BIOLOGICAL PRETREATMENT OF SUNFLOWER SEED HULL FOR ETHANOL PRODUCTION

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By

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CERTIFICATE

This is to certify that the thesis entitled **"BIOLOGICAL PRETREATMENT OF SUNFLOWER SEED HULL FOR ETHANOL PRODUCTION"** submitted by **Miss. SNEHA S. NAIR** in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **AGRICULTURAL MICROBIOLOGY,** College of Agriculture, Raichur, University of Agricultural Sciences, Raichur, is a record of research work done by her during the period of her study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar titles.

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Affectionately dedicated

to

Mom & Dad

for their unwavering faith in me…..

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With Regardful memories……

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RAICHUR

JUNE 2014 **(SNEHA S. NAIR)**

LIST OF ABBREVIATIONS

Abbreviation		Expansion
SSH	:	Sunflower Seed Hull
PDA	:	Potato Dextrose Agar
LBM	:	Lignolytic Basal Medium
CBM	:	CellulolyticBasal Medium
XBM	:	Xylolytic Basal Medium
RM	:	Rich Medium

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INTRODUCTION

I. INTRODUCTION

Excessive consumption of fossil fuels, particularly in urban areas, has resulted in generation of high levels of pollution during the last few decades. With the expansion of human population and increase of industrial prosperity, global energy consumption also has increased gradually. In view of these, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of the limited fossil fuel stock but also for safe and better environment (Chandel *et al.,* 2007). In this scenario, renewable sources such as biomass can be converted to fermentable sugars for the production of bio ethanol, an alcohol made by fermentation, to meet the growing consequences of global energy consumption.

The interest in biomass as an alternative source of energy has gained more momentum over the last century. According to Marszalek and Kaminski (2008), ethanol produced from renewable energy source is the most promising future biofuel. Hill *et al.* (2006) proposed that an alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, and be producible in sufficient quantities to make a meaningful impact on energy demands, it should also provide a net energy gain over the energy sources used to produce it. Bioethanol meets most of these demands.

Production of bioethanol from lignocellulosic biomass contains three major processes, including pretreatment, hydrolysis and fermentation. A fine combination of these three processes can result in high ethanol recovery. The purpose of pretreatment is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of the materials (McMillan, 1994). Literature reports a number of pretreatment options that has been tried for various biomass types. Several physical and chemical methods which can bring about these changes in the biomass have been employed for the pretreatment of different lignocellulosic wastes which includes steam explosion, use of alkali, acid treatment *etc.*

Apart from physical and chemical methods, biological processes, which involve microbial and enzymatic hydrolysis, have been effectively utilized for bioethanol production. Microbial pretreatment and hydrolysis which involves the use of microorganisms, mainly fungi, to degrade lignin and hemicellulose to obtain fermentable

sugars is also being considered as an effective pretreatment process. Several studies have shown that white-rot fungi are the most effective microorganisms for the pretreatment of lignocelluloses, which includes *Trichoderma viride, T. reesei*, *Fusarium solani*, *Aspergillus niger*, *A. terreus*, *Phanerochaete chrysosporium* and *Bacillus adusta* (Dashtban *et al*., 2009). Microbial pretreatment has been considered as an environment friendly approach, providing the benefit of inexpensive ethanol production. However, they suffer the drawback of the consumption of the hydrolyzed products (such as reducing sugars) due to cell growth. This limitation can be overcome in enzymatic hydrolysis, where in, lignocellulosic biomass is converted into fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms. There are several advantages of enzymatic hydrolysis, including little energy requirement and mild reaction conditions, high substrate specificity, high yield of sugars, and high hydrolysis efficiency. However, the high dosage of expensive cellulases for high hydrolysis yields remains another obstacle to cellulosic ethanol commercialization (Wyman, 2007). To make the process economically feasible, two or more effective pretreatment methods in combination can be carried out, which will disrupt the crystalline structure of lignocelluloses and make cellulose more accessible to the enzymes for the conversion of the polysaccharides into fermentable sugars. Manipulation of temperature, supplementation of surfactants, addition of polymers *etc.* can also effectively increase enzymatic hydrolysis of lignocelluloses due to a higher availability of enzymes for cellulose degradation. Once the lignocellulose has been broken down into fermentable sugars, the prime concern is ethanol production. Even though several microorganisms have been considered as ethanologenic microbes, the yeast, Saccharomyces *cerevisiae* and facultative bacterium *Zymomonas mobilis* are better candidates for industrial alcohol production (Gunasekaran and Raj, 1999). Traditionally, *Saccharomyces cerevisiae* has been used for the production of ethanol; however it has been associated with low alcohol tolerance and low productivity. *Z. mobilis*, a Gram negative bacterium possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance. *Zymomonas* grows and ferments glucose very fast and grows in high glucose and ethanol concentration (Swings and De-Ley, 1981). Some studies reported that after hydrolysis of lignocellulosic biomass, the produced pentose sugars (mainly D-xylose and L-arabinose) create problem in yeast alcohol fermentation because yeast strains lack the xylose utilization enzymes (Hahn-Hagerdahl *et al*., 2007). An exception to this is *Pichia stipitis.*

Unlike other yeasts, it has the ability to produce reductases that reduce D-xylose to xylitol.

Different agricultural wastes, rich in lignocellulose have been exploited over the years for production of bioethanol. Cellulose rich agricultural raw materials such as barley straw, sugar cane bagasse, cotton stalk, corn stalk, rice straw, sunflower seed hull etc. has been effectively used for bio ethanol production (Nigam, 2002).

Apart from these feed stocks, lignocellulosic biomass including industrial residues such as pulp and paper processing wastes and municipal solid wastes; energy crops such as switch grass *etc.* have also been used as potential feed stock for fuel ethanol (Mielenz, 2001; Kim *et al.*, 2002 and Kadam and McMillan, 2003). Lignocellulosic biomass considered for bioethanol production should have an appreciable percentage of cellulose and hemicellulose, when compared to lignin. Sunflower seed hull is such a source that can be utilized as efficient biomass owing to its high cellulose content (34.50%) (Dorrel and Vick, 1997). Sunflower is cultivated over a large area with production of more than 1.5 million metric tons in India generating large amounts of sunflower hulls during industrial processing of sunflower seeds. The hulls obtained as a waste product after de-hulling process have little commercial value and become a disposal problem because of their low bulk density (Sharma, 2004). Therefore, it is essential to develop efficient pretreatment methods to degrade this waste. Sunflower seed hulls have been exploited for ethanol production following alkali pre treatment and enzymatic saccharification (Sharma, 2004) omitting biological pretreatment strategies in combination, which would be a low cost strategy to the disposal problem of xylose rich sunflower seed hull. The present study was thus carried out to produce ethanol from xylose rich sunflower seed hull using biological treatment with local isolates of lignocellulosic fungi including enzymatic hydrolysis for saccharification followed by co fermentation using local bacterial and yeast isolates with the following objectives.

1. To analyze the proximate composition of sunflower seed hull.
2. To isolate and select local strains of white rot fungi for saccharification.
3. To hydrolyse the sunflower seed hull by biological pretreatment including enzymatic hydrolysis using local isolates of white rot fungi.
4. To produce ethanol from the fermentable sugars obtained during saccharification of sunflower seed hull using local and reference strains of *Zymomonas mobilis* and yeasts.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

A comprehensive review is mandatory in any research endeavor. This requires thorough effort on the part of the investigator to select relevant subject matter, to organize and to report it systematically. In the present investigation, an attempt has been made to study the production technology of bioethanol from sunflower seed hull. This chapter deals with a brief account of review of literature, which has direct and indirect bearing on the specific objectives of the investigation.

On account of limited global supply of oil, ethanol has emerged as an alternative for petroleum based liquid fuels. Now a days, its use in automobiles as an alternative fuel has attracted worldwide attention for its production on a large scale while maintaining the economic status of the country. In present state of energy crises, efforts are being made to reduce the dependence upon nonrenewable energy sources, one of which is fuel alcohol produced by fermentation of agricultural/agro industrial wastes and byproducts.

Bio-ethanol from lignocellulosic biomass is one of the important alternatives being considered due to the easy adaptability of this fuel to existing engines with an added advantage of higher octane rating than gasoline (Wheals *et al.*, 1999 and Grad, 2006). Lignocellulosic biomass is considered as the only foreseeable feasible and sustainable resource for renewable fuel

Various crop residues rich in lignocellulose, like wheat straw (Nigam, 2001), corn cob (Saracoglueken and Arslan, 2000), sunflower stalks (Sharma, 2004), water-hyacinth (Nigam, 2002) and sunflower seed hull (Sharma *et al.,* 2004) have been exploited for ethanol production. Sunflower hulls obtained as a waste product after de-hulling process have little commercial value and become a disposal problem because of their low bulk density. Therefore, it is essential to develop efficient pretreatment methods to degrade the lignin content in hulls making the cellulose available for hydrolysis.

2.1 Bioethanol

Bioethanol is an alcohol, made by, mostly from, carbohydrates produced in sugar or starch crops such as corn, sugarcane, or sweet sorghum**.** It is a water-free alcohol produced from the fermentation of sugar or converted starch. It is a colourless clear liquid with mild characteristic odour. It is volatile, miscible in both water and non-

polar solvents at ordinary conditions and has density of 0.792 g/cm^3 at 15.5 $^\circ$C (Purwadi, 2006 and Thomsen *et al.,* 2008).

With an ever increasing demand for energy and the fast depleting petroleum resources, globally there is an increased interest in alternative fuels; especially liquid transportation fuels (Lynd *et al*., 2008). In this scenario, renewable sources such as all petroleum-based fuels can be replaced by renewable biomass fuels such as bioethanol, bio-diesel, bio-hydrogen, etc., derived from sugarcane, corn, switch grass, algae, etc. According to Wheals *et al*. (1999) and Grad (2006), bioethanol from lignocellulosic biomass is one of the important alternatives being considered due to the easy adaptability of this fuel to existing engines and because this is a cleaner fuel with higher octane rating than gasoline.

Fermentation of sugars by yeast, the oldest synthetic chemical process used by man, is still of enormous importance for the preparation of ethyl alcohol. The sugars come from a variety of sources, mostly molasses from sugar cane or starch obtained from various grains. Though biomass itself is cheap, the cost of its processing is relatively higher. Technologies for biomass to ethanol conversion are also under preliminary stages of development.

