg per g and 85.74 per cent saccharification.

Table 50: Effect of different substrate concentrations on release of reducing sugars (mg g^{-1}) from corn husk 15 FPU per g

Incubation time (h)	Concentrations of Substrates			Mean
	2.5%	5.0%	7.5%	
2	346.13 (50.24)	271.35 (39.39)	170.38 (24.73)	**262.62**
4	413.18 (59.98)	363.93 (52.83)	230.54 (33.46)	**335.88**
8	545.76 (79.22)	477.61 (69.33)	284.09 (41.24)	**435.82**
12	584.00 (84.77)	555.54 (80.64)	405.10 (58.81)	**514.88**
24	590.67 (85.74)	559.23 (81.18)	407.69 (59.18)	**519.20**
Mean	**495.95**	**445.53**	**299.56**	

	SE±	CD (1%)
Incubation time (A)	5.43	21.12
Concentrations of substrate (B)	4.21	16.36
Interaction (A x B)	9.41	36.58

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 620 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

Table 51: Effect of different substrate concentrations on release of reducing sugars (mg g^{-1}) from corn stover 15 FPU per g

Incubation time (h)	Concentrations of Substrates			Mean
	2.5%	5.0%	7.5%	
2	353.75 (51.94)	283.54 (41.63)	175.46 (25.76)	**270.92**
4	424.61 (62.34)	374.21 (54.94)	235.61 (34.59)	**344.81**
8	564.61 (82.89)	473.97 (69.59)	322.72 (47.38)	**453.77**
12	584.58 (85.83)	562.31 (82.56)	397.325 (58.33)	**514.74**
24	587.81 (86.30)	566.18 (83.13)	398.27 (58.47)	**517.42**
Mean	**503.07**	**452.04**	**305.88**	

	SE±	CD (1%)
Incubation time (A)	3.49	13.59
Concentrations of substrate (B)	2.74	10.52
Interaction (A x B)	6.05	25.53

Figures in parentheses indicate per cent saccharification
Note : Initial cellulose content 613 mg g^{-1}
10 IU g^{-1} β-glucosidase and 5 U g^{-1} xylanase supplemented commonly

It was found to be on par with 12 h incubation period, 584.00 mg per g reducing sugar was 84.77 per cent saccharification and significantly superior over rest of the incubation periods.

The 5.0% substrate concentration resulted in a maximum of 559.23 mg per g reducing sugar release and 81.18 per cent saccharification at the end of 24 h incubation, however it was at par with 12 h incubation period, 555.54 mg per g reducing sugar and 80.64 per cent saccharification and significantly superior over rest of the incubation periods studied with respect to saccharification of sugars.

The 7.5% substrate concentration resulted in a maximum of 407.69 mg per g reducing sugar release corresponding to 59.18 per cent saccharification. It did not differ significantly over 12 h incubation period, which showed 405.10 mg per g reducing sugar release 58.81 per cent saccharification. Both the incubations were found to be significantly superior over 8, 4 and 2 h incubation periods at 7.5% substrate concentration with regards to release of reducing sugars.

4.12.5 Corn stover

The alkali pre-treated corn stover was saccharified by using commercial cellulose at 15 FPU per g substrate by varying substrate concentration over a different periods of incubation. The results obtained indicated significant differences with respect to release of reducing sugars due to factors incubation periods, substrate concentrations and the combined effect of incubation periods and substrate concentration.

The incubation periods as a factor influenced significantly in releasing this reducing sugar as indicated in Table 51 and Fig. 2. The mean higher reducing sugar release of 517.42 mg per g was recorded at the end of 24 h incubation period, which was found to be at par with 12 h incubation period (514.74 mg g^{-1}) and superior over 8, 4 and 2 h incubation periods with respect to release of reducing sugars.

The substrate concentration as a factor influenced significantly in releasing the reducing sugars. The mean higher reducing sugar release of 503.07 mg per g was recorded I 2.5% substrate concentration. It was found to be significantly superior over 5.0% (452.04 mg g^{-1}) and 7.5% (305.88 mg g^{-1}) with regards to release of reducing sugars.

The substrate concentrations and incubation periods as a combined factor revealed significant variation in releasing the reducing sugars. 2.5% substrate concentration recorded significantly the higher reducing sugar release at the end of 24 h incubation period. The reducing sugar recorded was 587.81 mg per g corresponding to 89.41 per cent saccharification. It was found to be on par with 12 h incubation period (584.58 mg g^{-1} and 88.87% reducing sugars and per cent saccharification and superior over rest of the incubation periods. 5% substrate concentration recorded a maximum of 566.18 mg per g reducing sugar and 84.87 per cent saccharification at 24 h after incubation, it was found to be on par with 12 incubation period with 562.31 mg per g reducing sugar and 84.26 per cent saccharification. They were significantly superior over rest of the incubation periods with respect to release of reducing sugars released.

7.5% substrate concentration resulted in a maximum of 398.27 mg per g reducing sugars and 59.22 per cent saccharification and at was significantly on par with 12 h incubation 397.32 mg per g reducing sugars and 56.57 per cent saccharification. The 24 and 12 h incubations were found to be significantly superior over 8, 4 and 2 h incubation periods with respect to release of reducing sugars in 7.5% substrate concentration.

4.13 Effect of different fermentation methods on bioethanol production and residual reducing sugars from different alkali pre-treated and commercial enzyme saccharified substrates

4.13.1 Separate Hydrolysis and Fermentation (SHF) on ethanol yield

The five different agro-residues *viz.*, sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were saccharified by using commercial cellulase enzyme and then fermented to bioethanol using six different ethanol fermenting microorganisms.

Table 52a: Effect of separate hydrolysis and fermentation (SHF) on ethanol yield (mg g^{-1}) from selected pre-treated and saccharified agro-residues

Sl. No.	Ethanol fermenting organisms	Saccharified agro-residues					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cereviseae*	262.93	246.93	232.00	222.40	220.27	**236.91**
2.	*Zymomonas mobilis*	241.60	228.27	214.40	205.86	219.20	**221.87**
3.	*Kluyveromyces marxianus*	232.00	217.07	202.14	196.80	202.13	**210.03**
4.	*Pachysolen tannophilus*	256.53	240.00	225.07	217.60	222.93	**232.43**
5.	*Pichia stipitis*	146.13	134.40	138.67	114.67	128.00	**132.37**
6.	*Candida shehatae*	155.73	145.07	137.24	123.73	130.67	**138.49**
7.	Control	1.82	1.51	1.42	1.46	1.50	**1.54**
	Mean	**185.25**	**173.32**	**164.42**	**154.65**	**160.67**	

		SE±	CD (1%)
	Cultures (A)	2.03	7.59
	Substrates (B)	1.71	6.41
	Interaction (A x B)	4.53	16.97

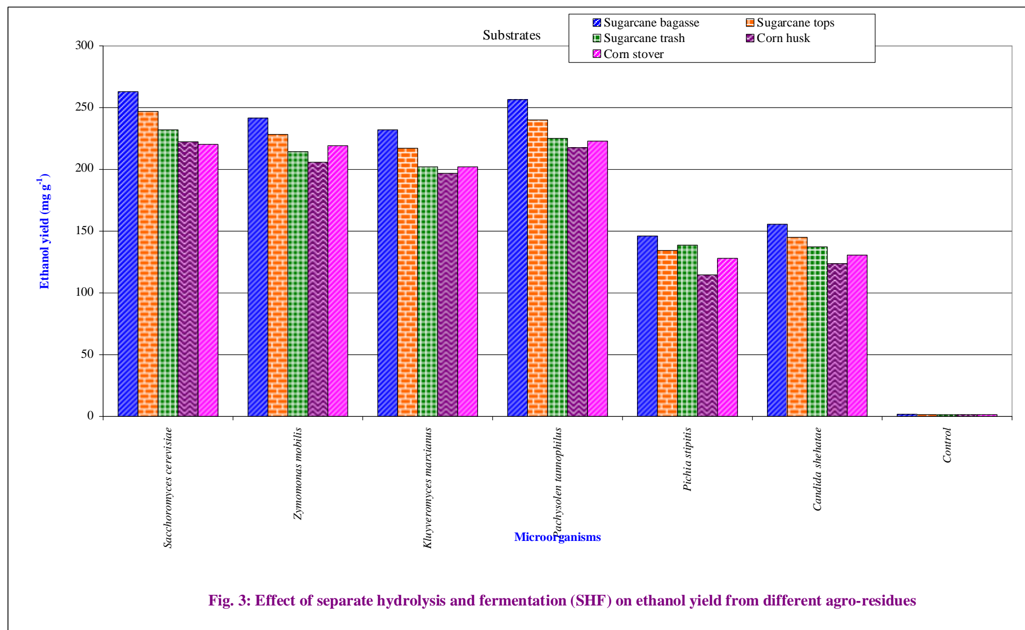


Fig. 3: Effect of separate hydrolysis and fermentation (SHF) on ethanol yield from different agro-residues

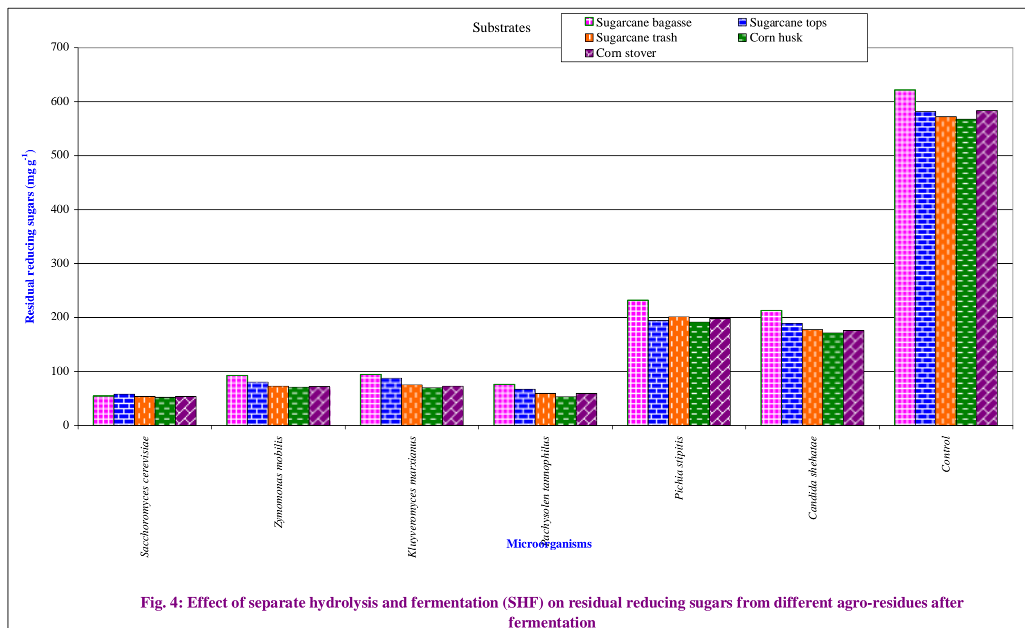


Fig. 4: Effect of separate hydrolysis and fermentation (SHF) on residual reducing sugars from different agro-residues after fermentation

The ethanol content in the fermented broth differed significantly with respect to type of microorganism involved in fermentation, type of agro-residue and the combined interaction of microorganisms and agro-residues.

The Table 52a and Fig. 3 indicates variations within the microorganisms involved in fermentation of ethanol. The significantly mean maximum ethanol content of 236.91 mg per g substrate was produced by the yeast *Saccharomyces cerevisiae*. The maximum ethanol produced by *S. cerevisiae* was found to be significantly at par with 232.43 mg per g ethanol produced by *Pachysolen tannophilus*. These two yeast cultures were significantly superior over other ethanol fermenting yeasts *viz*., *Kluyveromyces marxianus* (210.03 mg g^{-1}), *Pichia stipitis* (132.37 mg g^{-1}), *Candida shehatae* (138.49 mg g^{-1}) and over bacterial culture, *Zymomonas mobilis* (221.87 mg g^{-1}). The control treatment recorded the significantly lowest ethanol content of 1.54 mg per g.

The ethanol content in the agro-residues also differed significantly. The mean maximum ethanol content of 185.25 mg per g was observed in sugarcane bagasse. It was found to be significantly superior over other substrates interms of ethanol content. Followed by sugarcane bagasse, sugarcane tops (173.32 mg g^{-1}), sugarcane trash (164.42 mg g^{-1}), corn stover (160.67 mg g^{-1}) and corn husk (154.65 mg g^{-1}) stood in the decreasing order with respect to ethanol content.

The interaction between ethanol fermenting microorganisms and different substrates indicated significant differences in ethanol yield. The yeast *S. cerevisiae* produced a significantly the highest ethanol yield of 262.93 mg per g in bagasse, it was found to be on par with *P. tannophilus* (256.53 mg g^{-1}) also in bagasse and significantly superior over rest of the ethanol fermenting microorganisms with respect to ethanol yield.

The significant highest ethanol yield of 262.93 mg per g observed in case of bagasse, fermented by *S. cerevisiae* was found to be superior over al other agro-residues with regards to ethanol yield. It was also observed that *S. cerevisiae* recorded a significantly highest ethanol yield in all the agro-residues except in corn stover, where *P. tannophilus* produced highest ethanol yield (222.93 mg g^{-1}). The *S. cereviseae* and *P. tannophilus* were found to be at par with respect to ethanol yield in respective agro-residues and they were significantly superior over rest of the ethanol fermenting microorganisms. The lowest ethanol yield was observed in case of *P. stipitis* fermented agro-residues except for sugarcane trash, where the lowest ethanol yield was produced by *C. shehatae*.

4.13.2 Separate Hydrolysis and Fermentation (SHF) on residual reducing sugars

The residual reducing sugars present in the substrates that were subjected for SHF indicated significant variations due to ethanol fermenting microorganisms, the substrate and their combined interaction effects. The data is furnished in Table 52b and Fig. 4.

The residual reducing sugar content due to microbial fermentation varied significantly with different microorganisms. The significantly mean maximum residual reducing sugar content of 585.44 mg per g was observed with control treatment that was not inoculated with ethanol fermenting microorganisms. Among the microbial inoculations, the significantly mean maximum residual reducing sugar content of 203.76 mg per g was recorded with *P. stipitis*. It was on par with other pentose fermenting yeast *C. shehatae* (185.75 mg g^{-1}) and significantly superior over rest of the hexose fermenting microorganisms with respect to residual reducing sugar content.

The different substrates subjected for alcoholic fermentation also indicated significant variations in residual reducing sugar content. The mean maximum residual reducing sugar content of 198.02 mg per g was observed in sugarcane bagasse and it was significantly superior over other substrates subjected for ethanol fermentation. The mean lowest residual sugar content was recorded in the substrate corn husk with 168.37 mg per g reducing sugar.