2.2 Lignocellulose

Lignocellulose is the most abundant organic substance present in the biosphere. In nature, the annual production of biomass is estimated to be 10 to 50 $\times$ 10^9 tons (Chandel *et al*., 2010). Lignocellulosic biomass is being considered as the largest renewable energy resource all over the world and being promising and economically feasible carbohydrate source for the production of ethanol (Kim and Yun, 2006). Lignocellulose consists of three major components, constituting of cellulose, hemicellulose, and lignin which are closely associated with each other constituting the cellular complex of the vegetal biomass. Basically, cellulose forms a skeleton which is surrounded by hemicellulose and lignin. Cellulose is a high molecular weight linear homopolymer containing repeated units of cellobiose (two anhydrous glucose rings joined by β-1, 4 glycosidic linkages) (Klemm *et al*., 1998). The long-chain cellulose polymers are linked together by hydrogen bonds and Vander Wall's bonds, which cause the cellulose to be packed into micro fibrils (Apperley *et al*., 1998). Hemicellulose is a linear and branched heterogeneous polymer typically made up of five different sugars such as L-arabinose, D-galactose, D-glucose,

D-mannose, and D-xylose - as well as other components such as acetic, glucuronic and ferulic acids. When compared to cellulose, hemicelluloses differ by composition of sugar units, by the presence of shorter chains, by branching of main chain molecules etc. which makes the structure easier to hydrolyse than cellulose. Lignin is a very complex molecule constructed of phenyl propane units linked in a large three-dimensional structure. Three phenyl propionic alcohols exist as monomers of lignin: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is closely bound to cellulose and hemicellulose and its function is to provide rigidity and cohesion to the cell wall, to confer water impermeability to xylem vessels, and to form a physio-chemical barrier against microbial attack (Fengel and Wegener, 1989).

2.3 Lignocellulosic wastes

Various crop residues rich in lignocelluloses have been exploited for ethanol production. Cellulose rich agricultural raw materials such as barley straw (33.8%), sugar cane bagasse (40%), cotton stalk (33.7%), corn stalk (35%), rice straw (36%), sunflower seed hull (34.50%) *etc*. have been effectively used for bio ethanol production (Nigam, 2009).

Apart from these feed stocks, lignocellulosic biomass including industrial residues such as pulp and paper processing waste and municipal solid wastes; energy crops such as switch grass *etc*. have also been used as potential feed stocks for fuel ethanol (Mielenz, 2001; Kim *et al*., 2002 and Kadam and McMillan, 2003).

Cereal grains such as corn, barley, bajra, wheat, rice and tuber crops *viz*., potato, sweet potato and cassava have been evaluated and some, being extensively used for the production of fermented beverage. On dry weight basis, these grains contain around 60-75% (w/v) of starch hydrolysable to hexose with a significant weight increase (stochiometrically the starch to hexose ratio is 9:10) and these offer a good resource in fermentation processes (Jackman, 1987)

Pineapple wastes (containing 11.7% soluble sugars) was fermented for ethanol production by Bankoffi and Han (1990) and obtained 0.8% in 48 h.

Lynd *et al.* (1991) used waste paper and yard trash from US landfills as a substrate and obtained 400 billion liters of ethanol by microbial conversion of the sugar residues present in it. Sandhu *et al*. (1998) used rice straw as a substrate for ethanol production and

he obtained a maximum ethanol yield of 0.34 g/g based on sugar utilized after fermentation and fermentation efficiency of 66.87% was obtained.

Kim and Dale (2003) showed that wasted sorghum grains alone had potential to produce 1.4 GL of bioethanol along with sorghum straw it could produce 4.9 GL of bioethanol globally. Sharma *et al.* (2004) from his experiments on sunflower stalks and hulls concluded that the cellulose could be biotechnologically converted into bioethanol using *Saccharomyces cerevisiae* var. *ellipsoideus* under optimum condition of time 24 h, pH 5.0, 30 $^{\circ}$C with maximum ethanol yield 12.03 g/100 g sunflower stalk.

2.3.1 Sunflower seed hull

Sunflower Seed Hulls (SSH) are byproducts obtained by dehulling of sunflower seeds. Sunflower seeds contain about 20-30% hulls that are often removed before oil extraction due to deleterious effects on oil press, as they reduce the quality of oil. The hulls obtained as a waste product after de-hulling process have little commercial value and become a disposal problem because of their low bulk density (Sharma *et al.*, 2004). Therefore, it is essential to develop efficient pretreatment methods to degrade this waste.

SSH constitutes about 18-20% of the processed seeds. The main organic macronutrient of SSH are lipids, carbohydrates and proteins with highest percentage of the content being in the lignin and hemicellulose portion, with lignin comprising about 20-25% of the total dry weight (Dorrel and Vick, 1997). Reducing sugar is also an important part of seed coating, amounting to about 25%. Lipids and protein contents are around 5% and 4% respectively and 3% of the lipids are waxes (Cancalon, 1971). This chemical composition makes SSH an attractive material for microbial growth.

Ethanol production from sunflower seed hull hydrolysate has been evaluated using *Pichia stipitis* NRRLY-7124 by Okur and Saracoglu (2006) wherein, the hydrolysate prepared with 0.7 M H_2SO_4 was subjected to fermentation with *Pichia stipitis* NRRLY-7124. The highest ethanol accumulation, 9.66 g/l, and a yield of 0.41 g/g were achieved at the lowest tested flow rate, 2.28 v/v/minute, from 35 g reducing sugar per litre.

Sharma *et al.* (2004) conducted experiment on fermentation of enzymatic hydrolysate of sunflower hulls for ethanol production wherein, pretreated sunflower hulls hydrolyzed with *Trichoderma reesei* Rut C 30 cellulase showed 59.8% saccharifcation.

Enzymatic hydrolysate concentrated to 40 g/l reducing sugars was fermented with *Saccharomyces cerevisiae* var. *ellipsoideus* under optimum conditions of time (24 h), pH (5.0), temperature (30 $^{\circ}$C) and inoculum size, and it showed a maximum ethanol yield of 0.454 g/g. Ethanol production scaled up in 1 and 15 litre fermentors under optimum conditions revealed maximum ethanol yields of 0.449 and 0.446 g /g, respectively.

2.4 Lignocellulose degrading microorganisms

Microorganisms have evolved a capacity to modify and access lignocellulosic biomass to meet their metabolic needs. Itoh *et al*. (2003) used a variety of lignin-degrading white-rot fungi to treat wood chips prior to extracting lignin by an organosolvent method and demonstrated that improved ethanol yields were obtained from the solid fraction along with a 15% savings in electricity use.

A dozen fungal species *viz., Trichoderma viride, T. reesei, Fusarium solani*, *Aspergillus niger, A. terreus, Phanerocheate chrysosporium, Bacillus adusta* and *P. sanguineus* have been reported by Dashtban *et al*. (2009) which can effectively degrade lignocellulose to fermentable sugars.

Hatakka *et al*. (1983) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in 5 weeks. Similar conversion was obtained in the pretreatment by *Phanerochaete sordid* in 4 weeks (Ballesteros *et al*., 2006).

Antai (1985) exploited the use of actinomycetes for the degradation of lignocellulose wherein, he selected three *Streptomyces* strains, which were lignolytic, out of which one was most effective decomposer of lignocelluloses depleting 42% of lignin and 50% carbohydrate of lignocelluloses after 12 weeks of incubation. Similar works carried out in *Phanerochaete chrysosporium* by Bhattacharya *et al*. (1987) showed that the specific microorganism could degrade various lignolytic substances like jute sticks. Similar studies conducted by Donnely and Crawford (1988) on *S. viridosporus* showed that the organism when grown on lignocelluloses supplemented medium released p-coumaric acid and vanillic acid, which are intermediaries of lignin degradation.

Brown-rot fungal species *Coniophora puteana* and *Postia placenta* have been successfully used to improve glucose yields upon enzymatic saccharification of pine,

acting as a complete replacement for thermomechanical pretreatments (Ray *et al*., 2010). Recent studies on the effect of cellulolytic fungi on paddy straw conducted by Kadarmoidheen *et al*. (2012) showed that among the fungal isolates, *Trichoderma viride* was found to be the best in degrading cellulose of paddy straw which brought down the cellulose content of paddy straw of 34.82% to 16.12% followed by *Aspergillus niger* and *Fusarium oxysporum* which brought down the cellulose content to 21.30% and 23.32% respectively, in a period of 45 days of incubation.

2.5 Screening of lignocellulolytic fungi for enzymatic activity

Cellulase and laccase production with white rot fungi were measured by Zadrazil *et al.* (1993) using cellulose and sawdust, respectively, as the Carbon source. 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.

Koijam *et al.* (2000) studied cellulase producing ability of *Phanerochaete chrysosporium* strain and *Cladosporium* sp. BK-II on pretreated 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 °C. The optimized conditions of pH 5 (citrate buffer) had produced 2.8 and 2.4 Filter Paper Unit (FPU) activity by *P. chrysosporium* and *Cladosporium* sp.

Kumar *et al.* (2010) reported the production of higher amounts of FPU, endogluconase, beta-glucosidase activities in alkali pre-treated *Artimisea annua* by *Trichoderma citrinoviride* under growth conditions of 28 °C and 5.5 pH.

Naveenkumar and Thippeswamy (2013) conducted a study on isolation and screening of potential cellulolytic fungi from Areca nut husk waste and showed that highest cellulolytic activity was detected in ten isolates viz. *Aspergillus niger* (16 ± 0.6 mm), *Aspergillus terreus* (15 ± 0.5 mm), *Trichoderma viride* (13 ± 0.3 mm), *A. flavus* (12±0.3 mm), *Fusarium chlamydosporum* (10 ± 0.04 mm), *A. fumigatus* (9.5 ± 0.2 mm), *A. clavatus* (9.0 ± 0.05 mm), *Paecilomyces carneus* (8.0 ± 0.2 mm), *Penicillium chrysogenum* (7.2 ± 0.3 mm) and *A. wentii* (7.0 ± 0.2 mm) when compared to other fungi

whereas *Aspergillus*, *Penicillium*, *Heteroconium chaetospira* and *Staphylotrichum coccosporums* did not produce cellulase enzymes.

Kausar *et al.* (2011) conducted experiments on development of compatible lignocellulolytic fungal consortium for rapid composting of rice straw where in, a consortium of *Aspergillus niger* (F44) and *Trichoderma viride* (F26) were tested for in vitro biodegradation of rice straw. They concluded that the fungal consortium was able to decompose cellulose, hemicelluloses, lignin, and total carbon significantly ($p \pm 0.05$) over control. The C/N ratio was reduced to 19.5 from an initial value of 29.3 in three weeks of the biodegradation process, thus showing the potential of this method for use in large-scale composting of rice straw BK-II, respectively in eight days incubation.

Screening of wild-type fungal isolates for cellulolytic activity conducted by Doolotkeldieva and Bobusheva (2011) showed that, out of 17 strains of *Trichoderma*, *Aspergillus*, and *Penicillium* tested, *Penicillium* strain K-2-25 and *Trichoderma lignorium* strain T-22 had high cellulolytic activity. K-2-25 demonstrated the highest activity after 48 h of cultivation. T-22 also showed significant cellulolytic activity. *Penicillium* strain K-2-25 showed cellulolytic activity for 98 - 270 h during cultivation, and the amount of reduced glucose was 945 mg. *T. lignorium* T-22 was the second most active strain, with glucose reduction of 835 mg.