Table 52b: Effect of separate hydrolysis and fermentation (SHF) on residual reducing sugar (mg g^{-1}) from selected pre-treated and saccharified agro-residues

Sl. No.	Ethanol fermenting organisms	Saccharified agro-residues					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cereviseae*	54.96	58.36	53.96	52.53	53.96	**54.76**
2.	*Zymomonas mobilis*	92.90	80.89	73.32	71.21	72.36	**78.13**
3.	*Kluyveromyces marxianus*	94.60	88.00	75.44	70.19	73.20	**80.29**
4.	*Pachysolen tannophilus*	76.38	67.62	59.71	53.25	59.71	**63.34**
5.	*Pichia stipitis*	232.17	195.01	201.27	191.93	198.40	**203.76**
6.	*Candida shehatae*	213.49	189.77	177.56	171.81	176.12	**185.75**
7.	Control	621.64	582.11	572.31	567.64	583.49	**585.44**
	Mean	**198.02**	**180.25**	**173.37**	**168.37**	**173.89**	
		SE±			**CD (1%)**		
	Cultures (A)	2.03			7.60		
	Substrates (B)	1.72			6.42		
	Interaction (A x B)	4.54			17.00		

The influence of interaction of alcohol fermenting microorganisms and different substrates showed variations in residual reducing sugar content. The superior and significantly higher residual reducing sugar content, 621.64 mg per g was observed in control treatment in case of bagasse. Similarly, the control treatment indicated highest residual reducing sugar content for all the substrates. Among the alcohol fermenting microorganisms, *P. stipitis* showed the highest residual reducing sugar content in all the substrates, sugarcane bagasse (232.17 mg g^{-1}), sugarcane tops (195.01 mg g^{-1}), sugarcane trash (201.27 mg g^{-1}), corn husk (191.93 mg g^{-1}) and in corn stover (198.40 mg g^{-1}). It was found to be significantly highest over rest of the alcohol fermenting microorganisms in respective substrate with regards to residual reducing sugar content.

4.13.3 Simultaneous Saccharificaiton and Fermentation (SSF) on ethanol yield

The simultaneous saccharificaiton and fermentation of five agro-residues *viz*., sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover was carried out by using six different ethanol fermenting microorganisms. The ethanol production in the agro-residues, ethanol production ability of microorganisms as well as their combined interaction was found to differ with each other with regards to ethanol yield. The data is presented in Table 53a, Fig. 5 and Plate 5a.

The ethanol production as influenced by different ethanol fermenting microorganisms differed significantly. All the six ethanol fermenting microorganisms showed significant variation with each other in ethanol production abilities. The mean ethanol production ranged between 126.97 and 243.67 mg per g with the different ethanol fermenting microorganisms. The mean maximum ethanol production, 243.67 mg per g ethanol was observed with the yeast *S. cerevisiae*. It was found to be significantly highest when compared to all other ethanol fermenting microorganisms. The lowest mean ethanol production of 126.97 mg per g was noticed with *P. stipitis*.

The different agro-residues that were subjected for fermentation also showed significant variation in ethanol yield. The mean maximum ethanol yield of 184.07 mg per g was recorded in sugarcane bagasse, followed by sugarcane tops (175.59 mg g^{-1}), corn stover (164.25 mg g^{-1}), sugarcane trash (162.73 mg g^{-1}) and corn husk (162.37 mg/) with regards to ethanol yield. The substrate bagasse was found to be significantly superior over other substrates in ethanol production. The next best substrate was sugarcane tops. The sugarcane trash, corn husk and corn stover were observed to be at par with each other with regards to ethanol production and they were significantly lower as compared to sugarcane bagasse and sugarcane tops in yielding ethanol.

Significant differences were also noticed in the combined interaction of ethanol fermenting microorganisms and substrates with regards to ethanol yield. The 269.84 mg per g ethanol yield observed in sugarcane bagasse, fermented by *S. cerevisiae* was found to be significantly highest over other substrates, fermented by the same organism as well as over other ethanol fermenting microorganisms in the substrate sugarcane bagasse itself. The significantly lowest ethanol yield of 102.75 mg per g was observed in sugarcane trash, which was fermented by *C. shehatae*.

It was also observed that *S. cerevisiae* produced significantly highest ethanol in three different substrates, bagasse (269.84 mg g^{-1}), sugarcane tops (252.41 mg g^{-1}) and sugarcane trash (237.16 mg g^{-1}), whereas *Z. mobilis* produced highest ethanol yield in two substrates corn husk (231.29 mg g^{-1}) and corn stover (232.00 mg g^{-1}). However, the two microorganisms were on par with each other in ethanol yield in all the substrates, except in case of bagasse, where *S. cerevisiae* (269.84 mg g^{-1}) was significantly superior over *Z. mobilis* (251.78 mg g^{-1}) with respect to ethanol yield. *Z. mobilis* and *P. stipitis* were found to be on par with each other in producing ethanol within the substrates. The significantly lowest ethanol yield was noticed with *P. stipitis* in sugarcane bagasse (143.82 mg g^{-1}), sugarcane tops (135.29 mg g^{-1}), corn husk (117.23 mg g^{-1}) and in corn stover (118.22 mg g^{-1}) and with *C. shehatae* in sugarcane trash (102.75 mg g^{-1}).

Table 53a: Effect of simultaneous saccharification and fermentation (SSF) on ethanol yield (mg g^{-1}) from selected pre-treated and saccharified agro-residues

Sl. No.	Ethanol fermenting organisms	Saccharified agro-residues					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cereviseae*	269.84	252.41	237.16	227.50	231.41	**243.67**
2.	*Zymomonas mobilis*	251.78	244.78	235.75	231.29	232.00	**239.12**
3.	*Kluyveromyces marxianus*	219.63	214.17	213.69	212.70	209.49	**213.94**
4.	*Pachysolen tannophilus*	247.12	239.80	228.17	223.82	224.70	**232.72**
5.	*Pichia stipitis*	143.82	135.29	120.18	117.33	118.22	**126.97**
6.	*Candida shehatae*	154.49	141.15	102.75	122.49	132.45	**130.67**
7.	Control	1.82	1.51	1.42	1.46	1.50	**1.54**
	Mean	**184.07**	**175.59**	**162.73**	**162.37**	**164.25**	
		SE±			**CD (1%)**		
	Cultures (A)	0.94			3.53		
	Substrates (B)	0.80			2.98		
	Interaction (A x B)	2.11			7.89		

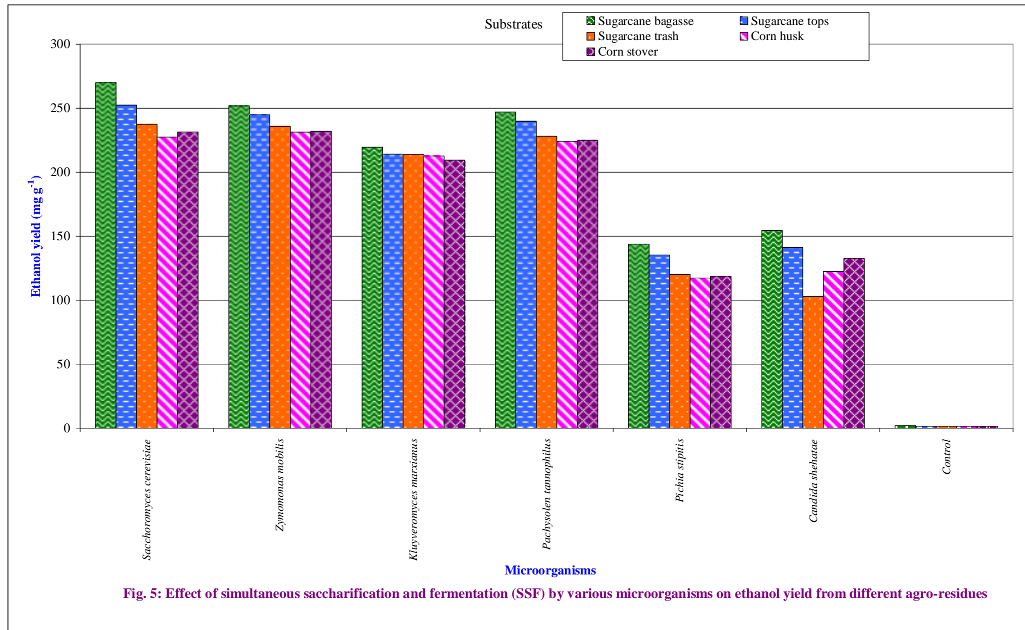


Fig. 5: Effect of simultaneous saccharification and fermentation (SSF) by various microorganisms on ethanol yield from different agro-residues

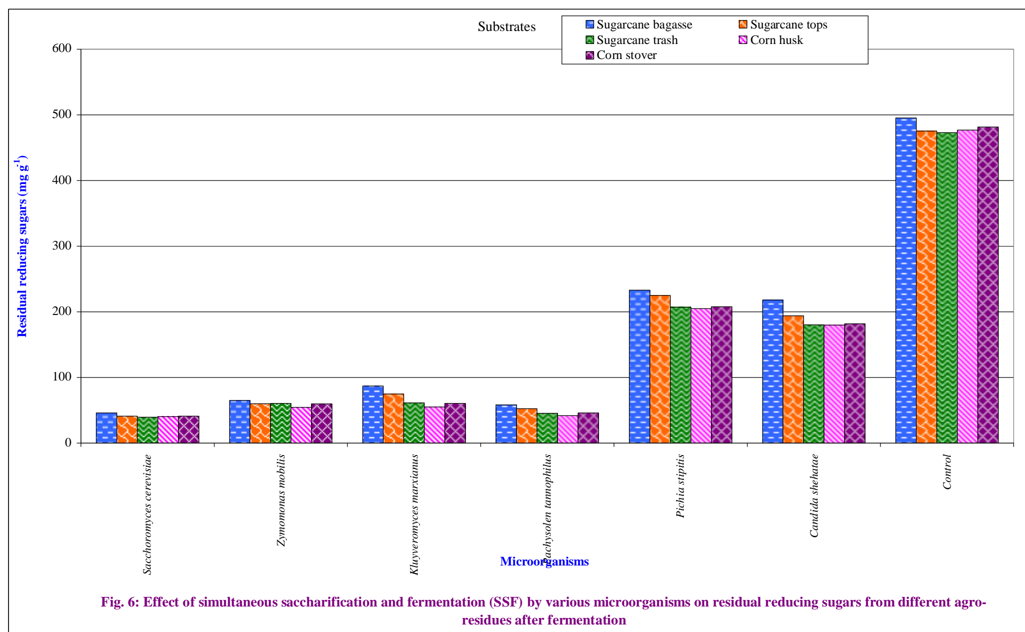


Fig. 6: Effect of simultaneous saccharification and fermentation (SSF) by various microorganisms on residual reducing sugars from different agro-residues after fermentation

4.13.4 Simultaneous Saccharification and Fermentation (SSF) on residual reducing sugars

The influence of alcohol fermenting microorganisms, different agro-residues and their combined interaction effects indicated significant variations in the residual reducing sugar content of fermented liquors. The results pertaining to residual reducing sugar content in furnished in Table 53b, Fig. 6 and Plate 5b.

The SSF of different agro-residues indicated significant variations in residual reducing sugar content. The mean maximum reducing sugar content of 480.40 mg per g was recorded in the control treatment that received no microbial inoculation. Among the microbial inoculations, inoculation of *P. stipitis* recorded the mean maximum residual reducing sugar content of 215.50 mg per g. It was found to be significantly superior over rest of the ethanol fermenting microorganisms with regards to residual reducing sugar content. The significantly lowest residual reducing sugar content, 41.56 mg per g was observed in the treatment inoculated with *S. cerevisiae*.

The residual reducing sugar content of the different agro-residues also showed significant variations. The mean maximum residual reducing sugar content of 171.73 mg per g was observed in the substrate sugarcane bagasse. It was found to be significantly superior over rest of the substrates. The significantly mean minimum 150.43 mg per g residual reducing sugar content was observed in the substrate corn husk.

The residual reducing sugar content due to microbial inoculations and different substrates differed differently. The significantly highest residual reducing sugar content of 495.31 mg per g was recorded with control treatment in case of sugarcane bagasse. Similarly, the control treatment indicated highest residual reducing sugar content in different substrates studied for ethanol production. The pentose fermenting yeast *P. stipitis* recorded significantly highest residual reducing sugars content for respective substrates. It was found to be significantly superior over all other alcohol fermenting microorganisms with regards to residual reducing sugar content. The significantly lowest residual reducing sugar content was observed with the yeast *S. cerevisiae* for all the respective agro-residues subjected for alcoholic fermentation.

4.13.5 Effect of co-fermentation on ethanol yield

Five different saccharified substrates were fermented to ethanol by using combinations of hexose fermenting and pentose fermenting microorganisms. The details of the treatments and the data is presented in Table 54a and Fig. 7. The ethanol production due to different substrates, coinoculation of glucose and xylose fermenting microorganisms and their combined interactions differed differently with respect to ethanol production.

The combined inoculation of hexose and pentose fermenting microorganisms showed significant variation in ethanol yield. The mean maximum ethanol yield of 250.95 mg per g ethanol was obtained with the coinoculation of *S. cerevisiae* and *C. shehatae*. It was found to be significantly superior over the next best combined inoculation of *P. tannophilus* and *P. stipitis* (236.45 mg g^{-1}) and over all other dual and tri inoculations of microorganisms with respect to ethanol yield. The lowest mean ethanol production of 147.66 mg per g was observed with combined inoculation of *S. cerevisiae*, *Z. mobilis* and *P. stipitis*.

The different saccharified substrates indicated significant variations in ethanol yield. The mean maximum ethanol yueld of 213.50 mg per g was observed in the substrate sugarcane bagasse. It was significantly superior over rest of the substrates with regards to ethanol yield. The significantly lowest ethanol yield (180.45 mg g^{-1}) was observed with corn husk substrate.

Variation in ethanol yield was also observed due to combined interaction of ethanol fermenting microorganisms and substrate. The significant and highest ethanol production was recorded in sugarcane bagasse, which was inoculated with *S. cerevisiae* and *C. shehatae* together. It was found to be significantly superior over all other agro-residues in yielding ethanol that were inoculated with various other double and triple microbial combinations.