Ponnambalam *et al.* (2011) conducted experiments on qualitative display and measurement of enzyme activity of isolated cellulolytic bacteria where in, six bacterial isolates were comparatively analysed for effective production of cellulase enzyme and concluded that, among the six bacterial isolates, a bacterium F was found to be an effective producer both qualitatively and quantitatively. It showed a cellulolytic activity of 23.9 mg of glucose/ml/minutes and developed a clearing zone of 1.9 cm compared to next effective producer having activity of 22.9 glucose/ml/minutes and clearing zone of 1.7 cm.

Isolation and preliminary screening of paddy straw degrading thermophilic fungi conducted by Nidhi and Urmila (2013) showed that, out of 80 isolates which were screened on Remazol Brilliant Blue (RBB) dye and guaiacol, a total of 68 fungi decolorized RBB during the growth and only 19 fungi showed redness zone on guaiacol.

Isolation, identification and screening of xylanase and glucanase-producing microfungi from degrading wood was carried out by Adesina and Onilude (2013), where in, all the fungal strains were screened for their ability to produce both glucanase and xylanase. Among the twenty isolates, *Trichoderma reesei* showed highest glucanase activity of 10.18 U/ml while *Fusarium compactum* showed highest xylanase activity of 9.33 U/ml. Shankarappa and Geeta (2013) conducted an experiment on Optimization of growth conditions for production of saccharolytic enzymes by cellulolytic fungi where, five cellulolytic fungi were screened for saccharolytic enzyme production. They observed that *T. reesei* showed highest FPU activity (2.23 U/ml), CMCase activity (3.68 U/ml), glucosidase activity (1.82 IU/ml) on cellulose and xylanase activity (3.65 U/ml) using sugarcane bagasse as carbon source followed by *T. viridae* with 2.21 U/ml of FPU activity, 2.90 U/ml CMCase activity, 1.56 IU/ml glucosidase activity and 2.75 U/ml xylanase activity on cellulose as carbon source when compared to *Aspergillus sydowii, A. awamori* and *P. chrysosporium.*

2.6 Pretreatment methods

Production of ethanol from lignocellulosic biomass contains three major processes, including pretreatment, hydrolysis, and fermentation. According to Cara *et al.* (2008) pretreatment of the biomass is a key process in the bioconversion of biomass to ethanol from an economic point of view. Chang and Holtzapple (2000) identified pretreatment as an important prerequisite to alter the biomass macroscopic and microscopic size and structure as well as its submicroscopic structural and chemical composition to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars. Pretreatment technologies are usually classified into physical, chemical, physicochemical and biological pretreatment methods.

2.6.1 Alkali pretreatment

Alkali pretreatment refers to the application of alkaline solutions such as NaOH, lime or ammonia to remove lignin and a part of the hemicellulose, and efficiently increase the accessibility of enzyme to the cellulose. Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier *et al.*, 2005). Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated (Mcdonald *et al.*, 1983).

Kong *et al.* (1992) reported that alkali removes acetyl groups from hemicellulose (mainly xylan), thereby reducing the steric hindrance of hydrolytic enzymes and greatly enhancing carbohydrate digestibility. They concluded that the sugar yield in enzymatic hydrolysis is directly associated with acetyl group content.

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 24 h) step to solubilize the hemicellulose and prepare the substrate, then a second alkaline/oxidative step (1% NaOH and 0.3% H_2O_2 for 24 h) solubilizes and oxidizes the lignin into low polluting compounds. The overall process was carried out at a low temperature (25-40 $^{\circ}$C) using a low concentration of chemicals, resulting in relatively low cost and waste liquor containing only trace of pollutant derived from the lignin.

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 minutes steaming. They obtained cellulose enriched rice bran and reported this process to be an optimum pre-treatment method for rice bran. Saha and Cotta (2006) showed that diluted alkaline peroxide treatment (7.5% H_2O_2, v/v; pH 11.5; 35 $^{\circ}$C; 24 h) is an efficient method for pretreatment of rice hulls, resulting in almost complete conversion (96%) of rice hulls to sugars after enzymatic hydrolysis. No measurable furfural and hydroxymethylfurfural (HMF) were detected in the process, which made it more fermentable/digestible compared to dilute-acid pretreatment.

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 $^{\circ}$C, 15 psi for 1 hour was found efficient in releasing of maximum cellulose in paddy straw 0.56 g/g (56.0%), wheat straw 0.593 g/g (59.3%) and bagasse 0.695 g/g (69.5%).

Pretreatment of switch grass using aqueous ammonia or NaOH was investigated by Gupta and Lee (2010) as a means to enhance the enzymatic digestibility. To increase the effectiveness of pretreatment, H_2O_2 was supplemented with ammonia or alkali. The composition of pretreatment liquid indicated that hemicellulose was solubilized during alkaline treatment and existed either in the form of oligomers or as Lignin Carbohydrate Complex (LCC).

2.6.2 Microbial pretreatment

Microbial pretreatment involves the use of microorganisms (mainly fungi) to degrade lignin and hemicelluloses but leave the cellulose intact (Kumar and Wyman. 2009 and Sanchez, 2009). Effective degradation of lignocellulosic biomass could be achieved by the use of efficient cellulolytic micro-organisms which can produce effective cellulolytic enzymes during hydrolysis. Several studies have shown that white-rot fungi are the most effective microorganisms for the pretreatment of lingocellulosic wastes, such as wood chips, wheat straw and softwood (Hatakka, 1993). Kerem *et al.* (1992) studied lignin degradation in the solid state fermentation of cotton stalks by comparing *Pleurotus ostreatus* and *Phanerochaete chrysosporium* wherein, he observed 55% degradation of organic components of cotton stalks with *Phanerochaete chrysosporium* treatment while *Pleurotus ostreatus* treated showed 20% degradation after 30 days of incubation.

Taniguchi *et al.* (2005) evaluated biological pretreatment of rice straw using four white-rot fungi (*Phanerochaete chrysosporium, Trametes versicolor, Ceriporiopsis subvermispora,* and *Pleurotus ostreatus*) on the basis of quantitative and structural changes in the components of the pretreated rice straw as well as susceptibility to enzymatic hydrolysis. He concluded that pretreatment with *P. ostreatus* resulted in selective degradation of the lignin rather than the holocellulose component, and increased the susceptibility of rice straw to enzymatic hydrolysis. Sharma (2004) reported that pretreated sunflower hulls hydrolysed with *T. reesei* Rut C 30 cellulase showed 59.8% saccharification (40 g/l reducing sugars).

Manjunath and Geeta (2007) studied the relative efficiency of different fungal cultures like *Aspergillus foetidus, Aspergillus niger, Phanerochaete chrysosporium* and *Trichoderma viride* on the degradation and saccharification of paddy straw for bioethanol production. Among fungal cultures *P. chrysosporium* was found to be superior in releasing fermentable sugars, which yielded 534 mg/ml and accounts to 53.40%. Simultaneously, per cent loss in total solids was also maximum in substrates inoculated with *P. chrysosporium*, *T. viride* and *A. niger*, which accounts to 24.0, 19.5 and 17.5% loss in total solids as compared to uninoculated, control and that inoculated with *A. foetidus*. A comparative study of ethanol production from microbial pretreated agricultural residues by Seema *et al.* (2007) showed that highest reducing sugar yield was seen in *A. niger* and *A. awamori* treated wheat straws producing 18 and 20 mg each per gram of the substrate respectively after 5 days of incubation.

Zhang *et al*. (2007) screened 35 isolates of white-rot fungi for the biological pretreatment of bamboo for enzymatic saccharification and observed that *Echinodontium taxodii* 2538, *Trametes versicolor* G20 and *Coriolus versicolor* B1 were the most promising white-rot fungi for highly selective lignin degradation and significant improvement of enzymatic saccharification.

The biological pretreatment of sugarcane straw was evaluated by screening eight micro organisms, including bacteria and fungi, for an incubation time of 30 days. The fungus *Aspergillus terreus* was found as the most effective strain, resulting in 92% reduction in the lignin content (Singh *et al*., 2008). Kumar and Pushpa (2012) conducted experiments on microbial pretreatment and saccharification of agricultural residues like rice straw, wheat straw and bagasse (raw materials) using a combination of two different fungi *P.chrysosporium* (MTCC-787) and *A. awamori* (MTCC-6652) and reported that, when compared to individual fungal treatment, the combination of two fungi with high concentration resulted in high yield of sugars. Among all treatments, highest reducing sugar yield was observed in case of *A. awamori* and *P. chrysosporium* treated bagasse (62.7 mg/g).

A recent study conducted by Nazarpour *et al*. (2013) on hydrolysis of rubber wood treated with *C. subvermispora*, *T. versicolor* and mixed culture for 90 days showed that there was an increase sugar yield of about 27.67%, 16.23%, and 14.20%, respectively, as compared with untreated rubber wood (2.88%).

Pretreatment can be carried out by incorporating two or more methods. Sawada *et al.* (1995) reported that maximum saccharification (74.8%) can be obtained when particle size of beech wood was 32-60 mesh and (treated or inoculated sample with *Phanerochaete chrysosporium* for 28 days followed by steam explosion at steam temperature of 215 $^{\circ}$C and steaming time of 6.5 minutes.

Vidya *et al.* (2006) studied the effect of irradiation on rice and wheat by gamma rays and electron beam. It was observed that irradiated samples were efficiently degraded by *P. chrysosporium.* The lignin biodegradation was monitored by the loss in dry weight and klason lignin and release of radioactivity from ^{14}C labeled lignin. The optimum dose required was 750 GY for gamma rays and 2500 GY for electron beam.

2.7 Saccharification by enzymes

Karunanandaa *et al.* (1992) studied the biodegradability of crop residues colonized by white-rot fungi wherein they studied the effect of 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 digestibility. From the experiments, they concluded that *Cyathus stercoreus* improved the *in vitro* cellulose digestibility of maize and rice straw by 37 and 45%, respectively and a minimum dry matter loss of 3.3% was recorded. The wild and mutant strains of *Phanerochaete* was shown to degraded both hemicellulose and cellulose. Mononmani and Sreekantiah, (1987) conducted experiment on enzymatic saccharification of sugarcane bagasse where they recorded a cellulose conversion yield of 90% in the enzymatic saccharification of 8% alkali-treated sugar-cane bagasse by using a mixture of cellulases (1.0 FPU/g substrate) from *Aspergillus ustus* and *Trichoderma viride*.

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 °C. It was found that the enzyme activity had a broad pH range between 3 to 9 and 41.2% of the original activity which was retained after heat treatment at 90 °C for 15 minutes.

Wen *et al*. (2004) conducted experiment for optimizing enzyme loading to hydrolyse lignocellulosic part of animal manure into fermentable sugars. The use of 13 FPU cellulose g-1 substrate and 5 IU ß-galactosidase/g substrate at 46 °C and pH 4.8 with Tween-80 at 2% resulted in 20% increase in glucose yield. A substrate concentration of 50 g/l was shown to favor glucose yield. Maximum glucose yield obtained was 11.32 g 100 g/l manure, corresponding to 40% cellulose conversion. 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/l cellulose. However, when the enzyme loading was reduced to 5 FPU, less than 27% 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 cellulose (less than 12 h) and low, FPU/g

(within 72 h) enzyme loadings. Qing *et al.* (2010) reported that xylose, xylan, and xylo-oligomers dramatically decrease conversion rates and yields of saccharification. Xylooligomers were shown to be 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 /l. At a higher concentration of 12.5 mg /l, xylooligomers lowered initial hydrolysis rates of Avicel by 82% and the final hydrolysis yield by 38%.

Jeya *et al.* (2010) reported that *Agaricus arvensis* a new basidiomycetous fungus produced the highest endoglucanase (EG), cellobiohydrolase (CBH) and glucosidase (BGL) activities of 0.3, 3.2 and 8 U/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 (293 mg/g substrate) was obtained at an enzyme concentration of 65 FPU/g substrate after optimization of the hydrolysis parameters.

2.8 Ethanol production

The saccharified biomass is used for fermentation by several microorganisms. A wide variety of microorganisms are known to produce ethanol as a product of carbohydrate fermentation. Microorganisms which have received attention in industrial alcohol production include a wide range of yeasts, some molds and a number of specialized bacteria. According to Stewart *et al.* (1983), the microorganism being employed in the production of fermentation ethanol should possess the following important characteristics; 1) rapid and relevant carbohydrate fermentation ability, 2) appropriate flocculation and sedimentation characteristics, 3) genetic stability, 4) osmotolerance (*i.e.,* the ability to ferment carbohydrate solutions), 5) ethanol tolerance and the ability to produce elevated concentration of ethanol, 6) high cell viability for repeated recycling and 7) temperature tolerance. In addition to these characteristics a good candidate should be able to use a wide range of sugar sources. Based on these criteria a number of yeasts and bacteria that produce significant quantities of ethanol have been studied.

2.8.1 Yeasts

Yeasts are the most suitable microorganisms for ethanol production. They can grow on variety of sugars with high substrate and ethanol tolerance. Gong *et al.* (1983)

tested 20 species of *Candida* 21 strains of *Saccharomyces* and 8 strains of *Schizosaccharomyces* for their ability to ferment D-xylose. Xylitol was produced in the range of 10 -15% by *Candida* strains with arabitol as the second major product. Ethanol was the major product with most of the *Candida* stains. In agricultural wastes, pentose sugars are present in larger proportion, which cannot be neglected if we want to increase the yield of ethanol and complete substrate utilization.

Abbi (1996) showed that *Pichia tannophilus*, *Candida shehatae*, *P. stipitis* and *Kluveromyces marxianus* are pentose utilizing yeasts. Other yeast investigated for their xylose-fermenting ability include *Brettanomyces*, *Clavispora*, *Schizosaccharomyces*, other species of *Candida* viz., *C. tenius*, C. *tropicalis*, *C. utilis*, *C. blankii*, *C. friedrichii*, *C. solani* and *C. parapsilosis* and species of *Debaromyces viz.*, *D. nepalensis* and *D. polymorpha*.

Srivastava *et al.* (1997) used three isolates of *Saccharomyces cerevisiae* for ethanol production from guava pulp and obtained maximum ethanol yield of 5.8 per cent w/v) co-immobilized culture of *S. cerevisiae* and *Candida shehatae* which could convert glucose and xylose simultaneously to ethanol productivity of 0.48 g/g of total sugar.

Ramanathan (2000) obtained an ethanol yield of 421 kg^{-1} of feed stock by fermentation of root crops namely cassava, potato, yam and sweet potato. Hari Krishna *et al.* (2001) carried out simultaneous saccharification and fermentation (SSF) to produce ethanol from lignocellulosic wastes (sugar cane leaves) using *Trichoderma reesei* cellulase and yeast cells. *Kluyveromyces fragilis* NCIM 3358 performed better than *Saccharomyces cerevisiae* NRRL-Y-132 in SSF process and resulted in high yields of ethanol (2.5-3.5% w/v) compared to *S. cerevisiae* (2.0-2.5% w/v). Increased ethanol yields were obtained when the cellulase was supplemented with ß- glucosidase.

Sreenath and Jeffries (2000) screened more than 40 yeast strains of *Pichia stipitis* and *Candida shehatae* 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 with *Pichia stipitis* and 34.0 ± 1.67 g/l with *Candida shehatae.*

Sreenath *et al.* (2001) investigated the ethanol production from alfalfa fibres using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and

fermentation (SSF) with and without liquid hot water (LHW) pretreatment. *Candida shehatae* FPL-702 produced 5 and 6.4 g/l ethanol with a yield of 0.25 and 0.16 g ethanol/g sugar respectively by SHF and SSF respectively from alfalfa fibre without pretreatment. With LHW pretreatment using SSF, *C. shehatae* FPL-702 produced 18 g/l ethanol, a yield of 0.45 g ethanol /g sugar from cellulosic solids. Using SHF, it produced 9.6 g/l ethanol, a yield of 0.47 g ethanol/g from raffinate. However, the soluble extract fraction containing hemicelluloses was poorly fermented in both SHF and SSF due to the presence of inhibitors. Addition of dilute acid during LHW pretreatment of alfalfa fibre resulted in fractions that were poorly saccharified and fermented. These results show that unpretreated alfalfa fibre produced a lower ethanol yield. Although LHW pretreatment can increase ethanol production from raffinate fibre fractions, it did not increase production from the hemicellulosic and pectin fractions.

Sharma *et al*. (2004) obtained maximum ethanol yield of 0.454 g/g (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 °C in 24 h time.

Studies by Hahn-Hägerdahl *et al*. (2007) reported that after hydrolysis of lignocellulosic biomass, the produced pentose sugars (mainly D-xylose and L-arabinose) create problem in yeast alcohol fermentation because yeast strains lack the xylose utilization enzymes (mainly xylose reductase and xylitol dehydrogenase). According to Jeffries (1982) pentose utilizing yeasts could convert xylose into xylitol in addition to ethanol.

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

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

2.8.2 Bacteria

Several bacteria, including *Clostridium* sp, *Zymomonas mobilis*, *Enterobacter arerogenes*, *Escherichia coli, Klebsiella oxytoca* have been considered as ethanologenic microbes. However, *Z. mobilis*, a Gram-negative bacterium, is considered as alternative organism to yeast in large-scale fuel ethanol production.

Comparative laboratory and pilot-scale studies on kinetics of batch fermentation of *Z. mobilis* versus a variety of yeast by have indicated the suitability of *Z. mobilis* over yeasts due to the following: its higher sugar uptake and ethanol yield, its lower biomass production, its higher ethanol tolerance, it does not require controlled addition of oxygen during the fermentation, and its amenability to genetic manipulations (Gunasekaran and Raj, 1999). However compared to the yeast, its utilizable substrate range is restricted to glucose, fructose, and sucrose. Also under anaerobic conditions, *Z. mobilis* produces by-products such as acetoin, glycerol, acetate, and lactate, which result in reduced production of ethanol from glucose (Gunasekaran and Raj, 1999). It is one of the few facultative anaerobic bacteria which metabolize glucose and fructose via the Entner-Deudoroff (ED) pathway, which is usually present in aerobic microorganisms. Glucose, fructose and sucrose are the only three carbon sources that *Z. mobilis* is able to utilize.

Simultaneous saccharification and fermentation (SSF) of cassava starch using *Z. mobilis* or *S. uvarum* ATCC 26602 was investigated by Poosaran *et al.,* (1985). They reported that *Z. mobilis* fermented the substrate considerably faster than *S. uvarum*, completing the fermentation in 20 h yielding 95% of the theoretical yield, while *S. uvarum* required a period of 33 h to complete fermentation with a yield of 90% of the theoretical value. Economics of fuel ethanol production from dry milled corn starch using *Zymomonas mobilis* was studied by Krishnan *et al*., (2000) and obtained ethanol yield of 24 g/l from a 15 million gallon per year capacity ethanol plant. Four strains of *Zymomonas mobilis* were screened by Panesar *et al.* (2006) for their ability to produce ethanol from molasses medium at pH 6.

Studies conducted by Manjunath and Geetha (2007) on the effectiveness of fungal pretreatment of bagasse for ethanol production by *Z. mobilis* and *Saccharomyces cerevisiae* yielded maximum ethanol of 614.4 mg/l and 480 mg/l, respectively followed by *Candida shehatae* (398.40 mg/l).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present study was undertaken in the Department of Agricultural Microbiology, College of Agriculture, University of Agricultural Sciences, Raichur on production of bio ethanol from sunflower seed hull. The materials used and the methods employed in the investigation are outlined below in detail.

3.1 General laboratory procedures

3.1.1 Glassware cleaning

For the entire laboratory work during the investigation, Borosil glasswares were used. New glasswares were washed with water and detergent followed by thorough rinsing with tap water and distilled water. Used glasswares were immersed in a cleaning solution (Potassium dichromate, 80 g; Distilled water, 300 ml; Sulphuric acid, 400 ml) overnight. On removal from the cleaning solution, the glasswares were thoroughly cleaned with water and detergent and finally rinsed in distilled water.

3.1.2 Sterilization

The glassware consisting of Petri dishes, pipettes, test tubes etc. were sterilized by placing them in hot air oven maintained at 180 $^{\circ}$C for two hours. Solid and broth media used in the experiment were sterilized by autoclaving at 121 $^{\circ}$C for 20 minutes.

3.2 Substrate

The sunflower seed hulls used in the present study were removed from sunflower seeds, procured from Main Agriculture Research Station, UAS, Raichur. The hulls were oven dried at 70 $^{\circ}$C to constant weight. Oven dried hulls then ground to 40 mesh were utilized in the study.

3.3 Substrate analysis

3.3.1 Moisture content

One gram of ground sunflower hull (W_1) was dried in hot air oven at 105 $^{\circ}$C for one h to its constant weight (W_2). The loss in weight gave moisture content.

$$\text{Moisture (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

3.3.2 Estimation of reducing sugars

Sugars by virtue of the presence of free or potentially free aldehyde (-CHO) or keto (>C=O) group in them, reduce certain metallic ions such as copper, bismuth, mercury, iron and silver in alkaline solution. The change can be quantified based on the amount of sugar present. Estimation of reducing sugars by Dinitrosalicylic acid method (DNSA) (Miller, 1959) is a quick method for the estimation of reducing sugars especially when the sample size is large. The aldehyde group of glucose converts 3, 5-dinitrosalicylic acid (DNSA) to 3-amino-5-nitrosalicylic acid, which is the reduced form of DNSA. The formation of 3-amino-5-nitrosalicylic acid results in a change in the amount of light absorbed, at wavelength 540 nm. The absorbance measured using a spectrophotometer is directly proportional to the amount of reducing sugar.