Table 53b: Effect of simultaneous saccharification and fermentation (SSF) on residual reducing sugar (mg g^{-1}) from selected pre-treated and saccharified agro-residues

Sl. No.	Ethanol fermenting organisms	Saccharified agro-residues					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cereviseae*	45.85	41.03	39.59	40.31	41.03	**41.56**
2.	*Zymomonas mobilis*	64.98	59.94	60.58	54.47	59.81	**59.96**
3.	*Kluyveromyces marxianus*	87.02	74.83	61.22	55.02	60.50	**67.72**
4.	*Pachysolen tannophilus*	58.28	52.53	45.34	41.75	46.06	**48.79**
5.	*Pichia stipitis*	232.89	224.98	207.02	204.86	207.74	**215.50**
6.	*Candida shehatae*	217.80	194.09	180.19	179.71	181.87	**190.73**
7.	Control	495.31	475.21	472.99	476.87	481.62	**480.40**
	Mean	**171.73**	**160.37**	**152.42**	**150.43**	**154.09**	
		SE±			**CD (1%)**		
	Cultures (A)	1.39			5.21		
	Substrates (B)	1.18			4.41		
	Interaction (A x B)	3.11			11.66		

Table 54a: Effect of co-fermentation on ethanol yield (mg g^{-1}) from selected pre-treated and saccharified agro-residues

Sl. No.	Ethanol fermenting organisms	Saccharified agro-residues					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cereviseae* + *Pichia stipitis*	186.13	171.73	165.15	161.25	166.04	**170.06**
2.	*Zymomonas mobilis* + *Pichia stipitis*	251.38	234.49	219.55	211.55	218.67	**227.13**
3.	*Kluyveromyces marxianus* + *Pichia stipitis*	237.33	220.27	207.82	201.78	205.16	**214.47**
4.	*Pachysolen tannophilus* + *Pichia stipitis*	260.80	243.38	230.04	222.05	225.96	**236.45**
5.	*Sacchoromyces cereviseae* + *Zymomonas mobilis* + *Pichia stipitis*	165.87	145.78	142.76	141.15	142.75	**147.66**
6.	*Sacchoromyces cereviseae* + *Candida shehatae*	278.40	262.75	241.42	232.36	239.82	**250.95**
7.	*Zymomonas mobilis* + *Candida shehatae*	251.20	235.91	220.45	209.42	216.18	**226.63**
8.	*Kluyveromyces marxianus* + *Candida shehatae*	243.73	227.02	208.18	205.15	262.75	**229.37**
9.	*Pachysolen tannophilus* + *Candida shehatae*	256.53	240.89	223.82	216.00	222.93	**232.04**
10.	*Sacchoromyces cereviseae* + *Zymomonas mobilis* + *Candida shehatae*	215.46	205.51	189.16	182.75	188.62	**196.30**
11.	Control	1.68	1.52	1.50	1.47	1.50	**1.54**
	Mean	**213.50**	**199.02**	**186.35**	**180.45**	**190.04**	
		SE±			**CD (1%)**		
	Cultures (A)	0.97			3.61		
	Substrates (B)	0.66			2.44		
	Interaction (A x B)	2.18			8.08		

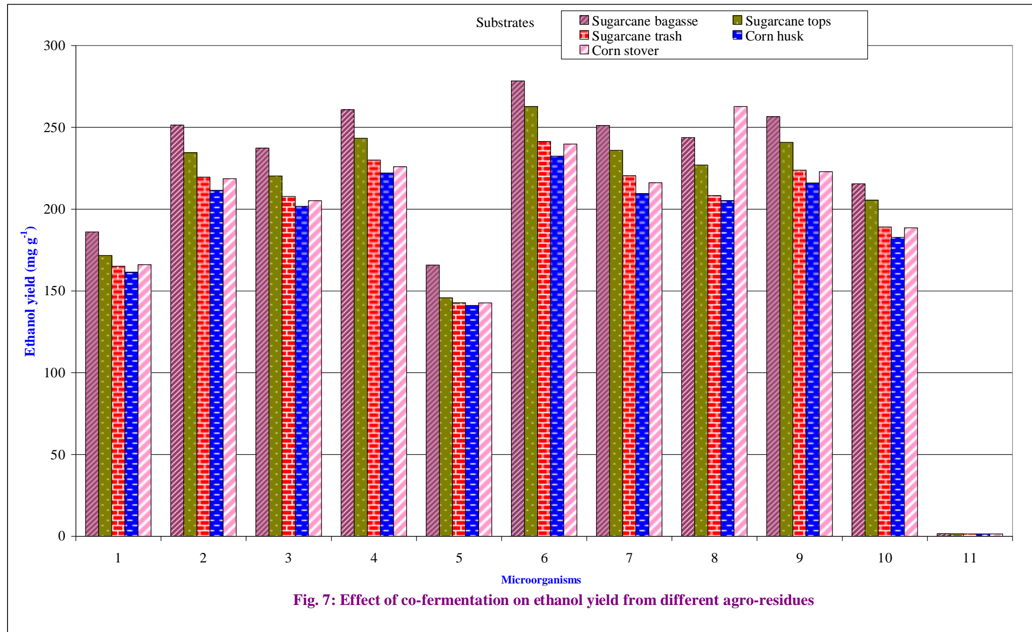


Fig. 7: Effect of co-fermentation on ethanol yield from different agro-residues

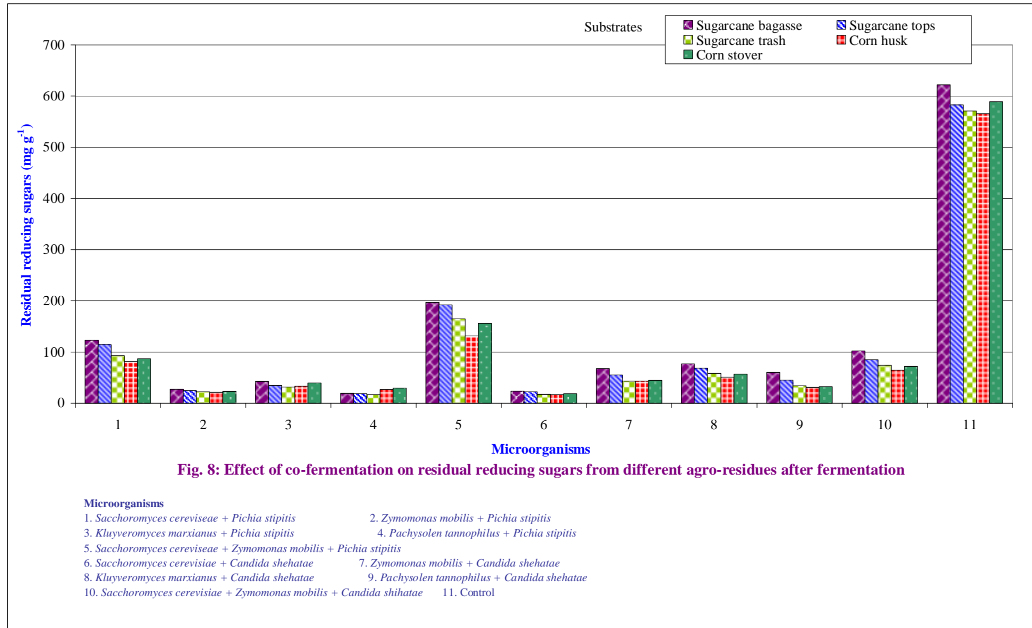


Fig. 8: Effect of co-fermentation on residual reducing sugars from different agro-residues after fermentation

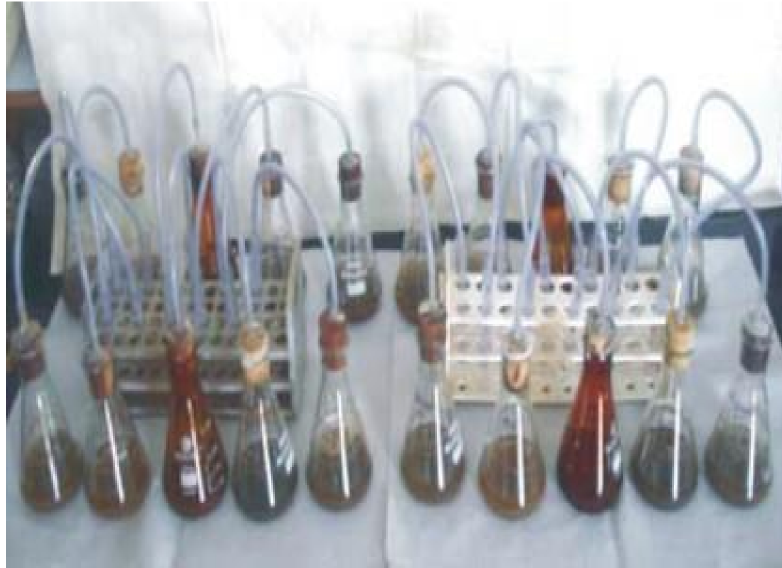


Plate 5a. Experimental setup of alcohol production

Plate 5b. Alcohol production using combined inoculations of *Saccharomyces cerevisiae* and *Candida shehatae*

Among the crorganisms used for alcohol fermentation, *S. cerevisiae* and *C. shehatae* co-inoculation resulted in highest ethanol yield in all the substrates, sugarcane bagasse (278.40 mg g^{-1}), sugarcane tops (262.75 mg g^{-1}), sugarcane trash (241.42 mg g^{-1}), corn husk (232.36 mg g^{-1}) and in corn stover (239.82 mg g^{-1}). The above co-inoculation of hexose and pentose fermenting microorganisms found to be significantly superior over all other combination of microbial inoculations in respective agro-residues with respect to ethanol yield. The significantly lowest ethanol yield was recorded with combined inoculation of *S. cerevisiae*, *Z. mobilis* and *P. stipitis* in all the saccharified agro-residues.

4.13.6 Co-fermentation on residual reducing sugar content

The data pertaining to co-fermentation of alcohol is presented in Table 54b and Fug. 8. The co-fermentation of hexose and pentose sugars present in different agro-residues indicated marked variations with respect to residual reducing sugar content. Both the individual factors, combined inoculation of microorganisms, different agro-residues as well as their combined interaction differed significantly with regards to residual reducing sugar content.

The combinations of microbial inoculations indicated variations in residual reducing sugar content. The mean maximum residual reducing sugar content, 586.13 mg per g was observed with uninoculated control treatment. The combined inoculation of microorganisms showed that the significant mean maximum residual reducing sugar content 168.22 mg per g was obtained with tri-inocualtion of *S. cerevisiae*, *Z. mobilis* and *P. stipitis*. It was found to be significantly superior over both dual and tri inoculation combinations of other ethanol fermenting microorganisms. The mean minimum residual reducing sugar content of 19.76 mg per g was observed with *S. cerevisiae* and *C. shehatae* dual inoculated treatment.

The variations in residual reducing sugar content in different agro-residues indicated that the mean residual reducing sugar content ranged between 96.90 and 123.87 mg per g. The significantly mean maximum (123.87 mg g^{-1}) and mean minimum (96.90 mg g^{-1}) residual reducing sugar content was observed in sugarcane bagasse and corn husk, respectively.

The interaction effect of coinoculation of alcohol fermenting microorganisms and different substrates varied differently with regards to residual reducing sugar content. The significantly highest residual reducing sugar content, 622.23 mg per g was recorded with control treatment in case of bagasse. It was found to be significantly superior over rest of the agro-residues. Also, the control treatment recorded significantly highest residual reducing sugar content in all the respective agro-residues tried for bioethanol production. The combined inoculation of *S. cerevisiae*, *Z. mobilis* and *P. stipitis* recorded the significantly highest residual reducing sugar contents within the substrates, sugarcane bagasse (197.20 mg g^{-1}), sugarcane tops (191.93 mg g^{-1}), sugarcane trash (164.62 mg g^{-1}), corn husk (131.33 mg g^{-1}) and corn stover (156.00 mg g^{-1}). It was found to be significantly superior over rest of the dual and tri inoculations of alcohol fermenting microorganisms with respect to residual reducing sugar content. The significantly lowest residual reducing sugar contents of 23.78, 22.35, 17.32, 16.60 and 18.75 mg per g was observed in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover respectively with dual inoculation of *S. cerevisiae* and *C. shehatae* (glucose and xylose fermenting yeasts).

4.14 Scale up production of bioethanol from sugarcane bagasse

The scaled up study was performed for bioethanol production using sugarcane bagasse under optimized conditions. 1) 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 121^{0}C temperature for 1 h; 2) Saccharificaiton with commercial cellulase enzymes (cellulase 15 FPU/g, β-glucosidase 10 IU/g and xylanase 5 U g^{-1}); 3) Fermentation by dual cultures (*S. cereviseae* and *C. shehatae*). One kg pre-treated sugarcane bagasse at 5% substrate concentration produced 223 g ethanol corresponding to 22.30 per cent (w/w) pre-treated substrate. The fermentation of sugarcane bagasse is presented in Plate 6.

Table 54b: Residual reducing sugars after co-fermentation of ethanol (mg g^{-1}) from selected pre-treated and saccharified agro-residues

Sl. No.	Ethanol fermenting organisms	Saccharified agro-residues					Mean
		Sugarcane bagasse	Sugarcane tops	Sugarcane trash	Corn husk	Corn stover	
1.	*Sacchoromyces cereviseae* + *Pichia stipitis*	123.01	114.32	92.77	81.27	87.02	**99.68**
2.	*Zymomonas mobilis* + *Pichia stipitis*	27.38	24.50	22.35	21.39	23.07	**23.74**
3.	*Kluyveromyces marxianus* + *Pichia stipitis*	42.61	34.56	31.69	33.13	39.59	**36.32**
4.	*Pachysolen tannophilus* + *Pichia stipitis*	19.47	18.75	16.60	26.66	29.53	**22.20**
5.	*Sacchoromyces cereviseae* + *Zymomonas mobilis* + *Pichia stipitis*	197.20	191.93	164.62	131.33	156.00	**168.22**
6.	*Sacchoromyces cereviseae* + *Candida shehatae*	23.78	22.35	17.32	16.60	18.75	**19.76**
7.	*Zymomonas mobilis* + *Candida shehatae*	67.62	55.40	43.19	43.19	44.62	**50.80**
8.	*Kluyveromyces marxianus* + *Candida shehatae*	77.05	68.43	58.28	51.09	56.84	**62.34**
9.	*Pachysolen tannophilus* + *Candida shehatae*	60.12	45.34	33.84	30.97	32.41	**40.54**
10.	*Sacchoromyces cereviseae* + *Zymomonas mobilis* + *Candida shehatae*	102.11	84.86	74.16	64.74	71.93	**79.56**
11.	Control	622.23	582.70	571.00	565.56	589.16	**586.13**
	Mean	**123.87**	**113.01**	**102.35**	**96.90**	**104.45**	
		SE±			**CD (1%)**		
	Cultures (A)	2.14			7.95		
	Substrates (B)	1.45			5.36		
	Interaction (A x B)	4.79			17.77		

Plate 6. Pilot scale bioethanol production from sugarcane bagasse substrate

5. DISCUSSION

Bioethanol production from agro residues holds a great potential in solving the energy crisis. Agro-residues are available in abundance, widely spread and cheap source. Although these feed stocks contain considerable amount of cellulose and hemicellulose, which form lignocellulosic complex, pre-treatments are required to release the sugars for fermentation to bioethanol.

The present study on bioethanol production from selected agro-residues involved several pre-treatment experiments to recover cellulose and hemicellulose, saccharification of polysaccharides and fermentation of the derived sugars into bioethanol by various microbial combinations. The results obtained during the course of study are being discussed in this chapter.

5.1 Initial chemical composition of agro-residues

The five agro-residues selected for bioethanol production studies differed in their composition of cellulose, hemicellulose and lignin contents (Table 1). Sugarcane bagasse contained the highest cellulose content of 0.353 per g followed by sugarcane trash (0.343 g g^{-1}), corn husk and sugarcane tops (0.333 g g^{-1}) and the least cellulose content of 0.323 per g in corn stover. The cellulose content in the substrates ranged between 0.333 and 0.353 g g^{-1}. Similarly, the hemicellulose content ranged between 0.227 and 0.293 per g while the lignin content ranged between 0.140 and 0.160 per g in the substrates. The difference in chemical composition of the agro-residues is due to variation in the composition of cell walls as it varies with plant species, tissue type and, region within the cell wall and development stages of the cell wall (Carpita and Mc Cann, 2000). Analysis of the substrates indicates that sugarcane bagasse and sugarcane trash contains high amounts of cellulose and could be potential substrates for ethanol production. The substrates were tested at different particle sizes in order to assess the extent of delignification and hydrolysis in releasing maximum reducing sugars.

5.2 Effect of different pre-treatments on weight loss of the substrates

The loss of weight due to effect of different pre-treatment methods employed such as alkali and heat (2.50% and 3.00% NaOH for 8 h at room temperature followed by autoclaving at 121^{0}C or 125^{0}C exposure temperatures for 15 min), autohydrolysis with and without dilute acid, 1.0% H_2SO_4 at 180^0 or 200^{0}C temperatures for 10 min) and pre-treatment with lignolytic fungi (*Phanerochaete chrysosporium*, *Pleurotus florida* and UASD-LF1) and their crude enzymes were found to differ significantly.