3.3.2.1 Reagents (Appendix A)

1. DNSA
2. Rochelle salt solution
3. Stock solution
4. Working standard solution

3.3.2.2 Preparation of standard graph

Ten to hundred µg of working standard solution was pipette out into a series of labelled test tubes and the volume was made up to 3 ml. A test tube with only 3ml of distilled water was maintained as blank. To all the test tubes 3 ml of DNSA reagent was added and mixed well. All tubes *viz.,* samples, standards and blank were kept on boiling water bath for 5 minutes to this reaction mixture, one ml of 40% Rochelle salt solution was added when it was still warm. Then the tubes were cooled and the absorbance in terms of optical density of the standards and sample were read at 540 nm using UV-Visible Spectrophotometer. The standard graph was plotted keeping glucose as standard and the amount of reducing sugars were estimated from the standard graph.

3.3.2.3 Extraction of plant sample

Two to five gram of sunflower seed hull cut into small pieces were plunged immediately in boiling alcohol and extracted. The extract was cooled, passed through double muslin layer cloth and grinded thoroughly with alcohol. The extract was then passed through muslin cloth and the extraction was repeated twice. The filtrate was pooled and filtered through Whatman no. 1 filter and the volume was made to a known value using ethanol. The filtrate was stored in refrigerator at 4 °C until further use.

3.3.2.4 Sample estimation

Initial volume of the sample was measured and noted down. One ml of the alcohol extract was heated in a water bath for 20 minutes to evaporate alcohol. The alcohol extract was diluted to 5 ml with distilled water. Known volumes (0.5, 1.5, 2.4 ml) were pipette out and diluted to 1 ml. To all the test tubes, 3 ml of DNSA reagent was added and mixed well. All tubes *viz.,* samples, standards and blank were kept on boiling water bath for 5 minutes. Following this, one ml of 40% Rochelle salt solution was added when the reaction mixture was still warm. The tubes were cooled and the absorbance in terms of optical density of the standards and sample were read at 540 nm using UV-Visible spectrophotometer. The standard graph was plotted with glucose as standard and the reducing sugar content was estimated from the standard graph.

3.3.3 Estimation of total sugars

Sugars are generally classified as reducing and non reducing sugars to estimate total sugars, the non reducing sugar is first converted to reducing sugars by acid hydrolysis and the reducing sugars are estimated by DNSA method.

3.3.3.1 Reagents (Appendix A)

1. 1 N HCl
2. 0.1N HCl
3. 1 N NaOH

3.3.3.2 Procedure

One ml of representative sample from each treatment and replication was taken in test tubes. One ml of 1 N HCl was added to each tube and placed them in boiling water for 15-20 minutes. Tubes were cooled and one drop of phenolphthalein indicator was added to each tube. To each tube 1 N NaOH was added till pink color appeared followed

by addition of 0.1 N HCl till pink color disappeared. Finally the contents in each tube were mixed well and volume was made up to 5 ml with distilled water. 0.5 ml representative sample taken from these tubes and total sugars was estimated by following DNSA method.

3.3.4 Estimation of protein

The protein content of culture broth was determined by Lowry *et al.* (1951) method with bovine serum albumin as standard. The aromatic amino acids present in the protein react with phosphomolybdo-phosphotungstate reagent to produce a blue coloured complex at 660 nm. The depth of the colour produced is proportional to the aromatic amino acids present in a given sample.

3.3.4.1 Reagents (Appendix A and B)

1. Alkaline copper reagent
2. Stock protein standard solution
3. Working standard solution
4. Folin-Ciocalteau reagent (FCR): 1 N

3.3.4.2 Procedure

Working standard solutions (20 -100 µg) were pipette out into labelled test tubes and the volumes were made up to 1 ml. A test tube with only 1 ml of distilled water was maintained as blank. To all the test tubes, 5 ml of alkaline copper reagent was added, mixed thoroughly and kept at room temperature for 10 minutes. 0.5 ml of 1 N FCR reagent was added to all test tubes and kept in dark for 30 minutes. Suitable aliquots of the alcohol extracted samples were treated as explained above. Absorbance of the standards and the sample were read against reagent blank at 750 nm. The amount of protein (mg/g) was calculated by referring to the standard graph prepared with bovine serum albumin as standard.

3.4 Isolation of local strains of white rot fungi from compost pits located in UAS, Raichur

3.4.1 Collection of compost samples

Compost samples were collected from mesophilic and thermophilic stages of compost from compost pits located in Raichur and Gulbarga.

Representative samples of farm yard manure from pits of 6 to 9 inch depth were taken and mixed well. Out of this sample, a known quantity of manure was added to 100 ml sterile water blanks and shaken well for 15 minutes. Then serial dilutions were made and 0.5 ml volumes of 10^5 dilutions were pipette on to Mandels's media (Appendix C) containing CMC (Carboxy Methyl Cellulose) as sole source of carbon and were incubated at 30 °C for three days. Fungi were isolated from mixed isolates from each plate and sub cultured on PDA (Appendix C).

3.5 Procurement of reference cultures

3.5.1 Fungal cultures

Table 1. Fungal cultures used for pretreatment and saccharification of SSH

Fungal cultures used in the study	**Source**
Phanerochaete chrysosporium NCIM 119	National Collection of Industrial Microorganisms (NCIM), Pune
Trichoderma viride NCIM 1195	NCIM, Pune
Aspergillus niger NCIM 793	NCIM, Pune
Aspergillus awamori PSF 10	UAS, Raichur

3.5.2 Maintenance of cultures

The fungal cultures used for microbiological pretreatment of the substrates were maintained on PDA.

3.5.3 Morphological characterization of fungal isolates

The morphological characters such as colour and type of hyphae and types of spore were studied following micro technique and were compared with reference strains.

3.6. Screening of fungal strains

3.6.1 Primary screening for cellulolytic fungi

From various isolates, cellulolytic fungi were screened using qualitative methods for determining lingo cellulolytic activity.

3.6.1.1 Cellulose agar clearance

Cellulolytic basal medium (CBM) was prepared by incorporating 4% (w/v) cellulose and 1.6% agar (Appendix C). The test fungus was inoculated on CBM plates and was incubated at 25 $^{\circ}$C in darkness for ten days. Formation of clear zone around the colony was taken as positive for the test.

3.6.1.2 Dye staining of Carboxymethyl Cellulose Agar (CMC agar)

CBM medium was prepared supplementing it with 2% (w/v) low viscosity Carboxymethyl cellulose and 1.6% (w/v) agar. The test fungi were inoculated on CMC plates at 25 $^{\circ}$C in darkness. When the colony diameter was 30mm, the agar plates were stained with 2% (w/v) aqueous Congo red for 15 minutes followed by destaining the plates with 1M NaCl for 15 minutes. Formation of yellow opaque area against a red color was taken as a positive test

3.6.1.3 Esculin plus Iron agar test

CBM medium supplemented with 0.5% (w/v) esculin, and 1.6% (w/v) agar was prepared. One ml of sterile 2% (w/v) aqueous ferric sulphate solution was added for each 100 ml CBM prepared. Test fungi were inoculated on esculin plus iron agar plates at 25 $^{\circ}$C in darkness. The plates were examined daily over 5 days. Formation of a black color around the colonies was taken as a positive test.

3.6.1.4 Dye staining with xylan agar

Xylanolysis Basal Medium (XBM) was prepared incorporating 4% (w/v) xylan and 1.8% (w/v) agar (Appendix C). Test fungi were inoculated on XBM plates and were

incubated at 25 °C in darkness. When the colony diameter was 30 mm, the agar plates were stained with 2% (w/v) aqueous Congo red for 15 minutes followed by destaining the plates with 1M NaCl for 15 minutes. Formation of yellow opaque area against a red color was taken as a positive test.

3.6.1.5 Bavendamm test

Lignolytic Basal Medium (LBM) was prepared supplementing 1.6% (w/v) agar (Appendix C). One ml of 20% (w/v) aqueous glucose and one ml of 1% (w/v) aqueous Tannic Acid solution separately sterilized were added. Test fungi were inoculated on XBM plates and were incubated at 25 °C in darkness and were examined for ten days. Formation of brown oxidation zone around colonies was taken as a positive test

3.6.2 Secondary screening

3.6.2.1 CMCase assay

3.6.2.1.1 Extraction of crude enzyme

The Carboxy methyl cellulose (CMC) broth containing 0.2% (w/v) CMC as a sole carbon/energy source was used for enzyme production. One ml of fungal culture was inoculated into the 50ml of the sterile CMC broth. The cultures were incubated in shaking for 7 days at room temperature (25 °C - 30 °C). Cultures were harvested by centrifugation at 6000 x g for 15 minute and the cell free culture supernatants were used as crude enzyme source.

3.6.2.1.2 Reagents (Appendix A and B)

1. Carboxymethyl cellulose 1% (w/v)
2. Citrate buffer of pH 4.8 (0.1 M)
3. DNSA reagent

3.6.2.1.3 Preparation of Standard curve

In eight test tubes, different volumes of glucose standard solution were pipette out. To each test tube 3 ml of DNSA reagent was added followed by addition of one ml of 40% Rochelle salt solution the tubes were cooled. The absorbance was read at 540 nm against blank using spectrophotometer.

3.6.2.1.4 Enzyme estimation

One ml of appropriately diluted enzyme solution was incubated for 30 minutes with 1 ml of 1.0% CMC (substrate) and 1 ml of 1.0 M citrate buffer of pH 4.8 at 50 °C. The reaction was terminated by adding 3 ml of DNSA reagent and the mixture was boiled for 5 minutes and cooled followed by addition of one ml of 40% Rochelle salt solution the tubes were cooled The absorbance was read at 540 nm. This absorbance was translated by plotting against standard curve to get μ mol of glucose to calculate units of enzyme activity.

$$\text{Enzyme Activity (IU/ml/minutes)} = \frac{\text{Absorbance of enzyme solution x standard factor}}{\text{Time of incubation (minutes)}}$$

Whereas,

$$\text{Standard factor} = \frac{\text{Concentration } (\mu M/ml) \text{ of standard}}{\text{Absorbance at 540 nm}}$$

One unit of enzyme activity is defined as the amount of glucose (μM) released per ml enzyme solution per minute. The concentration of glucose (mg/ml) was plotted against respective absorbance values at 540 nm to draw glucose standard curve.

3.6.2.2 Xylanase activity

3.6.2.2.1 Extraction of crude enzyme

XBM containing 0.8% birch wood xylan as sole carbon was used for enzyme production. One ml of fungal culture with a spore count of 10^4 was inoculated into 50 ml of sterile XBM broth. The cultures were incubated in shaking (at 120 rpm) for six days. Samples were taken after 72 h; the cell free supernatant was recovered by centrifuging at 12000 rpm at 40 °C for 15 minutes.

3.6.2.2.2 Reagents (Appendix A and C)

1. Xylose 0.1% (w/v)
2. Citrate Phosphate buffer of pH 4.8 (0.1 M)
3. DNSA reagent

4. Rochelle salt solution (40%)

3.6.2.2.3 Standard curve

In eight test tubes, different volumes of xylose standard solution were pipette out. To each test tube 3 ml of DNSA reagent was added followed by addition of one ml of 40% Rochelle salt solution the tubes were cooled. The absorbance was read at 540 nm against blank using spectrophotometer. The concentration of xylose expressed in mg/ml was plotted against respective absorbance values at 540 nm to draw xylose standard curve

3.6.2.2.4 Enzyme estimation

One ml of appropriately diluted enzyme was taken in a test tube. One ml of citrate phosphate buffer was added and mixed and the mixture was incubated at 50 °C for 30 minutes. The reaction was terminated by adding 3 ml of DNSA reagent and the mixture was boiled for 5 minutes and cooled followed by addition of one ml of 40% Rochelle salt solution the tubes were cooled The absorbance was read at 540 nm. This absorbance was translated by plotting against standard curve to get µ mol of glucose to calculate units of enzyme activity.

$$\text{Enzyme Activity (IU/ml/minutes)} = \frac{\text{Absorbance of enzyme solution x standard factor}}{\text{Time of incubation (minutes)}}$$

3.6.3 Cellulase enzyme production studies:

Cellulase enzyme production was studied by FPU (Filter Paper Unit) assay (Ghosh 1987)

3.6.3.1 Lignocellulolytic Enzyme production

Cellulase production by *T. viride, A. awamori, A. niger* and *P. chrysosporium* were estimated under submerged batch conditions using basal medium supplemented with one percent cellulose. One hundred ml basal medium was dispensed into each of 250 ml Erlenmeyer flasks containing 1.0 g cellulosic substrate (pure cellulose). The flasks were autoclaved at 15 psi for 20 minutes, cooled at room temperature and inoculated with 10 ml fungal culture. The inoculated flasks were incubated at 28 °C on a rotary shaker (150 rpm) for 12 days.

3.6.3.2 Preparation of reagents (Appendix A)

1. Citrate buffer of pH 4.8 (0.1 M)
2. DNSA reagent

3.6.3.3 Procedure

One ml of 0.05 M sodium citrate having pH 4.8 was added to a test tube. To this, 0.5 ml of enzyme and one strip of Whatman No.1 filter paper (weighing 50 mg) were added. (The filter paper strip has to be pushed down whenever it winds up the test tube). The tube along with blank was kept in a water bath at 50 °C for 60 minutes. After 60 minutes, the tubes were taken out and DNSA method was followed further to account for the amount of sugars released by the Cellulase. Three ml of DNSA reagent was added to the tubes. The tubes were kept on a vigorously boiling water bath for 5 minutes. One ml of 40% Rochelle salt solution was added when the tubes were still warm. After cooling, 1.5 ml of distilled water was added to equalize the reaction mixture to a volume of 7 ml. The contents were mixed well and the filter paper pulp was allowed to settle down. After the filter paper had settled down, the color formed was read at 540 nm using spectrophotometer. The amount of sugars in terms of glucose was known by referring to standard graph of glucose. One unit of enzyme activity is the one mole of reducing sugar in terms of glucose released per minute. Enzyme activity was measured in IU/ ml.

The isolates identified as potential lignocellulose degraders through primary and secondary screening were sub cultured and maintained on PDA until further use.

3.7 Isolation of ethanological bacteria

Twenty five ml of sugarcane juice was inoculated into RM broth (Rogers *et al.,* 1982) using 4% sucrose instead of 1% glucose with 100 mg/l of antifungal agent (Appendix C). After incubation at room temperature for 24 h, 5 ml Schiff's reagent was added to 10 ml aliquot. Samples which produced purple color, characteristic of *Zymomonas sp.* were plated on RM broth tubes and incubated at 30 °C for 24 h. Isolates which released gas upon shaking were screened for biochemical characterization.

3.7.1 Procurement of reference strains

Table 2. Microbial cultures used for ethanol from SSH

Cultures used in the study	**Source**
Pichia stipitis NCIM 3498	NCIM, Pune
Zymomonas mobilis NCIM 2915	NCIM, Pune
Saccharomyces cerevisiae NCIM 3095	NCIM, Pune

3.7.2 Maintenance of cultures

The yeast cultures were maintained on MGYP (Malt Extract Glucose Yeast Extract Peptone) medium (NCIM catalogue) and *Zymomonas mobilis* was maintained on the selective medium (Gunasekaran and Raj, 1999).

3.8 Identification and characterization of bacteria isolate

Identification and characterization of bacterial cultures were performed based on cultural, morphological and biochemical tests following the standard protocol given by Aneja (2003).

3.8.1 Cultural characteristics

The cultural characteristics such as colony shape, edge, elevation colour etc. were recorded following standard procedure.

3.8.2 Morphological characteristics

3.8.2.1 Gram's staining

The isolates were smeared and heat fixed on glass slides. The slides were flooded with crystal violet for one minute. Excess dye was washed gently in tap water and the slide was wiped dry against a paper towel. The smear was treated with Gram's iodine for one minute by washing with iodine, then adding more iodine and leaving it on the smear until the minute is over. The slides were again washed with tap water and drained carefully. The slides were washed with 95% alcohol for 30 seconds, washed with tap water and counterstained with 0.25% Safranin for 30 seconds. The slide was washed, drained, blotted, and examined under oil.

3.8.2.2 Motility test

A drop of fresh culture suspension of the bacterial isolate was prepared separately in nutrient broth and was placed on a clean cavity slide on which a cover slip was placed. The motility was then observed under phase contrast fluorescent microscope.

3.8.3 Biochemical tests

3.8.3.1 Catalase test (Blazevic and Ederer, 1975)

Nutrient agar slants were inoculated with test organisms and were incubated at 30 $^{\circ}$C for 24 h. After incubation the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for production of gas bubbles. The occurrence of gas bubbles was scored positive for catalase activity.

3.8.3.2 Hydrogen sulphide production (Cowan and Steel, 1970)

Bacterial isolates were inoculated to test tubes containing 5 ml of sterile medium and incubated at room temperature at 28 $^{\circ}$C. The test tubes were observed for H_2S production. The formation of black ring in the medium was taken as positive for H_2S production.

3.8.3.3 Methyl red test (Seeley and Vandemark, 1981)

Test culture containing MR-VP broth (Appendix C) were sterilized and inoculated with the test cultures. The tubes were incubated at 28 ± 2 °C for 48 h. After incubation five drops of methyl red indicator was added to each tube and gently shaken. The production of red color was taken as positive and production of yellow color was taken as negative for the test.

3.8.3.4 Voger Proskauer test (Seeley and Vandemark, 1981)

To the pre-sterilized tubes containing MR-VP broth test cultures were inoculated. The tubes were incubated for 48 h at 37 °C. After incubation ten drops of Baritt's reagent A was added and gently shaken followed by addition of ten drops of Baritt's reagent B. The development of rose color in the broth was taken as positive for the test.

3.8.3.5 Carbohydrate (sugar) fermentation

The ability of bacterium to ferment various carbohydrates using glucose, fructose, sucrose and lactose glucose were determined by growing the isolate in liquid standard medium containing 1% (w/v) of the particular carbohydrate as described (Obire, 2005).

Durham-tubes were inverted into the culture tubes for gas collection. Incubation was at 30 °C for 24 h and uninoculated broths were used as control.

3.9 Isolation of ethanological yeasts

One ml of aliquot of grape juice was taken aseptically into test tubes. These samples were serially diluted 10-fold in sterilized distilled water. One ml of the serially diluted sediment was inoculated by streaking on plates of standard media (media were supplemented with chloramphenicol (0.05 mg/l) and incubated at 28 °C for 24 h (Okafor, 1975).

3.10 Identification of yeast isolates

Colonies suspected to be yeast were isolated from the spread plates and purified by streaking on freshly prepared media and incubated for 1 day at 30 °C. Isolates from such fresh plates were subjected to the following tests for the characterization; morphology and surface characteristics.

3.10.1 Cultural characteristics

The cultural characteristics such as colony shape, edge, elevation colour etc. were recorded.

3.10.2 Biochemical characteristics

3.10.2.1 Cyclohexamide test

Cyclohexamide test was conducted to differentiate *Saccharomyces* from *Candida.* The cultures were isolated on Sabouraud Dextrose Agar incorporating 10 µg/ml of cyclohexamide and incubated 30 °C for 10 days.

3.10.2.2 Flocculation test

The isolates were inoculated in 10 ml of YPG broth and incubated at 30 °C for 3 days. After incubation, tubes were agitated to observe the flocculation formed (Thais *et al*., 2006).

3.10.2.3 Fermentative capacity test

The ability of yeast to ferment various carbohydrates using glucose, fructose, sucrose and lactose were determined by growing the isolate in liquid standard medium

containing 1% (w/v) of the particular carbohydrate as described (Obire, 2005). Durham-tubes were inverted into the culture tubes for gas collection. Incubation was carried out at 30 °C for 24 h and uninoculated broths were used as control.

3.11 Evaluation of yeast and bacterial isolate for ethanol fermentation

To evaluate ethanol fermentation by different strains of *Pichia stipitis*, *Saccharomyces cerevisiae* and *Z. mobilis*, 100 ml of rich medium (RM) containing 5 g/L of glucose was places into 100 ml of Erlenmeyer flasks. After inoculation with 5% (v/v) seed culture, the cultures were incubated at 30 °C without agitation for 24 h. The yield of ethanol was used to assess the fermentation performance. The ethanol yield was determined by colorimetry. The isolates identified as potential ethanological agents through primary and secondary screening and comparison with reference strains were sub cultured and maintained on MYPG slants (Appendix C) until further use.

3.12 Sodium hydroxide pre treatment

Sodium hydroxide pre treatment of the sunflower seed hulls were carried out according to the procedure given by Sharma (2004). To the flask containing 2.5 g of ground sunflower hulls; 50 ml of sodium hydroxide solution (1.5%) was added. The flasks were autoclaved at 120 °C for 1.5 h. The solids recovered after filtration were repeatedly washed with distilled water until pH 7.0 and was oven dried at 60 °C.

3.13 Saccharification

3.13.1 Saccharification with fungal consortia

Ten gram / l of powdered hull was taken in conical flask containing 200 ml of Mandle's medium. The plugged conical flasks were sterilized at 15lbs for 20 minutes. Each flask was inoculated with 4-5 discs (1 cm diameter) of each fungus separately. For consortia, each flask was inoculated with 4-5 discs of different fungi. These flasks were incubated at room temperature for 5 days on an orbital shaker at 180 rpm. After five days, mycelium was separated by filtration through Whatman No.1 filter paper. The same procedure was followed for alkali pre treated SSH.