The alkali and heat pre-treatment methods, the substrates and their particle sizes did not differ significantly but resulted in the highest weight loss when compared to other treatment methods (Table 2). It was revealed that the treatment 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 125^{0}C (1 h) resulted in highest loss of weight for the particle size 0.5 mm in respective substrates, sugarcane bagasse (0.568 g g^{-1}), sugarcane tops (0.532 g g^{-1}), sugarcane trash (0.548 g g^{-1}), corn husk (0.555 g g^{-1}) and corn stover (0.547 g g^{-1}) when compared to the other combinations of alkali and heat pre-treatments. The increased weight loss observed with alkali and heat is due to increased digestibility of the substrates as it causes swelling effect of Na^+ from the NaOH (due to counter ionic action of Na^+) on the carboxilic groups around the lignocellulosic backbone (Lawther and Runcang, 1996). Similar results were reported by Kodali and Pogaku, (2006) in rice husk (50 mesh size) where they obtained weight loss between 74 and 78 per cent with 1 N to 5 N sodium hydroxide solution and 10 minutes steam pre-treatment. The maximum weight loss observed in the smallest particle is probably due to more surface area to volume ratio for the chemical and heat treatment to act upon.

The autohydrolysis pre-treatment with 1.0% acid and exposure at 200^{0}C temperature for 10 min produced significantly the highest weight loss in case of particle size 0.5 mm in all the substrates, where the observed loss of weight was 0.517 g (sugarcane bagasse), 0.507 g (sugarcane tops), 0.502 g (sugarcane trash), 0.530 g (corn husk) and 0.507 g (corn stover) (Table 6). It was significantly high when compared to other autohydrolysis pre-treatments.

The highest weight loss observed with 1.0% acid and 200^0 C exposure temperature for 10 min is explained by the strong hydrolytic action of acid that solubilize hemicellulose into monomeric sugars (arabinose, galactose, glucose, mannose and xylose) and soluble oligomers, along with degradation of lignin (Sun and Cheng, 2005) as well as the high steam pressure that rapidly explode the substrate to produce reactive fibre (Laser *et al.,* 2002). The maximum weight loss observed in the smallest particle is probably due to more surface area to volume ratio for the action of acid and heat treatment on smaller size substrates.

The pre-treatment with lignolytic fungi and their crude enzymes revealed that significantly the maximum weight loss was observed with inoculation of lignolytic fungi *Phanerochaete chrysosporium* treatment in 0.5 mm particle size in respective substrates at the end of 30 days incubation. However the loss of weight observed with *P. chrysosporium* inoculation was on par with inoculation of other fungi, *P. florida* and UASD-LF1 at the end of 30 days incubation in 0.5 mm particle size substrates with regard to weight loss (Table 10 and 14). The loss in weight of the substrates is attributed to non selective and rapid degradation of the organic components by the lignolytic fungi (Kerem *et al*., 1992). Also, Valaskova *et al.,* (2007) reported the degradation of leaf litter up to 38%, 23% and 32% respectively by lignolytic fungi *Gymnopus sp., Hypoloma fasciculare* and *Rhodocollybia butyracea* isolated from quercus petraea forest respectively in 12 weeks.

Zohar *et al.* (1992) *Pleurotus ostreatus* and *Phanerochaete chrysosporium* produced 20% and 55% degradation of organic compounds in cotton stalks in 30 and 15 days, respectively under solid state fermentation. The losses of weight observed with these pre-treatments were comparatively less than alkali and heat pre-treatments. The loss of weight in different substrates inoculated with crude enzymes was found to be insignificant irrespective of particle size and incubation periods (Table 14). The insignificant weight loss observed with crude enzymes of different fungi after 10 days incubation is probably attributed to the lower enzymatic activity during prolonged periods of incubation as the stability of enzymes is controlled by temperature, pH, substrate concentration and production of inhibitory compounds *etc.*

5.3 Effect of different pre-treatments on cellulose content of the substrates

The cellulose content as influenced by different pre-treatment methods employed *viz.* alkali and heat (2.50% and 3.00% NaOH for 8 h at room temperature followed by autoclaving at 121^0C or 125^0C exposure temperatures for 15 min), autohydrolysis in presence and absence of dilute acid, 1.0% H_2SO_4 at 180^0 or 200^0C temperatures for 10 min) and pre-treatment with lignolytic fungi (*Phanerochaete chrysosporium*, *Pleurotus florida* and UASD-LF1) and their crude enzymes showed significant differences.

The combined interaction of alkali and heat, substrates and their particle size were not significant with respect to cellulose content (Table 3). However, the highest cellulose content was observed in the treatment combination of 3.0% NaOH coupled with 121^0C temperature and particle size of 0.5 mm in sugarcane bagasse (0.633 g g^{-1}), corn husk (0.620 g g^{-1}), sugarcane tops (0.613 g g^{-1}) and in corn stover (0.613 g g^{-1}). All these substrates exhibited the highest per cent increase in cellulose content over their initial value due to this pre-treatment, sugarcane bagasse (79.32), sugarcane tops (84.08%), corn husk (86.19%) and corn stover (87.46%). The treatment 2.5% NaOH coupled with 125^0C temperature exposure showed the highest cellulose content of 0.620 g per g in case of sugarcane trash with a per cent increase in cellulose content at 80.76 per cent.

The higher cellulose content observed with 3.0% NaOH coupled with 121^0C temperature heat treatment is attributed to effective delignification of the substrates by the optimized alkali and heat treatment. Similar results were reported by Bjerre *et al.* (1996), they obtained about 85 per cent (w/w) glucose convertible cellulose by alkaline hydrolysis of wheat straw with 20g NaOH L^{-1} at 170^0C for 5-10 m pre-treatment and also Raghavendra (2006) reported the cellulose recovery of 56, 59.3 and 69.5 per cent respectively in paddy straw, wheat straw and sugarcane bagasse with NaOH treatment at (3% on particle size of 5 mm) with residential time of 8 h (ambient temp.) followed by autoclaving at 121^0C, 15 psi for 1 h.

The effect of autohydrolysis with and without acid indicated that the highest cellulose content was found in the treatment 1.0% H_2SO_4 coupled with autohydrolysis at 200^{0}C temperature for 10 min in the particle size 0.5 mm (Table 7). At this combination the cellulose content and the per cent increase in cellulose of the substrates observed was 0.647 g per g and 83.29 per cent in sugarcane bagasse, 0.627 g per g and 88.29 per cent in sugarcane tops, 0.627 g per g and 82.80 per cent in sugarcane trash, 0.620 g per g and 86.19 per cent in corn husk and 0.627 g per g and 91.71 per cent in corn stover, respectively. It was found to be almost similar with that of alkali and heat pre-treatment (3.0% NaOH coupled with 121^{0}C temperature) with respect to cellulose recovery.

The higher recovery of cellulose with 1.0% H_2SO_4 coupled with autohydrolysis at 200^{0}C temperature for 10 min is attributed to the strong hydrolytic ability of acid to digest the hemicellulose fraction of biomass and depolymerization effect of steam explosion. Excoffier *et al.* (1991) reported the highest recovery of glucose from steam explosion of wood chips impregnated with sulphuric acid (0.4%) at 225^{0}C for 120s. similarly, Soderstorm *et al.*, (2003) reported the improved sugar yield from steam exploded soft wood by two-step pre-treatment of temperatures between 180^0 C and 220^0 C for 2, 5 and 10 m residence time in presence of H_2SO_4 (1% and 2%). The increase in cellulose content with alkali plus heat and autohydrolysis with acid is relative to the loss of hemicellulose and lignin in the pre-treated substrates.

The effect of lignolytic fungi and their crude enzymes on cellulose content showed that the cellulose content was increased in all the substrates over a period of 30 days incubation over the initial values (Table 11). The trend of increase in cellulose content was observed with decrease in particle size, where particle size of 0.5 mm was found to contain more cellulose than particles size of 1.0 mm and 10.0 mm.

The inoculation of *Phanerochaete chrysosporium* fungi produced the cellulose yield of 0.427, 0.407, 0..400, 0.413 and 0.413 g per g in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover respectively with particles size of 0.50 mm in 30 days incubation period. The observed per cent increase in cellulose content were 20.96, 22.22, 16.62 24.02 and 26.69 per cent respectively with sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover. The increase in cellulose content with inoculation of lignolytic fungi is because of the degradation of lignin to carbon dioxide and water (Kirk and Farrel, 1987), as the white rot fungi possess many kinds of lignin degrading enzymes and release cellulose and hemicellulose during delignification process (Buswell *et al.* 1995, Leontievsky *et al.* 1997 and Vishwanath *et al.* 2008).Although part of the cellulose is used up by the fungi for the synthesis of cellular material. It could be noted that the cell wall of fungal biomass containing cellulose would have contributed for the increase in cellulose content.

Also, all the fungi (*P. chrysosporium* and *P. florida* and UASD-LF1) were found to be on par with respect to cellulose yield. The similar trend with respect to cellulose content was noticed with crude enzymes of different fungal inoculations (Table 15). The inoculation of crude lignolytic enzymes did not yield higher cellulose recovery when compared to the alkali plus heat and autohydrolysis pre-treatments or fungal delignification, it could be due to lesser activity of the crude lignolytic enzymes in hydrolyzing non-sugar components of the substrates under ambient conditions. Further, the increase in cellulose content due to crude lignolytic enzymes could be due to very less or absence of cellulose activity since it was selectively produced on the media that encourages ligninase enzyme production.

5.4 Effect of different pre-treatments on hemicellulose content of the substrates

The hemicellulose content of the substrates varied differently due to various pre-treatment methods *viz.* alkali and heat (2.50% and 3.00% NaOH for 8 h at room temperature followed by autoclaving at 121^{0}C or 125^{0}C exposure temperatures for 15 min), autohydrolysis in presence and absence of dilute acid, 1.0% H_2SO_4 at 180^0 or 200^{0}C temperatures for 10 min) and pre-treatment with lignolytic fungi (*Phanerochaete chrysosporium*, *Pleurotus florida* and UASD-LF1) and their crude enzymes showed significant differences. The alkali and heat as well as autohydrolysis with or without acid pre-treatments had decreased the hemicellulose content where as lignolytic fungi and their crude enzymes inoculation increased the himicellulose content.

Hemicellulose content of the substrate as influenced by alkali and heat pre-treatments were found to decrease in different substrates (Table 4). The increased concentration of alkali and high temperature of exposure had decreased the hemicellulose content in all the substrates and also the concentration of the treatment was more pronounced in smaller size particle with respect to hemicellulose loss. The highest hemicellulose content was observed in the treatment that received lower concentration of alkali and low temperature exposure (2.50% NaOH coupled with 121^0C temperature exposure) in case of particle size 10.00 mm for all the substrates. Sugarcane bagasse (0.247 g g^{-1}), sugarcane tops (0.220 g g^{-1}), sugarcane trash (0.233 g g^{-1}), corn husk (0.240 g g^{-1}) and corn stover (0.233 g g^{-1}) showed least reduction in hemicellulose content of 15.70, 3.08, 5.67, 5.14 and 10.38 per cent, respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover.

The lowest hemicellulose content was observed in the treatment that received higher concentration of alkali and high temperature of exposure (3.00% NaOH coupled with 125^0C temperature exposure) for the particle size 0.50 mm for all the substrates, sugarcane bagasse (0.147 g g^{-1}), sugarcane tops (0.133 g g^{-1}), sugarcane trash (0.153 g g^{-1}), corn husk (0.133 g g^{-1}) and corn stover (0.147 g g^{-1}) had shown highest reduction in hemicellulose content of 49.83, 41.41, 38.06, 47.43 and 43.46 per cent, respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover. Since acetyl bond between hemicellulose and lignin would have lost upon pre-treatment resulting in destruction of hemicellulose with minimum loss of cellulose (Koulas *et al.*, 1993) Similar observations were reported by Bjerre *et al.*, (1996) in wheat straw pre-treated with 2% alkali at 170^0C for 5-10 min and Curerli *et al.*, (2002) in wheat straw pre-treated with 1% alkali between 25 and 40^0 C in 24 h.

Autohydrolysis in presence or absence of acid showed variations in hemicellulose content of different substrates and their particle size. It is evident from the Table 8 that autohydrolysis of the substrates with high steam temperature in presence of acid had decreased the hemicellulose content and again the decrease was more in small sized particles of all substrates. It was observed that the least reduction in hemicellulose content ranging from 5.67 to 8.87 per cent was noticed with the treatment autohydrolysis without acid at 180^0C temperature for the particle size 10.0 mm in all the substrates.

The highest per cent decrease in hemicellulose was seen in the particle size, 0.5 mm which was subjected for 1% H_2SO_4 plus autohydrolysis at 200^0C. The maximum reduction in hemicellulose content observed with this treatment was 47.78, 44.05, 48.58, 49.80 and 51.15 per cent for the substrates sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover, respectively. It was observed to be very high when compared to alkali and heat pre-treatments. The autohydrolysis impregnated with acid treatment is known to solubilize hemicellulose almost completely. Nguyen *et al.*, (1998) reported solubilisation of 90-95% hemicellulose from acid imprignated steam explosion (0.4% sulphuric acid, 200^0 C - 230^0 C, 1-5 m) of soft wood. Also, Saha and Bothast (1999) reported solubilization of xylan in corn fibre that was exposed to 170^0C temperature pre-treatment.

With regard to hemicellulose content due to inoculation of lignolytic fungi and their crude enzymes, a marginal increase in hemicellulose was observed. The hemicellulose content of the substrates as influenced by inoculation of lignolytic fungi and their crude enzymes and incubation over a period of 30 days did not differ significantly. The different pre-treated substrates showed the per cent increase in hemicellulose with a range between zero and 11.60 for both lignolytic fungi and their crude enzymes inoculation (Table 12 and 16).

It was observed that the crude enzyme inoculation had increased the hemicellulose content in the initial stages (8.10- 11.50%) and later decreased over 30 days incubation period (4.78-7.51%) in *P. chrysosporium*. The decrease in hemicellulose content due to lignolytic enzyme treatment is supported by the observations of Rodriguez *et al.*, (2003) who observed 48 per cent decrease in hemicellulose with lignolytic enzyme of *P. chrysosporium* in grape cluster stems.

5.5 Effect of different pre-treatments on lignin content of the substrates

The lignin content of the substrates showed variations due to various pre-treatment methods such as alkali and heat, autohydrolysis with and without dilute acid, 1.0% H_2SO_4 at 180^0 or 200^0C temperatures for 10 min) and pre-treatment with lignolytic fungi (*Phanerochaete chrysosporium*, *Pleurotus florida* and UASD-LF1) and their crude enzymes showed significant differences. The physical and chemical pre-treatments (alkali plus heat and autohydrolysis with or without acid) had decreased very high quantities of lignin as against biological pre-treatments (lignolytic fungi and their crude enzymes inoculations).

The lignin content in the substrates due to alkali and heat pre-treatments was found to decrease in different substrates (Table 5). Probably, the increased concentration of alkali and high temperature of exposure had decreased the lignin content in all the substrates and also to the severity of the treatment was higher on smaller size particles with respect to lignin loss.

The lowest lignin content was observed in case of 0.5 mm particle size treated with 3.00% NaOH with 125^0C temperature exposure. The highest delignification observed with alkali and heat is due to increased digestibility of the substrates as the hydroxyl ion (OH^-) break lignin –lignin bonds and dissolve lignin (Koulas *et al*., 1993) and oxidation loss of lignin polymer to its aromatic derivatives. Kodali and Pogaku (2006) reported the lignin loss of 80.16 per cent with 10N sodium hydroxide held in steam for 10 min in case of rice husk.

The highest lignin content was observed in the treatment that received 2.50% NaOH along with 121^0C temperature exposure for all the substrates at 10.00 mm particle size with per cent reduction in lignin content between 42.80 and 52.14.

The reduction in lignin as influenced by autohydrolysis (with and without acid) was found to be very high (Table 9). The highest reduction in lignin content was observed in the treatment with 1% H_2SO_4 plus autohydrolysis at 200^0C temperature for all the substrates with particle size 0.50 mm. The observed lignin reduction was 80.71, 78.98, 70.63, 71.43 and 73.80 per cent, respectively for sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover. The highest lignin loss observed with 1.0% acid and 200^0 C exposure temperature for 10 min is due to the strong hydrolytic action of acid that causes degradation of lignin (Sun and Cheng, 2005) as well as the high steam pressure that rapidly explode or depressurize the substrate to produce reactive fibre (Laser *et al.,* 2002).

The reduction in lignin with regard to inoculation of lignolytic fungi and their crude enzymes was found to be less when compared to alkali and heat as well as autohydrolysis with acid (Table 13). The inoculation of lignolytic fungi showed the highest reduction between 28.57 and 30.07 for the particle size 0.50 mm, 23.57 and 30.07 for 1.00 mm and 12.50 and 19.29% for 10.0 mm with *P. florida* in 30 days incubation. All the lignolytic fungi showed similar range of lignin reduction in 30 days incubation. The variations in lignin reduction observed with different substrates could be due to differential lignin content in the raw substrates.

The inoculation of crude enzymes of lignolytic fungi recorded the highest reduction between 19.61 and 28.57 for the particle size 0.50 mm, 12.74 and 21.43 for 1.0 mm and 7.14 and 14.34 per cent for 10.0 mm with *P. chrysosporium* in 30 days incubation (Table 17). The delignification observed with crude enzymes of lignolytic fungi was on the lesser side as against fungi inoculation itself. This could be due to loss of activity of lignin degrading enzymes over long periods of incubation. Rodriguez *et al*., (2003) reported the lignin loss of 20% with *P. chrysosporium* ligninase enzymes in grape cluster stems.

The evaluation of different pre-treatment methods indicated alkali pre-treated of 3% NaOH (8 h incubation) followed by autoclaving at 1 h (121^0C) for the particle size 0.5 mm to be very good in terms of cellulose and hemicellulose recovery. Among the substrates, sugarcane bagasse was found to be an ideal substrate for ethanol production.

5.6 Effect of different cellulolytic fungal inoculation on release of reducing sugars from alkali pre-treated substrates

Substrates *viz.* Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were pre-treated with 3.0% NaOH for 8 hours at room temperature followed by autoclaving at 121^{0}C for 1 hour were saccharified using cellulolytic fungi. The release of reducing sugars due to inoculation of different cellulolytic fungi resulted in variations in release of reducing sugars.

The inoculation of 2% inoculum showed the maximum reducing sugar release in 8 days incubation period (in few cases – sugarcane bagasse and sugarcane tops with *Trichoderma reesei*) with respective substrates. However, the inoculation of 4% and 6% inoculum levels resulted in maximum release of reducing sugars in just 6 days incubation period in the respective pre-treated substrates. The release of highest amounts of reducing sugars with 4% and 6% inoculum level of *T. reesei* in 6 days when compared to 2% inoculum was observed in all the substrates. The 4% and 6% inoculation would have shortened the lag phase and favoured in the faster growth of the fungi resulting in secretion of higher amounts of cellulase enzymes that could saccharify the substrates in comparatively quick time.

Zayed and Meyer (1996) obtained reducing sugar yield of 27.0 g (54%) from 50 g delignified wheat straw (1% (w/v) NaOH autoclaved for 1 hr at 121^{0}C) by using *Trichoderma viridae* from delignified wheat straw within 3 days at 25-30° C.

Further the inoculation of substrates with *T. reesei* cellulolytic fungi yielded the highest reducing sugar release as compared to other cellulolytic fungal inoculation in respective pre-treated substrates. The variations in release of reducing sugar due to inoculation of different fungi was reported by Narothama Prasad and Geeta, (2011). They obtained the maximum reducing sugars of 31.45 mg g^{-1} in sugarcane bagasse, 26.41 mg g^{-1} in paddy straw with *T. viridae* and 24.48 mg g^{-1} in wheat straw with *T. reesei* inoculation for 7 days. Raghavendra (2006) saccharified the alkali delignified substrates using efficient *Trichoderma reesei* and observed reducing sugar yield of 22.3 mg g^{-1} (2.2%), 25.56 mg g^{-1} (2.56%) and 26.26 mg g^{-1} (2.62%) respectively in delignified paddy straw, wheat straw and sugarcane bagasse.

It was observed from the Table 18, 19, 20, 21 and 22 that the inoculation of *T. reesei* at 4% yielded the higher amounts of reducing sugars and per cent saccharification in 6 days incubation in respective pre-treated substrates such as in sugarcane bagasse (173.33 mg g^{-1} and 24.64%), sugarcane tops (171.39 mg g^{-1} and 25.16%), sugarcane trash (180.50 mg g^{-1} and 26.50%), corn husk (185.84 mg g^{-1} and 26.98%) and corn stover (182.98 mg g^{-1} and 26.87%). The inoculation of 4% *T. reesei* fungi for 6 days incubation was found to be on par with its 8 days incubation as well as with inoculation of 2% *T. reesei* and 6 days incubation, inoculation of 6% *T. reesei* for 6 or 8 days incubation. Patel *et al.*, (2007) reported the release of 32.50 mg g^{-1} reducing sugars in rice husk in 5 days with *A. awamorii* and *P. chrysosporium* coculture.

The inoculation of 4% *T. reesei* and 6 days incubation was also found to be on par with inoculation of *T. viridae* at 4% and 8 days incubation. The rest of the fungi and their inoculum levels were less efficient in releasing the reducing sugars.

5.7 Optimization of parameters for secretion of crude cellulase enzymes

The effect of different temperatures (*viz.*, 25, 30, 35 and 40^0 C), different pH (*viz.* 5.0, 5.5, 6.0 and 6.5 pH) on the growth of cellulolytic fungi to secrete cellulase enzyme were studied.

The crude enzyme extracts obtained from lignolytic fungal cultures grown at different temperatures and pH indicated significantly higher FPU activity at 30^{0}C in case of *T. reesei* crude enzyme extract with 2.31 U of FPU activity than other crude enzyme extracts (Table 23). Also, all the crude enzyme extracts of different fungi were found to be significantly superior at 30^{0}C when compared to 25, 35 and 40^{0}C with respect to FPU activity.

Similarly the crude enzyme extracts (different fungi) obtained at 6.0 pH growth conditions in higher FPU activity in all the crude enzyme extracts of different fungi than 5.0, 5.5 and 6.5 pH growth conditions (Table 24) with respect to secretion of cellulase enzyme.

The different cellulolytic fungi crude enzymes extracted at 30^0C temperature and 6.0 pH were assayed for FPU activity at different temperatures (30, 40 and 50^0C) and different pH (4.6, 4.8 and 5.0) using sodium citrate and sodium acetate buffers.

The highest FPU activity (2.40 U) was observed at 50^0 C temperature followed by *T. viridae* at 50^0C. The temperature 50^0 C was found to be optimum for all the crude enzyme extracts of cellulolytic fungi for higher FPU activity (Table 25).

The citrate buffer (0.05 M) of pH 4.8 recorded significantly the highest FPU activity of 2.38 U in *T. reesei* crude enzyme extract. It was found to be on par *T. viridae* crude enzyme also at pH 4.8 (citrate buffer). The results indicated that citrate buffer of pH 4.8 was found to be optimum for higher FPU activities in all the crude enzyme extracts (Table 26).

5.8 Enzyme activities of different crude enzyme extracts at optimized conditions

The five cellulolytic fungal cultures were grown at temperature 30^0C and pH 6.0 for 8 days using different alkali pre-treated substrates as carbon source and analyzed for FPU activity, CMCase activity, β-glucosidase activity and xylanase activity. This condition also resulted in production of protein between 0.475 and 0.530 mg g^{-1}. The *T. reesei* crude enzyme extract showed the higher FPU activity (2.23 U ml^{-1}), CMCase activity (3.68 U ml^{-1}), β-glucosidase activity (1.82 IU ml^{-1}) on cellulose and xylanase activity (3.65 U ml^{-1}) on sugarcane bagasse (Tables 27-31). Rest of the crude enzyme extracts of cellulolytic fungi were found to be significantly lower in all the enzyme activities studied. Also *A. sydowii* and *A. awamorii* crude enzymes failed to show detectable amount of xylanase on all the substrates evaluated.

Koijam *et al.,* (2000) studied cellulase production ability of *Phanerochaete chrysosporium* strain and *Cladosporium* sp. BK-II on pre-treated rice husk, wheat bran and sugarcane bagasse and found wide range of pH stability for the cellulase between 2.5 and 6.5 with optimum pH between 4.5 and 5.0 at 50^0 C. Hari *et al.* (2010) reported the production of higher amounts of FPase, endogluconase, β-glucosidase activities in alkali pre-treated *Artimisea annua* by *Trichoderma citrinoviride* under growth conditions of 28^0C and 5.5 pH. The optimized conditions of. 8 pH (citrate buffer) had produced 2.8 and 2.4 FPU activity by *P. chrysosporium* and *Cladosporium* sp. BK-II respectively in eight days incubation.

5.9 Effect of different crude enzymes of cellulolytic fungi on release of reducing sugars

5.9.1 Effect of different concentrations of crude enzymes

The alkali pre-treated substrates *viz.* Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were subjected for saccharification at 5% substrate concentration using different concentrations of crude cellulase enzymes (10, 20 and 30 U) extracted from 4 different fungi (*T. viridae*, *T. reesei*, *A. sydowii* and *A. awamorii*). The use of different concentrations of crude enzymes expressed their activity differently with regard to release of reducing sugars.

The release of reducing sugars as influenced by various concentrations of crude cellulose enzymes indicated that the inoculation of 20 U per g was optimum for all the crude enzyme extracts of different fungi studied. As per the data presented in Table 32, 33, 34, 35 and 36 the use of 20 U/g enzyme concentration had resulted in maximum release of reducing sugar in 24 h incubation period. The extension of incubation period up to 48 h with 20 U per g enzyme concentration did not release significant amounts of reducing sugars. The crude enzyme extract of *T. reesei* when used at 20 U per g showed the significantly superior amounts release of reducing sugars and per cent saccharification respectively in sugsrcane sugarcane bagasse (477.45 mg g^{-1} and 67.88%), sugarcane tops (428.68 mg g^{-1} and 62.94%), sugarcane trash (437.45 mg g^{-1} and 64.22%), corn husk (411.26 mg g^{-1} and 59.70%) and in corn stover (421.83 mg g^{-1} and 61.32%).

The present results are supported by the observations of Hatakka (1993) who reported 41% reducing sugars yield after enzymatic saccharification with *Trichoderma reesei* cellulase from alkali pre-treated wheat straw (2%, NaOH w/v) at 115^0 C for 10 min.

The inoculation of 20 U per g, *T. reesei* crude enzyme after 24 h incubation did not increase the reducing sugars significantly when the incubation period was extended up to 48 h. Further, the use of 30 U per g enzyme concentration for 24 h or 48 h incubation also failed to increase the reducing sugars beyond that produced by 20 U per g enzyme concentration. The 20 U and 30 U enzyme concentrations although are different they produced on par saccharification and they attained saturation levels almost equal for a given pre-treated substrate as 68 per cent in sugarcane bagasse, 63 per cent in sugarcane tops, 64 per cent in sugarcane trash, 61 per cent in corn husk and 61 per cent in corn stover. It could be due to inhibition of cellulase activity by the reducing sugars produced and other derivatives released by the crude enzyme extract. Holtzaple *et al.* (1990) had reported the inhibition of *T. reesei* cellulase by cellobiose and glucose during the saccharification. The study indicated the cellulase activity of mear 37 per cent (of its potential) in presence of 9.2 per cent cellobiose and 55 per cent glucose concentration.

The trend of reducing sugars released observed with 20 U per g enzyme concentration with *T reesei* crude enzyme inoculation was exhibited in all the crude enzyme extracts of different fungi. It was also observed that the release of reducing sugars was significantly highest with higher concentrations of enzyme (30 U) in the initial stages but had reached saturation point in 24 h incubation with release of reducing sugars and found on par with 20 U enzyme concentration in 24 h incubation. These results suggest that the optimum crude enzyme concentration required for saccharification with crude enzymes of different fungi was 20 U per g substrate in all the pre-treated substrates.

The release of reducing sugars and per cent saccharification with crude enzymes of saccharolyitic fungi other than *T. reesei* (Tables 32, 33, 34, 35 and 36) was found to be significantly lesser in all the pre-treated substrates irrespective of enzyme concentrations.

5.9.2 Effect of different concentrations of substrates

The alkali pre-treated substrates (Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover) were subjected for saccharification by varying the substrate concentrations (2.50%, 5.0% and 7.50%) with 20 U per g substrate crude cellulose enzymes obtained from four different fungi. The substrate concentrations resulted in significant differences with respect to release of reducing sugars in all the pre-treated substrates studied.

The release of reducing sugars as influenced by different substrate concentrations indicated significant differences. The data presented in Table 37, 38, 39, 40 and 41 showed that the use of 2.50% substrate concentration had resulted in significantly higher amounts of reducing sugar release in 24 h incubation in all the pre-treated substrates.

The release of reducing sugars and per cent saccharification by the *T. reesei* crude cellulase enzyme in different substrates were achieved 478.67 mg g^{-1} and 68.06 per cent in sugarcane bagasse, 442.13 mg g^{-1} and 64.91 per cent in sugarcane tops, 437.56 mg g^{-1} and 64.24 per cent in sugarcane trash, 420.80 mg g^{-1} and 61.08 per cent in corn husk and 428.42 mg g^{-1} and 61.08 per cent in corn stover, respectively. However, the release of reducing sugars was the highest at 48 h incubation period with 2.50% substrate concentration and it was found to be on par with 24 h incubation. Also, the use of 5.0% substrate concentration at both 24 h and 48 h incubations which were on par with 2.50% substrate concentration. The use of 7.5% substrate concentration resulted in significantly the lowest release of reducing sugars in all the pre-treated substrates.

With respect to release of reducing sugars due to cellulolytic fungi crude enzymes in different pre-treated substrates, *T. reesei* crude enzymes were found to be superior at all concentrations of substrates, followed by *T. viridae* crude cellulase enzyme. Both the enzyme sources were found to be on par with each other with respect to release of reducing sugars in all the pre-treated substrates at a given substrate concentration (2.5%, 5.0% and 7.50%). The crude enzymes of other fungi (*A. sydowii* and *A. awamorii*) were observed to be less efficient in releasing the reducing sugars in different substrates. Perhaps the higher concentration of substrate had limited enzyme activity resulting in lower release of reducing sugars.