3.13.2 Saccharification with enzyme

The residue of SSH obtained after pre treatment were enzymatically saccharified using crude enzyme filtrate of *T. viride, A. niger, A. awamori* and *P. chrysosporium* by the method of Sharma (2004).

3.13.2.1 Procedure

One gram of pretreated substrate was separately mixed with 25 FPU/g of crude enzyme obtained from different efficient cultures. The total volume was made up to 25 ml using citrate buffer (0.1 M, pH 4.8). Saccharification was carried out under shaking conditions (150 rpm). Samples were withdrawn after intervals of 12 h, centrifuged at 5000 rpm for 20 minutes and the supernatant was analyzed for reducing sugars by the method of Miller (1959).

3.14 Ethanol production

3.14.1 Cultures

The bioconversion of hydrosylates into ethanol was carried out using efficient strains identified through secondary screening and their reference strains. .

3.14.2 Inoculum Preparation

3.14.2.1 *Saccharomyces cerevisiae*

The inoculum of *S.cerevisiae* was prepared in GYE broth (Appendix C). A loopful of 24 hours yeast culture was transferred to 100 ml broth in 250 ml Erlenmeyer flasks and incubated at 30 °C for 24 hours on rotary shaker at 150 rpm. A cell count of 2.1 x 10^8 cells/ml was obtained.

3.14.2.2 *Pichia stipitis*

A loopfull of 36 hours old *Pichia stipitis* culture was aseptically inoculated into Erlenmeyer flasks each containing 100 ml of GYE broth and was incubated for 36 h at 30 °C under shake flask conditions at 150 rpm until a cell count of 2.1 x 10^8 cells/ ml was obtained.

3.14.2.3 *Zymomonas mobilis*

A loopful of 24 hour old yeast culture was transferred to 100 ml broth in 250 ml Erlenmeyer flasks and incubated at 30 °C for 9 h on rotary shaker at 150 rpm until a cell count of 2.1 x 10^8 cells/ ml was obtained.

3.14.3 Ethanol fermentation

3.14.3.1 Procedure

Flasks containing 50 ml of the fermentation medium containing fungal pretreated and enzyme pretreated hydrosylates amended with glucose (40 g), yeast extract (3 g), malt extract (3g) peptone (5g) per litre were inoculated with 2% (v/v) each of *Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis* and their respective reference strains. Fermentation was carried out at 30^0C and ethanol was estimated at every 12 h interval up to 60 h. The flasks were kept in aerobic condition for 24 h and after 24 h, anaerobic condition was created by plugging flasks with rubber cork, making provision for trapping carbon dioxide.

The fermentation efficiency (%) was calculated as

$$\frac{\text{Ethanol yield obtained}}{\text{Theoretical maximum ethanol yield from sugar substrate}} \times 100$$

(Theoretical maximum ethanol yield from sugar substrate= 0.51 g ethanol per gram sugar)

3.14.3.2 Ethanol Estimation

The ethanol was estimated colorimetrically as described by Caputi *et al.* (1968).

3.14.3.2.1 Reagents (Appendix A and B)

1. Potassium dichromate ($K_2Cr_2O_7$) 0.23 N
2. Preparation of stock solution : It was prepared by mixing 12.6 ml of analytical grade ethanol (789 mg/ml) with little amount of distilled water and making up the volume to 100 ml using distilled water, this gives 100 mg ethanol/ml.

3.14.3.2.2 Procedure

Three ml of representative sample from each treatment was transferred to 250 ml round bottom flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74-75 $^{\circ}$C The distillate was collected in 25 ml of 0.23 N $K_2Cr_2O_7$ reagents, which was kept at the receiving end. The distillate containing alcohol was collected till total volume of 45 ml was obtained. Similarly, standard (20-100 mg ethanol) were mixed with 25 ml of $K_2Cr_2O_7$ separately. The distillate containing alcohol

was collected till total volume of 45 ml was obtained. These samples and standards were kept in water bath at 600 for 20 minutes, cooled and volume was made up to 50 ml with distilled water. The optical density of the distillate was measured at 600 nm using UV-Visible spectrophotometer. The standard curve was plotted considering the concentration against absorbance. The residual sugar was also estimated by Nelson and Somogyi's method (Nelson, 1944).

3.15 Statistical analysis of data

The results obtained were analysed statistically using Completely Randomized Design (CRD) with 3 replications per treatment. The data was subjected to one way analysis of variance (ANOVA) following the principles outlined by Snedecor & Cochran (1969).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

In the present investigation of bioethanol production from sunflower seed hull by lignocellulosic fungi, microbial pretreatment was conducted to increase the accessibility of cellulose. Further, the saccharification of cellulose was achieved employing cellulolytic fungi and their crude enzyme extracts followed by fermentation of derived sugars by ethanological yeasts and bacteria to produce bio ethanol. The results obtained from these experiments are presented in this chapter.

4.1 Initial composition of agro-residues

The sunflower seed hulls (Plate 1) utilized in the present investigation were characterized for its proximate composition. The moisture content was estimated to be 8.5%. The hulls were analyzed with 1.26 mg/g total sugar, 0.27 mg/g reducing sugar and 4.50% protein content on dry weight basis (Table 3).

4.2 Isolation of lignocellulolytic fungi from compost pits

A total of 50 lignocellulolytic fungi were isolated from compost pits at different stages of composting on Mandel's media supplemented with 1% CMC and were temporarily designated as LCDF (Ligno Cellulose Degrading Fungi) 1-50. All the isolates were then purified under sterile working conditions and the pure cultures were stored at 5 $^{\circ}$C for further use. The details of the location and number of lignocellulolytic fungi isolated from different compost samples are mentioned in Table 4 which shows that compost at its mesophilic stage, harbored more lignocellulolytic fungi than that in thermophilic stage.

A total of 42 lignocellulolytic fungi were isolated from compost pits, at various stages of composting, located at UAS, Raichur. Seven lignocellulolytic fungi were isolated from mesophilic stage of indigenous compost pits, while three were isolated from

Plate 1. Sunflower seed hull used in the study

thermophilic stages located in Raichur. Fifteen lignocellulolytic fungi were isolated from mesophilic stage from enriched compost located in Raichur and four were isolated from mesophilic stage. Ten and three lignocellulolytic fungi were isolated from vermi compost pits located in UAS, Raichur. Five and three lignocellulolytic fungi were isolated from maturation and thermophilic stages respectively from indigenous composts located in Gulbarga (Table 4).

Table 3. Proximate composition of sunflower seed hull

Parameters	Values
Moisture content (% wet basis)	8.5
Reducing sugars (mg/g)	027
Total sugars (mg/g)	1.20
Protein (% of dry mass)	4.50

Table 4. Lignocellulolytic fungi isolated from different compost samples

Name of district	Type of compost	Stage of compost	No of fungal sp. isolated
Raichur	Indigenous Compost	Mesophilic	7
		Thermophilic	3
	Vermi Compost	Mesophilic	10
		Thermophilic	3
	Enriched Compost	Mesophilic	15
		Thermophilic	4
Gulbarga	Indigenous Compost	Maturation	5
		Thermophilic	3
Total			**50**

4.3 Morphological Characterization of fungal isolates

All the fungi isolated were subjected to morphological characterization for identification (Plate 2) (Table 5). Out of the 50 isolates, fifteen fungi showed light green and yellowish colonies scattered in Czapeck Dox Agar media. There were no concentric rings observed in the colony and microscopic examination revealed globose conidia with septate, hyaline hyphae. These fungi were identified as *Trichoderma viride* based on the morphological characterization suggested by Sha *et al.* (2012). Fifteen isolates which developed white with dusty yellow sporulating colonies with dark brown gloves conidia and hyaline septate hyphae were identified as *Aspergillus niger* based on the morphological characterization as suggested by Sharma and Pandey (2010).

Ten isolates which developed white colonies with a brownish sporulating area with gloves conidia and septate hyphae were identified as *Aspergillus awamori* predicated basedon the morphological characterization suggested by Perrone *et al.*, 2011. Eight isolates were identified as *Phanerochaete chrysosporium* based on colony characters suggested by Gilman (1944), which appeared to be white, mycelium appressed to agar surface, which felt like texture becoming loosely cottony with age. They exhibited thick-walled chlamydospores with septate hyphae. Other organisms identified were *Trichoderma harzianum* and *A. flavus* based on colony characters.

Table 5. Morphological identification of fungal isolates from different types of compost

Name of isolate	Cultural characteristics (as on CDA)	Type of conidia	Type of hyphae	Tentative identity	Strain No.
LCDF 1	Single green and yellowish concentric ring with a cluster of yellow conidia around the point of inoculum. Some white conidia appear to grow scarcely towards the edges.	Conidia globose to subglobose	Septate, hyaline hyphae	*T. harzianum*	
LCDF2	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 1
LCDF 3	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*Aspergillus niger*	CAN 1
LCDF 4	White with brownish sporulating area			*Aspegillus awamori*	CAA 1
LCDF 5	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age. Chlamydospores abundant, spherical to oblong, terminal,	Chlamydospores, thick-walled spores	Septate hyphae	*Phanerochaete chrysosporium*	CPC 1
LCDF 48	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 2
LCDF 7	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 2
LCDF 8	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 3

LCDF 9	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age. Chlamydospores abundant, spherical to oblong, terminal,	Chlamydospores, thick-walled spores	Septate hyphae	*P.chrysosporium*	CPC 2
LCDF 10	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age. Chlamydospores abundant, spherical to oblong, terminal,	Chlamydospores, thick-walled spores	Septate hyphae	*P. chrysosporium*	CPC 3
LCDF 11	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate*, hyaline* hyphae	*T. viride*	CTV 3
LCDF 12	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate*, hyaline* hyphae	*T. viride*	CTV 4
LCDF 13	White with brownish sporulating area			*A.awamori*	CAA 2
LCDF 14	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 4
LCDF 15	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 5
LCDF 16	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 6