Koijam *et al.,* (2000) hydrolysed rice husk, wheat bran and sugarcane bagasse with crude extract of *Phanerochaete chrysosporium* strain and *Cladosporium* sp. BK-II and obtained maximum reducing sugars in 60 h incubation for 2.5 g, 5.0 g and 10.0 g substrate concentration. However, the amount of reducing sugars per gram of cellulose was the highest at 2.5% (w/v) concentration. It was also observed that per cent saccharification depended on substrate concentration. The reducing sugar concentration showed a declining trend as the substrate concentration increased. The maximum reducing sugars released was 80 mg g^{-1} cellulose with *Phanerochaete chrysosporium* cellulase and 75 mg g^{-1} with *Cladosporium* sp. BK-II at 2.5% (w/v) concentration.

5.10 Effect of commercial cellulase enzymes on release of reducing sugars

5.10.1 Effect of different concentrations of commercial cellulase enzymes

The alkali pre-treated substrates (sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover) were subjected for saccharification at 5% substrate concentration using different concentrations of commercial cellulase enzymes (10, 15 and 20 U) along with 10 IU g^{-1} β-glucosidase and 5 U per g xylanase. The use of different concentrations of cellulase enzymes differed significantly with regard to release of reducing sugars.

The release of reducing sugars as influenced by various concentrations of commercial cellulase enzymes showed that the inoculation of 15 U per g was optimum for higher saccharification of the pre-treated substrates. The data outlined in Table 42, 43, 44, 45 and 46 reveal that the use of 15 U per g enzyme concentrations had resulted in higher amounts of reducing sugar release at 12 h incubation period in all the pre-treated substrates. The extension of incubation period up to 24 h with 15 U per g enzyme concentration did not increase the release of reducing sugars in significant amounts.

In general the commercial cellulase when used at 15 U per g yielded significantly superior amounts of reducing sugars release and per cent saccharification respectively in sugarcane bagasse (655.32 mg g^{-1} and 93.17%), sugarcane tops (615.23 mg g^{-1} and 90.33%), sugarcane trash (576.28 mg g^{-1} and 84.61%), corn husk (555.30 mg g^{-1} and 80.61%) and in corn stover (550.37 mg g^{-1} and 80.80%).

The inoculation of 15 U per g, commercial cellulase enzyme after 12 h incubation did not increase the reducing sugars significantly even when the incubation period was extended up to 24 h. Similarly, the use of 20 U per g enzyme concentration for 12 h or 24 h incubation also failed to increase the reducing sugars yield beyond the reducing sugars release produced by 15 U per g enzyme concentration in all the pre-treated substrates studied.

It was also observed that the release of reducing sugars were significantly highest with higher concentrations of enzyme (20 U) in the initial stages but the saccharification had reached saturation point at 12 h incubation in release of reducing sugars and found on par with 15 U enzyme concentration at 12 h incubation. These results suggest that the optimum commercial cellulase enzyme concentration required for saccharification of different pre-treated substrates was 15 U per g substrate. Kumar *et al.* (2010) reported similar kind of reports with steam pre-treated substrates where use of 20 FPU per g cellulase resulted in almost 90 per cent saccharification within 12 h of incubation.

Sattler *et al.*, (1989) hydrolysed Sigmacell 50 (20 g L^{-1}) and steam pre-treated poplar wood with commercial cellulase by using different concentrations of enzyme (5, 10, 15, 37.5 75 and 100 FPU g^{-1}) and reported that the structural properties *viz.* adsorption properties, pore size and accessibility determine the enzyme hydrolysis. Sreenath *et al.* (1999) reported release of 51 g fermentable sugar from 100 g alfalfa fibre at 50^0 C using mixture of cellulase and pectinase consisting of 25 filter paper units and 800 polygalacturanase units.

Wen *et al.* (2004) have saccharified the animal manure with optimized enzyme loading of 13 FPU cellulose g^{-1} substrate and 5 IU β-galactosidase g^{-1} substrate at 46^0C and pH 4.8 and obtained maximum glucose yield of 11.32 g 100 g^{-1} manure, corresponding to 40 per cent cellulose conversion.

5.10.2 Effect of different concentrations of substrates

The alkali pre-treated substrates *viz.* sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were saccharification by varying the substrate concentrations (2.50%, 5.0% and 7.50%) with 15 U per g commercial cellulase enzymes along with 10 IU g^{-1} β-glucosidase and 5 U per g xylanase. The substrate concentrations resulted in significant differences with respect to release of reducing sugars in all the pre-treated substrates studied.

The release of reducing sugars as influenced by different substrate concentrations indicated significant differences. The data presented in Table 47, 48, 49, 50 and 51 indicate that the use of 2.50% substrate concentration had resulted in significantly higher amounts of reducing sugar release in 12 h incubation in all the pre-treated substrates.

The reducing sugars released and the per cent saccharification achieved with 2.5% substrate concentration in 12 h incubation by the use of commercial cellulase enzyme in different substrates were 675.73 mg g^{-1} and 96.08 per cent in sugarcane bagasse, 643.94 mg g^{-1} and 94.54 per cent in sugarcane tops, 605.28 mg g^{-1} and 88.87 per cent in sugarcane trash 584.00 mg g^{-1} and 84.77 per cent in corn husk and 584.58 mg g^{-1} and 85.83 per cent in corn stover achieved respectively. However, the highest release of reducing sugars was found in 24 h incubation period with 2.50% substrate concentration in case of sugarcane tops, sugarcane trash, corn husk and corn stover but it was on par with 12 h incubation. Also, the use of 5.0% substrate concentration at both 12 h and 24 h incubations were found on par with 2.50% substrate concentration. The use of 7.50% substrate concentration resulted in significantly less release of reducing sugars in all the pre-treated substrates.

The saccharification studies indicated significantly the highest release of reducing sugars with 2.50% substrate concentration from the initial stages and reached saturation along with 5.0% substrate concentration in just 12 h incubation period when compared to 7.50% substrate concentration in all the substrates. The reducing sugar concentration showed a declining trend as the substrate concentration increased. The lowest per cent saccharification observed in 7.50% substrate concentration as the per cent saccharification is depended on substrate concentration (Koijam *et al.,* 2000). Although the 7.5 per cent substrate concentration (mg g^{-1}), but the concentration of sugars per unit volume was more or less same with that of 5 per cent substrate concentration and further saccharification was not observed in both 7.5 per cant and 5 per cent substrate concentration, at this point of sugar concentration, the cellulase activity would have been inhibited completely by the end products of saccharification (Holtzapple *et al.*, 1990).

The different saccharification methods studied in the present investigation showed saccharification with commercial cellulase supplemented with β-glucosidase and xylanase (15 FPU : 10 IU : 5 U, respectively) on alkali pre-treated substrates as an ideal method for maximum saccharification. Again among the substrates, sugarcane bagasse produced highest reducing sugar release in 12 h incubation period.

5.11 Effect of different fermentation methods on bioethanol production and residual reducing sugar content

The five different alkali pre-treated and saccharified agro-residues *viz.*, sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were fermented to bioethanol using six different ethanol fermenting microorganisms individually as separate hydrolysis and fermentation (SHF) and combinations of hexose and pentose fermenting microorganisms (Co-fermentation) and use of cellulase enzymes and alcohol fermenting microorganisms together as simultaneous saccharification and fermentation (SSF) on alkali pre-treated substrates. The ethanol produced by different methods and microorganisms differed significantly.

5.11.1 Separate Hydrolysis and Fermentation (SHF)

The yeast *S. cereviseae* produced significantly the highest ethanol yield of 262.93 mg per g in sugarcane bagasse, followed by *P. tannophilus* (256.53 mg g^{-1}) also in sugarcane bagasse and they were significantly superior over rest of the ethanol fermenting microorganisms with respect to ethanol yield (Table 52a).

S. cereviseae recorded the highest ethanol yield in all the agro-residues except in corn stover, In corn stover, *P. tannophilus* produced the highest ethanol yield (222.93 mg g^{-1}). The *S. cereviseae* and *P. tannophilus* were found to be at par with respect to ethanol yield in respective agro-residues and they were significantly superior over rest of the ethanol fermenting microorganisms. The lowest ethanol yield was observed in case of pentose fermenting yeasts *P. stipitis* and *C. shehatae* in all the substrates.

The highest ethanol yield observed in different fermented substrates indicates the efficiency of the organism to produce high concentrations of ethanol. Similar results were reported by Harpreet *et al.* (1998) in alkali pre-treated rice straw which was saccharified by *Trichoderma reesei* cellulase with ethanol yield of 34 per cent. Sharma *et al.* (2004) obtained maximum ethanol yield of 0.454 per g (45.4%) from enzymatically hydrolysed sunflower hulls having 40.0 g per litre reducing sugars by fermentation with *Saccharomyces cerevisiae* var. *ellipsoideus* under optimum conditions of time 24 h, pH 5.00, temperature 30.0^{o}C.

The highest ethanol yield observed in the substrates is supported by the work of Raghavendra (2006), who reported ethanol production of 49.13 mg g^{-1} (4.91%), 54.27 mg g^{-1} (5.47%) and 57.67 mg g^{-1} (5.76%) by *Pachysolen tannophylus* (NCIM-3445) respectively from delignified, saccharified paddy straw, wheat straw and sugarcane bagasse respectively.

Among the alcohol fermenting microorganisms used for alcohol fermentation, *S. cereviseae* showed the lowest residual reducing sugar content in all the substrates, sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and in corn stover. The lowest residual reducing sugar observed reflects about the relative ability of the organism to utilize reducing sugars for higher ethanol production (Table 52b).

5.11.2 Simultaneous Saccharification and Fermentation (SSF)

The SSF with *S. cereviseae* recorded significantly the highest ethanol in three different substrates, sugarcane bagasse (269.84 mg g^{-1}), sugarcane tops (252.41 mg/) and sugarcane trash (237.16 mg g^{-1}), whereas *Z. mobilis* produced the highest ethanol yield in other two substrates corn husk (231.29 mg g^{-1}) and corn stover (232.00 mg g^{-1}) (Table 53a). These two microorganisms were on par with each other in ethanol yield in all the substrates, except in case of sugarcane bagasse, whereas *S. cereviseae* (269.84 mg g^{-1}) was significantly superior over *Z. mobilis* (251.78 mg g^{-1}). The significantly lowest ethanol yield was noticed with *P. stipitis* in sugarcane bagasse (143.82 mg g^{-1}), sugarcane tops (135.29 mg g^{-1}), corn husk (117.23 mg g^{-1}) and in corn stover (118.22 mg g^{-1}) and with *C. shehatae* in sugarcane trash (102.75 mg g^{-1}).

The ethanol yield observed with *S. cereviseae* and *Z. mobilis* by SSF was slightly more than the ethanol yield obtained by SHF with the same microorganisms in all the substrates. This could be due to efficient conversion of the sugars released by cellulase enzymes. The higher ethanol yield in SSF with *S. cereviseae* and *Z. mobilis* could be due to efficient conversion of sugars released by cellulase enzymes into ethanol by ethanol fermenting microorganisms, whereas, SSF with other microorganisms, the conversion of sugars into ethanol is almost similar to that of SHF. It could be reasoned that these microorganisms possess lesser ethanol fermenting abilities.

Walsum *et al.* (1996) reported 90 per cent conversion liquid hot water (LHW) pre-treated lignocellulosics to ethanol in 2-5 days at enzyme loadings of 15-30 FPU g^{-1} using *Saccharomyces cerevisiae* in presence of *Trichoderma viride* cellulase. Similarly Hari Krishna *et al.* (2001) performed simultaneous saccharification and fermentation (SSF) of pre-treated sugar cane leaves in presence of *Trichoderma reesei* cellulase using two different yeast cultures. It was found that *Kluyveromyces fragilis* NCIM 3358 performed better and produced high yields of ethanol (2.5-3.5% w/v) than *Saccharomyces cerevisiae* NRRL-Y-132 (2.0-2.5% w/v) in SSF process. Also, increased ethanol yields were obtained when the cellulase was supplemented with β-glucosidase.

The residual reducing sugar content due to microbial inoculations and different substrates differed differently. The significantly lowest residual reducing sugar content was found with *S. cereviseae* in all the substrates. The significantly lowest residual reducing sugar content observed could be due to higher rate of sugar utilization by the yeast *S. cereviseae* (Table 53b).

5.11.3 Co-fermentation

Among the microorganisms used for alcohol fermentation, *S. cereviseae* and *C. shehatae* co-inoculation resulted in the highest ethanol yield in all the substrates, sugarcane bagasse (278.40 mg g^{-1}), sugarcane tops (262.75 mg g^{-1}), sugarcane trash (241.42 mg g^{-1}), corn husk (232.36 mg g^{-1}) and in corn stover (239.82 mg g^{-1}). The above co-inoculation of hexose and pentose fermenting microorganisms were significantly superior over all other combinations of microorganisms in respective agro-residues with respect to ethanol yield. The significantly lowest ethanol yield was recorded with the combined inoculation of *S. cereviseae*, *Z. mobilis* and *P. stipitis* in all the saccharified agro-residues (Table 54a).

The ethanol yield observed in combined inoculation with different hexose and pentose fermenting microorganisms was more than the ethanol yield observed with both SHF and SSF. Again, the co-inoculation with *S. cereviseae* and *C. shehatae* was more than any other microbial combinations used. This trend observed with all substrates could be attributed for better compatibility between hexose fermenting *S. cereviseae* and pentose fermenting *C. shehatae* that would have converted both hexose and pentose sugars more efficiently in all the substrates. The increased uptake of xylose and glucose, mannose and galactose sugars by *P. tannophilus* was reported by Sreenath *et al*., (2001).

Contrary to this, the co-inoculation with *S. cereviseae* and *P. stipitis* had resulted in decreased amount of ethanol production than the ethanol production observed with only *S. cereviseae* either as SHF or as SSF. This could be due to inhibitory action of *P. stipitis* on *S. cereviseae* that would have hindered the growth as well as ethanol fermenting ability of *S. cereviseae.* Amutha and Gunasekaran (2000) fermented liquefied cassava starch by monoculture and co-culture using *Saccharomyces diastaticus* and *Zymomonas mobilis* and reported the ethanol yield of 0.24 per g with *Saccharomyces diastaticus* monoculture and ethanol yield of 0.34 per g ethanol with the mixed culture fermentation. *Z. mobilis* has higher sugar uptake and ethanol yield and known to divert very less sugars to its biomass production compared to other yeasts (Gunasekaran and Chandraraj, 1999).

The significantly lowest residual reducing sugar content of 23.78, 22.35, 17.32, 16.60 and 18.75 mg per g was observed in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover respectively with dual inoculation of *S. cereviseae* and *C. shehatae* (glucose and xylose fermenting yeasts). It shows that these organisms efficiently converted the sugars to bioethanol. The combined inoculation of *S. cereviseae*, *Z. mobilis* and *P. stipitis* recorded the significantly highest residual reducing sugar contents in all the substrates. This could be due to incompatibility among the microorganisms to grow together and to cause fermentation of ethanol (Table 54b).

The fermentation (alkali pre-treated and saccharified substrates with commercial cellulase enzyme) with combined inoculation of *S. cerevisiae* and *C. shehatae* to be the efficient method. Sugarcane bagasse produced the highest ethanol content.

5.12 Pilot scaled study on production of bioethanol from sugarcane bagasse

The bioethanol of 223 g per kg (223 mg g^{-1}) was produced from pre-treated sugarcane bagasse under optimized conditions obtained, which was lower than the ethanol produced (278.40 mg g^{-1}) in small scale fermentation (5.0 g substrate in 100 ml) by the same microorganisms in presence of citrate buffer (4.8 pH of 0.5 molar solution). It could be reasoned that in absence of buffer the commercial cellulase enzyme might have not saccharified the cellulose to the maximum extent resulting in lesser quantity of ethanol production and the saccharification using commercial cellulase enzymes was done at 37^0C rather than 50^0C required for optimum enzymatic activity of the cellulase. Similar observations were made by Ballerini *et al.* (1994) with poplar wood and Sharma *et al.* (2004) with sunflower hulls in scaled up fermentation.

The scaled up production of bioethanol using the best observed pre-treatment and saccharification produced maximum ethanol by co-fermentation with *S. cereseae* and *C. shehatae.* The method is described in the form of Fig. 9 and 10.

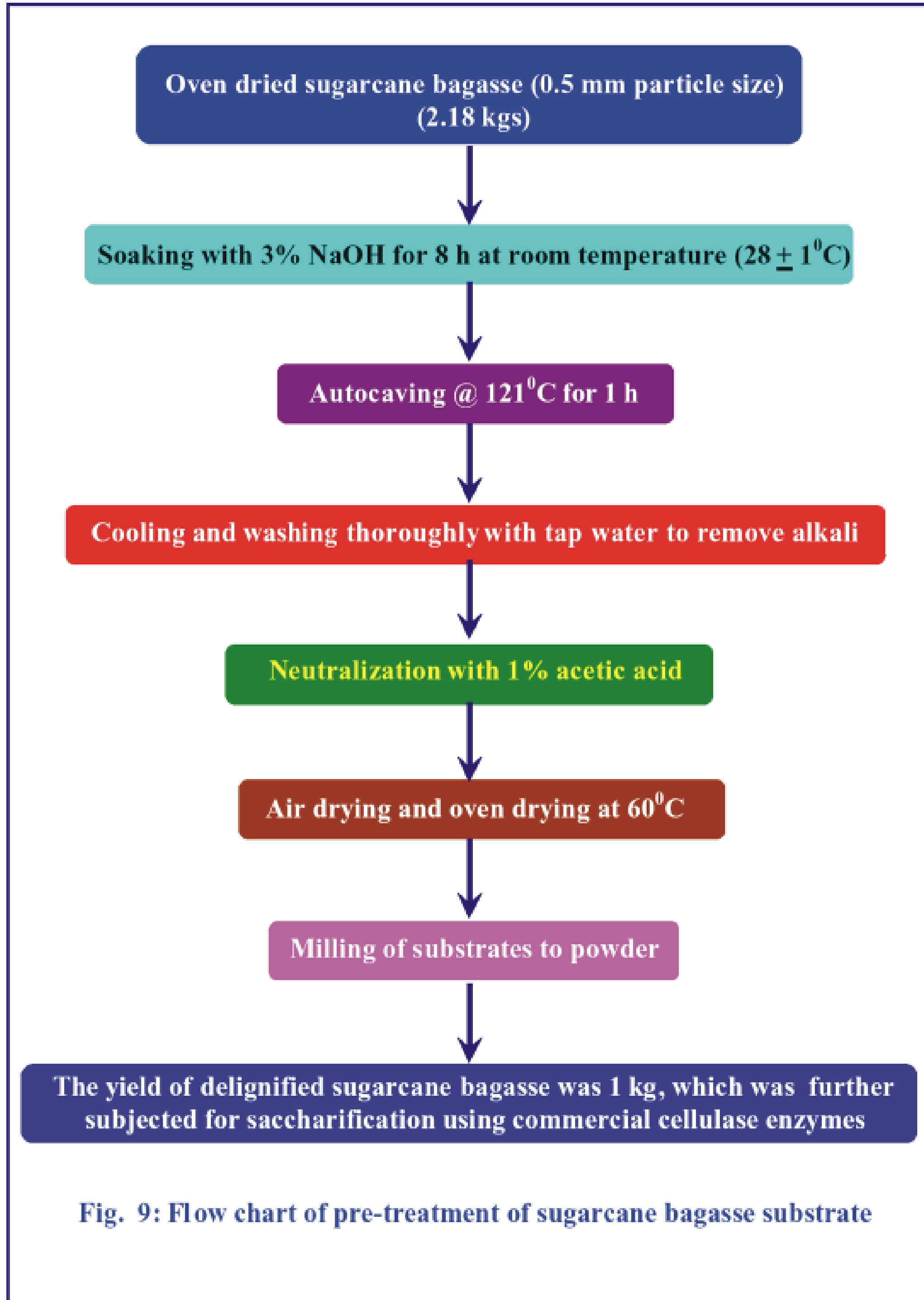


Fig. 9. Flow chart of pre-treatment of sugarcane bagasse substrate

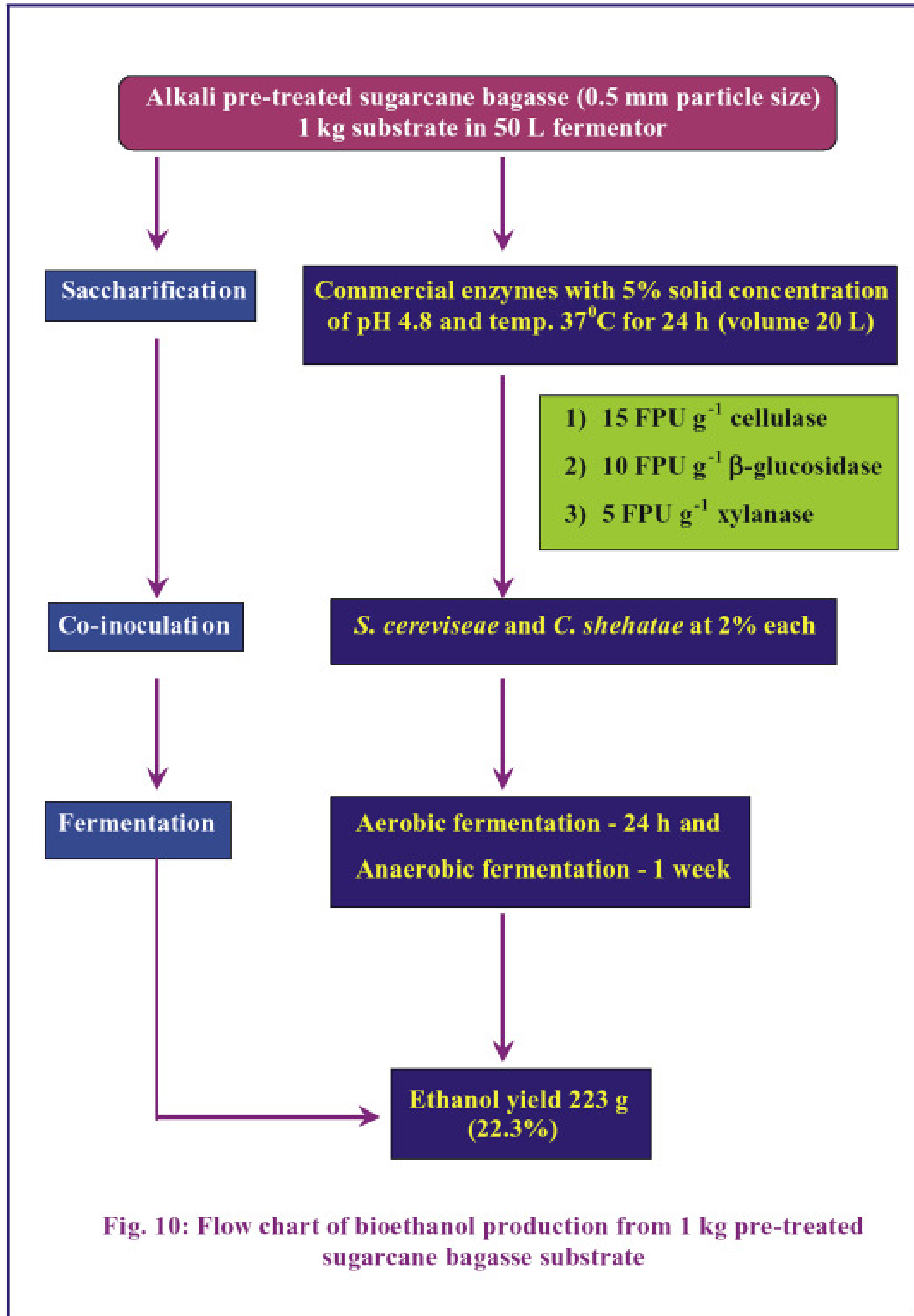


Fig. 10. Flow chart of bioethanol production from 1 kg pre-treated

Future line of work

Studies on the following aspects may be attempted.

1. Microwave pre-treatment for delignification of agro-residues.
2. Isolation and screening of microorganisms for maximum cellulase activity and purification of enzyme.
3. Isolation and screening of microorganisms for efficient xylose fermentation.
4. The use of immbolized enzymes for saccharification.
5. Isolation and screening of microorganisms for combined activities of saccarification and ethanol production.

6. SUMMARY AND CONCLUSIONS

The utilization of renewable lignocellulosic agro-residues has been attracting interest because of depletion of fossil fuel reserves and more recently, increasing environmental and political pressure. The present investigation on bioethanol production from selected agro-residues was attempted with objectives of achieving higher recovery of fermentable sugars through delignification and saccharification followed by fermentation of derived sugars into bioethanol. The results obtained from various experiments are summarized in this chapter.

Pre-treatment of ago-residues used in the study

Five agro-residues namely sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were subjected for various methods of pre-treatments with particle sizes of 0.50 mm, 1.0 mm and 10.0 mm. The different substrates analyzed for chemical composition had cellulose, hemi cellulose and lignin contents in the range between 0.327 and 0.353, 0.227 and 0.293 and 0.140 and 0.160 g per g, respectively.

The use of 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 125^0C (1 h) resulted in highest loss of weight in the particle size 0.5 mm in all the substrates, sugarcane bagasse 0.568 g per g. sugarcane tops 0.532 g per g, sugarcane trash 0.548 g per g, corn husk 0.555 g per g, and corn stover 0.547 g per g.

The highest cellulose recovery was achieved with the treatment combination of 3.0% NaOH coupled with 121^0C temperature with particle size 0.5 mm in sugarcane bagasse (0.633 g g^{-1}), corn husk (0.620 g g^{-1}), sugarcane tops (0.613 g g^{-1}) and corn stover (0.613 g g^{-1}). The treatment 2.5% NaOH coupled with 125^0C temperature exposure resulted in highest cellulose content of 0.620 g per g in case of sugarcane trash.

The highest hemicellulose loss was observed in the treatment 3.0% NaOH coupled with 125^0C temperature exposure in the particle size 0.50 mm in all the substrates, highest reduction in hemicellulose content of 49.83, 41.41, 38.06, 47.43 and 43.46 per cent, respectively in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover.

The same treatment also resulted in the highest loss of lignin content in case of 0.50 mm particle size with per cent reduction of 80.71 in sugarcane bagasse, 78.98 in sugarcane tops, 75.00 in sugarcane trash, 76.43 in corn husk and 73.76 per cent in corn stover.

The treatment combination of 3.0% NaOH coupled with 121^0C temperature with particle size 0.5 mm was found to the best for the recovery of both cellulose and hemicelluloses after pre-treatment in sugarcane bagasse (0.813 g g^{-1}), sugarcane tops (0.786 g g^{-1}), sugarcane trash (0.806 g g^{-1}), corn husk (0.806 g g^{-1}), and corn stover (0.806 g g^{-1}).

The autohydrolysis pre-treatment with 1.0% acid and 200^0C exposure temperature for 10 min produced the highest weight loss in case of particle size 0.5 mm in all the substrates with loss of weight of 0.517 in bagasse, 0.507 in sugarcane tops, 0.502 in sugarcane trash, 0.530 in corn husk and 0.507 in corn stover.

The treatment with 1.0% acid and 200^0C exposure temperature for 10 min also resulted in higher recovery of cellulose content of 0.647 g per g in sugarcane bagasse, 0.627 g per g in sugarcane tops, 0.627 g per g in sugarcane trash, 0.620 g per g in corn husk and 0.627 g per g in corn stover. The cellulose recovered with this treatment showed almost identical values with that of 3.0% NaOH coupled with 121^0C temperature treatment.

The treatment, 1% H_2SO_4 plus autohydrolysis at 200^0C had decreased the hemicelluloses content in all the pre-treated substrates with a maximum reduction in 0.50 mm particles of the substrates. The per cent reduction in hemicelluloses content was 47.78, 44.05, 48.58, 49.80 and 51.15 in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and in corn stover respectively.

The treatment, 1% H_2SO_4 plus autohydrolysis at 200^0C had decreased the lignin contents of sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover by 80.71, 78.98, 70.63, 71.43 and 73.80 per cent, respectively in particles of size 0.50 mm.

The pre-treatment with lignolytic fungi and their crude enzymes revealed the maximum weight loss with inoculation of lignolytic fungi *Phanerochaete chrysosporium* treatment in 0.5 mm particle size in respective substrates at the end of 30 days incubation. However the loss of weight observed with *Phanerochaete chrysosporium* inoculation was at par with other fungi inoculation, *Pleurotus florida* and UASD-LF1 at the end of 30 days incubation in 0.5 mm particle size substrates. Whereas the loss of weight in different substrates due to inoculated of crude enzymes of lignolytic fungi was found to be insignificant irrespective of particle size and incubation periods.

The inoculation of *Phanerochaete chrysosporium* fungi produced the cellulose yield of 0.427, 0.407, 0..400, 0.413 and 0.413 mg per g in sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover respectively with particles size of 0.50 mm in 30 days incubation period but found to be at par with other fungi inoculation, *P. florida* and UASD-LF1. The similar trend was observed for cellulose content with crude enzymes of different fungi inoculation.

The hemicellulose content of the substrates as influenced by inoculation of lignolytic fungi and their crude enzymes and incubation over a period of 30 days did not show much variation from the initial values with observed increase in hemicelluloses content between zero and 11.60 per cent for both lignolytic fungi and their crude enzymes inoculation.

The inoculation of lignolytic fungi *Pleurotus florida* had resulted in a maximum reduction of lignin content between 28.57 and 30.07 per cent in the particles of size 0.50 mm, between 23.57 and 30.07 per cent in 1.00 mm and between 12.50 and 19.29 per cent in 10.0 mm over a period of 30 days incubation. All the lignolytic fungi (*P. chrysosporium P. florida* and UASD-LF1) were found to be on par with respect to delignification. Whereas the inoculation of crude enzymes of lignolytic fungi recorded the highest reduction between 19.61 and 28.57 for the particle size 0.50 mm, 12.74 and 21.43 for 1.00 mm and 7.14 and 14.34 per cent for 10.0 mm with *P. chrysosporium* derived crude enzyme in 30 days incubation.

Optimization of parameters for crude cellulase enzymes

The growth of cellulolytic fungi at 30^0C temperature and 6.0 pH with cellulose as the carbon source over a period of 8 days was found to be ideal for secretion of maximum cellulase enzyme for all the fungi. Whereas for the activity of crude cellulase enzymes 50^0C reaction temperature with citrate buffer of pH 4.8 (0.05 M) were found to be optimum for all the crude enzymes extracted from *Trichoderma viridae, Trichoderma reesei, Aspergillus sydowii, Aspergillus awamorii* and *Phanerochaete chrysosporium.*

The crude enzyme extract from *T. reesei* showed highest FPU activity (2.33 U ml^{-1}), CMCase activity (3.68 U ml^{-1}), β-glucosidase activity (1.82 IU/ ml) on cellulose and xylanase activity (3.65U ml^{-1}) on sugarcane bagasse as carbon source. The next best fungal crude enzyme extract observed was from *T. viridae* with 2.21 U ml^{-1} of FPU activity, 2.90 U ml^{-1} CMCase activity, 1.56 IU ml^{-1}, β-glucosidase activity and 2.75 U ml^{-1} xylanase activity on cellulose as carbon source.

Saccharification studies

The pre-treatment with 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 125^0C (1 h) resulted in the highest recovery of both cellulose and hemicelluloses with particle size 0.5 mm in sugarcane bagasse (0.813 g g^{-1}), sugarcane tops (0.786 g g^{-1}), sugarcane trash (0.806 g g^{-1}), corn husk (0.806 g g^{-1}), and corn stover (0.806g g^{-1}). The substrates obtained from this treatment is used for further saccharification studies using different cellulolytic fungi, crude enzymes of different cellulolytic fungi and commercial cellulase enzymes.

The inoculation of *T. reesei* fungus at 4% under solid state fermentation had yielded the superior amounts of reducing sugars and per cent saccharification in 6 days incubation in respective pre-treated substrates such as in sugarcane bagasse (173.33 mg g^{-1} and 24.64%), sugarcane tops (171.39 mg g^{-1} and 25.16%), sugarcane trash (180.50 mg g^{-1} and 26.50%), corn husk (185.84 mg g^{-1} and 26.98%) and in corn stover (182.98 mg g^{-1} and 26.87%). The use of 6% inoculum or extension of incubation period up to 8 days was not significant in releasing the reducing sugars.

The inoculation of *T. reesei* crude enzyme extract at 20 U per g concentration along with 5.0% substrate concentration and 24 h incubation was found optimum for higher saccharification. This combination of crude enzymes produced significantly the superior amounts of reducing sugars release and per cent saccharification respectively in sugarcane bagasse (477.45 mg g^{-1} and 67.88%), sugarcane tops (428.68 mg g^{-1} and 62.94%), sugarcane trash (437.45 mg g^{-1} and 64.22%), corn husk (411.26 mg g^{-1} and 59.70%) and in corn stover (421.83 mg g^{-1} and 61.32%). The use of 30 U per g or extension of incubation period up to 48 h did not release significant amount of reducing sugars and stood on par with 20U per g enzyme concentration and 24 h incubation period.

Saccharification of alkali pre-treated substrates (Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover) with variations in substrate concentrations (2.50%, 5.0% and 7.50%) with 20 U per g substrate crude cellulase enzymes had yielded highest reducing sugars and per cent saccharification with 2.5% substrate concentration in 24 h incubation by the use of *T. reesei* crude cellulase enzyme in different substrates (478.67 mg g^{-1} and 68.06% in sugarcane bagasse, 442.13 mg g^{-1} and 64.91% in sugarcane tops, 437.56 mg g^{-1} and 64.24% in sugarcane trash, 420.80 mg g^{-1} and 61.08% in corn husk and 428.42 mg g^{-1} and 61.08% in corn stover), reducing sugars released and per cent saccharification respectively but found to be on par with 5.0% substrate concentration at both 24 h and 48 h incubations.

The use of commercial cellulase enzyme with 15 U per g concentration along with β-glucosidase and Xylanase enzymes at 10 and 5 U per g, respectively on alkali pre-treated substrates (Sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover) at 5% substrate concentration was found to be optimum for higher saccharification. This combination produced significantly the highest reducing sugars release and per cent saccharification respectively in sugarcane bagasse (655.32 mg g^{-1} and 93.17%), sugarcane tops (615.23 mg g^{-1} and 90.33%), sugarcane trash (576.28 mg g^{-1} and 84.61%), corn husk (555.30 mg g^{-1} and 80.61%) and in corn stover (550.37 mg g^{-1} and 80.80%) in 12 h incubation period. The use of 20 U per g commercial cellulase enzyme concentration along with β-glucosidase (10 IU g^{-1}) and Xylanase (5 U g^{-1}) or extension of incubation period up to 24 h stood on par with 15 U per g commercial cellulase enzyme concentration along with β-glucosidase (10 IU g^{-1}) and Xylanase (5 U g^{-1}) and 12 h incubation in releasing the reducing sugars.

Saccharification of different alkali pre-treated substrates with 15 U per g commercial cellulase enzyme along with β-glucosidase (10 IU g^{-1}) and Xylanase (5 U g^{-1}) on different concentrations of substrates (2.50%, 5.0% and 7.50%) resulted significantly highest release of reducing sugars and per cent saccharification of 675.73 mg g^{-1} and 96.08 per cent in sugarcane bagasse, 643.94 mg g^{-1} and 94.54% in sugarcane tops, 605.28 mg g^{-1} and 88.87 per cent in sugarcane trash 584.00 mg g^{-1} and 84.77 per cent in corn husk and 584.58 mg g^{-1} and 85.83 per cent in corn stover, achieved respectively in all the substrates having 2.5% substrate concentration in 12 h incubation. The use of 2.50% substrate concentration with 12 h incubation stood on par with the use 5.0% substrate concentration at both 12 h and 24 h incubations.

The use of 15 U per g concentration of commercial enzyme and 12 h incubation period at 5.0% solid loading had produced significantly highest saccharification in all the alkali pre-treated substrates than the saccharification caused by either crude enzymes of different fungi or inoculation of cellulolytic fungi.

Fermentation of alcohol

The alkali pre-treated and commercial cellulase enzyme saccharified substrates *viz.*, sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover were fermented to bioethanol by using six different ethanol fermenting microorganisms individually as separate hydrolysis and fermentation (SHF) and in combinations of hexose and pentose fermenting microorganisms (Co-fermentation) and by inoculating cellulase enzymes and alcohol fermenting microorganisms together as simultaneous saccharification and fermentation (SSF).

The yeast *S. cerevisiae* produced significantly the highest ethanol yield in four of the substrates when inoculated to the respective broths after saccharification with the production of 262.93 mg per g in sugarcane bagasse, 246.93 mg per g in sugarcane tops, 232.00 mg per g in sugarcane trash, 222.40 mg per g and in corn husk whereas in corn stover highest ethanol yield (222.93 mg g^{-1}) was produced by *P. tannophilus*. Both yeasts were found to be on par in respective substrates in causing ethanol fermentation.

The lowest residual reducing sugar content was found in all the substrates fermented by *S. cerviseae* in all the substrates with residual reducing sugar contents of 54.96 mg g^{-1} in sugarcane bagasse, 58.36 mg g^{-1} in sugarcane tops, 53.96 mg g^{-1} in sugarcane trash, 52.53 mg g^{-1} in corn husk and in 53.96 mg g^{-1} corn stover due to separate hydrolysis and fermentation (SHF).

The simultaneous saccharification and fermentation of pre-treated substrates with commercial cellulase enzyme and inoculation of *S. cerevisiae* recorded significantly the highest ethanol in three different substrates, sugarcane bagasse (269.84 mg g^{-1}), sugarcane tops (252.41 mg g^{-1}) and sugarcane trash (237.16 mg g^{-1}), whereas in other two substrates corn husk (231.29 mg g^{-1}) and corn stover (232.00 mg g^{-1}) SSF with commercial cellulase enzyme and *Z. mobilis* produced highest ethanol yield.

The lowest residual reducing sugar content was found in all the substrates fermented by *S. cerevisiae* with residual reducing sugar contents of 45.85 mg g^{-1} in sugarcane bagasse, 41.03 mg g^{-1} in sugarcane tops, 39.59 mg g^{-1} in sugarcane trash, 40.31 mg g^{-1} in corn husk and in 41.03 mg g^{-1} corn stover due to simultaneous saccharification and fermentation (SSF).

The combined inoculation of *S. cerevisiae* and *C. shihatae* on pre-treated and saccharified substrates resulted in highest ethanol yield in all the substrates, sugarcane bagasse (278.40 mg g^{-1}), sugarcane tops (262.75 mg g^{-1}), sugarcane trash (241.42 mg g^{-1}), corn husk (232.36 mg g^{-1}) and in corn stover (239.82 mg g^{-1}).

The ethanol produced by combined inoculation of *S. cerevisiae* and *C. shihatae* in different pre-treated and saccharified substrate was comparatively superior than the ethanol produced either by separate hydrolysis and fermentation or simultaneous saccharification and fermentation.

The lowest residual reducing sugar content was observed with the treatment combined inoculation of *S. cerviseae* and *C. shihatae* for all the substrates with residual reducing sugar contents of 23.78 mg g^{-1} in sugarcane bagasse, 22.35 mg g^{-1} in sugarcane tops, 17.32 mg g^{-1} in sugarcane trash, 16.60 mg g^{-1} in corn husk and in 18.75 mg g^{-1} corn stover due to simultaneous saccharification and fermentation

The pre-treatment with 3.0% NaOH (8 h incubation at room temp.) followed by autoclaving at 125^0C (1 h) followed by saccharification by commercial cellulase enzyme (M/s. Maps India Ltd), β-glucosidase (M/s. SRL Chemicals) and Xylanase (M/s. Sameerwadi sugar industry, Bagalkot, Karnataka) at 15 FPU per g, 10 IU per g and 5 U per g respectively with 5.0% substrate concentration and fermentation with dual inoculation with *S. cerevisiae* and *C. shihatae* yeasts produced highest ethanol in all the agro-residues used for bioethanol production. With this protocol produced bioethanol was 278.40 mg g^{-1} in sugarcane bagasse, 262.75 mg g^{-1} in sugarcane tops, 241.42 mg g^{-1} in sugarcane trash, 232.36 mg g^{-1} in corn husk and 239.82 mg g^{-1} in corn stover.

The scaled up study on bioethanol production with pre-treated sugarcane bagasse under optimized conditions produced 223 g bioethanol from one kg pre-treated substrates with a corresponding yield of 22.30 per cent bioethanol from pre-treated sugarcane bagasse.

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Appendix I: Media composition

Selective media for *Zymomonas mobilis* (NCIM Catalogue, India, 2002)

Yeast Extract	: 10 g
Peptone	: 10 g
Sucrose	: 10 g
Agar	: 18 g
Distilled water	: 1000 ml

Malt Extract Glucose Yeast Extract Peptone (NCIM Catalogue, India, 2002)

Yeast Extract	: 3 g
Malt Extract	: 3 g
Peptone	: 5 g
Glucose	: 10 g
Agar	: 18 g
Distilled water	: 1000 ml
pH	: 6.4 – 6.8

Potato Dextrose Agar (PDA) (NCIM Catalogue, India, 2002)

Potato	: 200 g
Dextrose	: 20 g
Agar	: 20 g
Distilled water	: 1000 ml
pH	: 6.0

200 g of peeled potatoes were cut into small pieces and suspended in 1000 ml distilled water and steamed for 30 min. The extract was obtained by filtering through muslin cloth and final volume made up to 1000 ml with distilled water.

Fermentation medium (Yu and Zhang, 2004)

Urea	: 0.64%,
KH_2PO_4	: 0.2%
$MgSO_4.7H_2O$	: 0.1%

Mandel's basal salt for cellulase production (Mandel and Weber, 1969)

KH_2PO_4	: 2.0 g
$(NH_4)_2SO_4$	: 1.4 g
Tween 80	: 0.50 ml (0.05%)
Urea	: 0.30 g
$MgSO_4.7H_2O$	: .0.30 g
$CaCl_2$	: 0.30 g
Trace mineral solution	: 1.0 ml
Carbon source (cellulose)	: 10.0 g
Distilled water	: 1000 ml

Trace mineral solution

$MnSO_4$	: 1.56 g
$FeSO_4$	: 5.00 g
$ZnCl_2$	: 1.67 g
$CoCl_2$	: 2.00 g
Distilled water	: 1000 ml

Nutrient solution for biological pre-treatment (Singhania *et al.*, 2006)

$(NH_4)_2 \cdot SO_4$	: 10.0 g
KH_2PO_4	: 3.0 g
$MgSO_4 \cdot 7H_2O$	: 0.50 g
$CaCl_2 \cdot H_2O$	: 0.50 g
Distilled water	: 1000 ml

Kirk's basal liquid medium (Kirk *et al.*, 1978)

Glucose	: 3.0 g
KH_2PO_4	: 1.0 g
NaH_2PO_4	: 0.26 g
$(NH_4)_2SO_4$	: 0.317 g (2.4 mM)
$MgSO_4.7H_2O$	: 0.5 g
$CuSO_47H_2O$	: 0.05 mg
2, 2-dimethylsuccinic acid	: 2.2 g
$CaCl_2.2H_2O$	: 74 mg
$ZnSO_4.7H_2O$	: 6 mg
$FeSO_4.7H_2O$	: 5 mg
$MnSO_4.4H_2O$	: 5 mg
$CoCl_2.6H_2O$	: 1 mg
Vitamin solution	: 500 µl
Veratryl alcohol	: 1 mM
Distilled water	: 1000 ml
pH	: 4.5

STUDIES ON BIOETHANOL PRODUCTION FROM SELECTED AGRO-RESIDUES

SHANKARAPPA, T. H. **2011** **Dr. GEETA G. SHIRNALLI**
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ABSTRACT

The present study was conducted on bioethanol production from selected agro-residues with the objectives of achieving higher recovery of fermentable sugars through delignification and saccharification followed by fermentation of the derived sugars to bioethanol. Five agro-residues namely sugarcane bagasse, sugarcane tops, sugarcane trash, corn husk and corn stover of particle sizes 0.5, 1.0 and 10.0 mm were delignified by various pre-treatment methods such as alkali, autohydrolysis and lignolytic fungi. The treatment combination of 3.0% NaOH with 121^{0}C temperature for one h on particle size 0.5 mm resulted in the highest recovery of cellulose and hemicelluloses in sugarcane bagasse (0.813 g/g), sugarcane tops (0.786 g/g), sugarcane trash (0.806 g/g), corn husk (0.806 g/g) and corn stover (0.806 g/g). The delignified substrates were further saccharified using cellulolytic fungi and their crude enzymes and commercial cellulase enzymes. Among these, the commercial cellulase enzyme with 15 U/g along with β-glucosidase (10 U/g) and Xylanase (5 U/g) enzymes at 5% substrate produced significantly highest reducing sugars with per cent saccharification in sugarcane bagasse (93.17%), sugarcane tops (90.33%), sugarcane trash (84.61%), corn husk (80.61%) and in corn stover (80.80%) in 12 h of incubation period. The pre-treated substrates were fermented to bioethanol by using six microorganisms. The combined inoculation of *Saccharomyces cerevisiae* and *Candida shihatae* on pre-treated substrates resulted in the highest ethanol yield in all the substrates, sugarcane bagasse (278.40 mg/g) followed by sugarcane tops (262.75 mg/g), sugarcane trash (241.42 mg/g), corn stover (239.82 mg/g) and corn husk (232.36 mg/g). The scaled up study on bioethanol production with sugarcane bagasse under optimized conditions produced 223 g bioethanol from one kg pre-treated substrate. Thus, it can be concluded that the substrates need to be alkali pretreated followed by treatment with commercial enzymes and further conversion to ethanol. The combined inoculation of the yeast cultures yields maximum ethanol.