LCDF 17	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age. Chlamydospores abundant, spherical to oblong, terminal,	Chlamydospores, thick-walled spores	Septate hyphae	*P.chrysosporium*	CPC 4
LCDF 18	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 7
LCDF 19	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 8
LCDF 20	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 9
LCDF 21	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age.	Chlamydospores, thick-walled spores	Septate hyphae	*P.chrysosporium*	CPC 5
LCDF 22	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 5
LCDF 23	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 6
LCDF 24	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age.	Chlamydospores, thick-walled spores	Septate hyphae	*P.chrysosporium*	CPC 6
LCDF 25	Single green and yellowish concentric ring with a cluster of yellow conidia around the point of inoculum. Some white conidia appear to grow scarcely towards the edges.	Conidia globose to subglobose	Septate, hyaline hyphae	*T. harzianum*	
LCDF 26	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely	Chlamydospores, thick-walled spores	Septate hyphae	*P.chrysosporium*	CPC 7

	cottony with age.				
LCDF 27	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, *hyaline* Hyphae	*T. viride*	CTV 10
LCDF 28	granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age	Chlamydospores are terminal or intercalary, hyaline, smooth or rough-walled	Hyaline and coarsely roughened	*A. flavus*	
LCDF 29	White with brownish sporulating area			*A. awamori*	CAA 3
LCDF 30	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, *hyaline* Hyphae	*T. viride*	CTV 11
LCDF 31	White with brownish sporulating area			*A.awamori*	CAA 4
LCDF 32	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, *hyaline* Hyphae	*T. viride*	CTV 12
LCDF 33	White with brownish sporulating area			*A.awamori*	CAA 5
LCDF 34	Colonies white, mycelium appressed to agar surface, felt like texture becoming loosely cottony with age.	Chlamydospores, thick-walled spores	Septate hyphae	*P.chrysosporium*	CPC 8
LCDF 35	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 13
LCDF 36	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	Septate, hyaline hyphae	*T. viride*	CTV 14

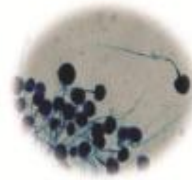
LCDF 37	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*Aspergillus niger*	CAN 7
LCDF 38	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*Aspergillus niger*	CAN 8
LCDF 39	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*Aspergillus niger*	CAN 9
LCDF 40	White with brownish sporulating area			*A.awamori*	CAA 7
LCDF 41	White with brownish sporulating area			*A.awamori*	CAA 8
LCDF 42	Light green and yellowish conidia scattered throughout the plate. There were no concentric rings formed	Globose	septate, hyaline hyphae	*T. viride*	CTV 15
LCDF 43	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 10
LCDF 44	White with brownish sporulating area			*A. awamori*	CAA 9
LCDF 45	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 11
LCDF 46	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 12
LCDF 47	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 13
LCDF 48	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 14
LCDF 49	White with brownish sporulating area			*A. awamori*	CAA 10
LCDF 50	White with dusty yellow sporulating area	Globose, dark brown	Hyaline septate hyphae	*A. niger*	CAN 15

LCDF: Ligno Cellulose Degrading Fungi, CAN: Compost *Aspergillus niger*, CAA: Compost *Aspergillus awamori*, CTV: Compost *Trichoderma viride*, CPC: Compost *Phanerochaete chrysosporium.*

Trichoderma viride on Czapeck Dox

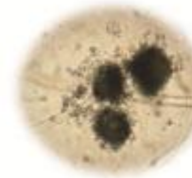
Hyphal characteristics of *T. viride* (100 X)

Aspergillus niger on Czapeck Dox Agar



Conidiospores of *A. niger*

Aspergillus awamori on Czapeck Dox



Conidiospores of *A. awamori* (100 X)

Phanerochaete chrysosporium on Czapeck Dox Agar

Chlamydospores of *P. chrysosporium* (100 X)

Plate 2. Lignocellulolytic fungal cultures isolated from compost pits

4.4 Screening of fungal strains

4.4.1 Primary screening

The fungal isolates were selected by plate assay to evaluate the lignocellulolytic properties like cellulolytic, xylanolytic, and polyphenoloxidase activity. Cellulolytic activities of the fungal isolates were determined by the following technique *viz.,* cellulose agar clearance, dye staining of CMC and colouration on Esculin plus Iron agar (Plate 3) (Table 6). The diameter of clearing zone for each isolate is shown in Table 6. Out of 48 isolates, 38 produced clear zone. Among isolates of *Trichodema viride*, isolate CTV 5 recorded maximum clearance zone (26 mm) when compared to reference strain NCIM 1195 (22 mm). Among isolates of *Aspergillus niger*, isolate CAN 3 recorded maximum clearance (7 mm) when compared to reference strain NCIM 793 (6 mm). Isolate CPC 3 recorded maximum clearance zone (4 mm) comparable to reference strain NCIM 1197 (4 mm) among isolates of *Phanerochaete chrysosporium*. Among isolates of *Aspergillus awamori*, isolate CAA 1 produced a clearance zone (3.2 mm) comparable to reference strain PSF (3.2 mm).

Screening of cellulolytic fungal isolate for cellulose was performed based on the diameter of the clearing zone surrounding the colony of Carboxy Methyl Cellulose (CMC) medium (Plate 3). Among the 48 isolates, 38 isolates have shown CMCase activity. Among isolates of *T. viride*, isolate CTV 5 recorded maximum clearance zone (10mm) when compared to reference strain NCIM 1195 (9mm). Among isolates of *A. niger*, isolate CAN 3 recorded maximum clearance (9mm) when compared to reference strain NCIM 793 (7mm). Among isolates of *P. chrysosporium*, isolate CPC 3 recorded maximum clearance zone (3mm) comparable to reference strain NCIM 1197 (4mm). Among isolates of *A. awamori*, isolate CAA 1 produced a solubulization zone (4.2 mm) comparable to reference strain PSF 10 (4.4 mm). All the 48 fungal isolates showed positive results for glucosidase production.

The ability of fungal isolates to degrade xylan; was studied by Xylanolytic assay. All 48 isolates produced clearance zone, when treated with Congo red. However isolate CTV 5 showed the highest clearance zone (38mm) among isolates of *Trichodema viride*, when compared to reference strain NCIM 1195 (35mm). Isolate CAN 3 recorded

maximum clearance (13mm) which was comparable to reference strain NCIM 793 (15mm) among isolates of *Aspergillus niger*. Among isolates of *Phanerochaete chrysosporium*, isolate CPC 3 recorded maximum clearance zone (5mm) comparable to reference strain NCIM 1197 (4mm). Isolate CAA 1 of *A. awamori* showed the highest clearance zone, compared to reference strain PSF (3mm).

All the 48 isolates were tested for polyphenol oxidase activity by Bavendamm test, on lignolytic basal medium wherein, all the fungi developed positive result.

4.4.2 Secondary screening

All the lignocellulolytic fungi were further subjected to secondary screening by analyzing their enzymatic activity *viz.,* CMCase, Xylanase and FPU assay. Among all the isolates of *Trichoderma viride*, maximum CMCase activity (0.72 U/ml) was obtained from isolate CTV 5 which was significantly higher than all the isolates, including the reference strain, NCIM1195. Similar pattern was noted for xylanase activity (Table 7) where in, CTV 5 showed maximum xylanase activity (5.52 U/ml) which was significantly higher than all other isolates. However, for FPU assay, CTV 15, CTV 1, CTV 2 and reference strain NCIM1195 showed activity on par with CTV 5 (2.16 U/ml).

In screening the individual isolates of *Aspergillus niger* for their relative enzymatic activity (Table 8). Isolate CAN 3 showed highest Xylanase activity (3.29 U/ml) when compared to reference strain NCIM 793 (3.05 U/ml). CTV 2, CTV 3 and CTV 15 also showed activity on par with CAN 3. The isolate also recorded maximum FPU activity (1.78 U/ml) when compared to reference strain NCIM 793 (1.78 U/ml). CTV 2, CTV 3 and CTV 14 also showed xylanase activity on par with CAN 3. However, the reference strain NCIM 793 recorded maximum CMCase activity (0.63 U/ml) when compared to CAN 3 (0.62 U/ml) which was on par with reference strain.

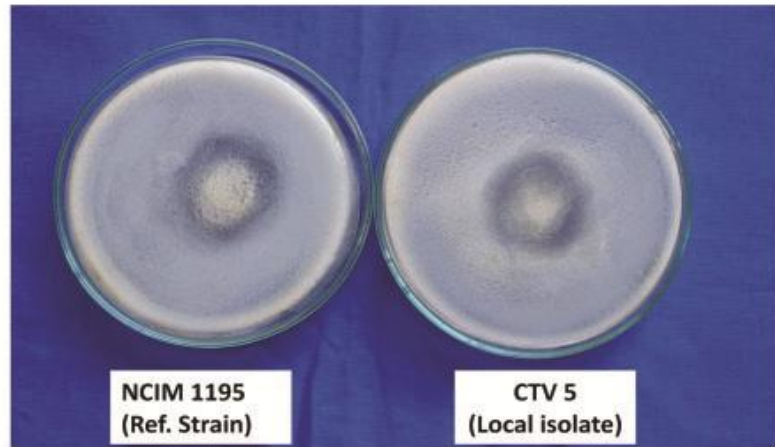


Plate 3 a. ***Trichoderma viride*** **showing cellulose clearance on cellulolytic basal medium**

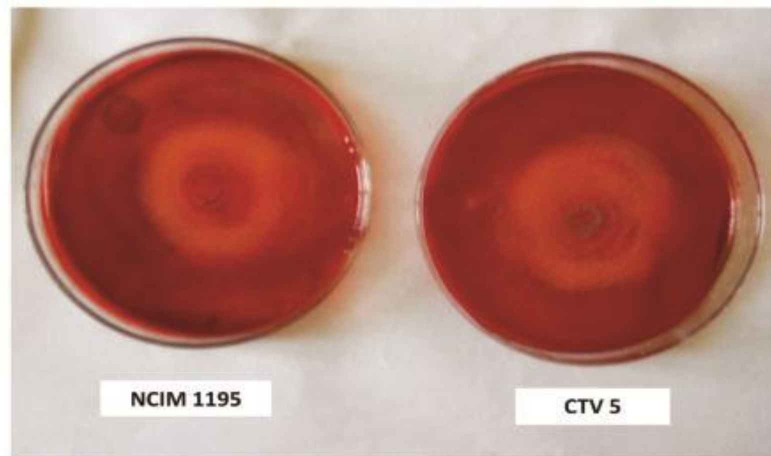


Plate 3 b. ***T. viride*** **showing CMCase activity on cellulolytic basal medium**

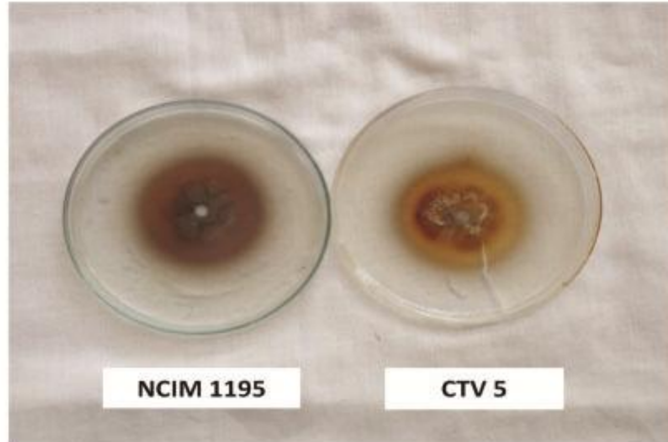


Plate 3 c. *T. viride* showing β glucosidase activity on cellulolytic basal medium

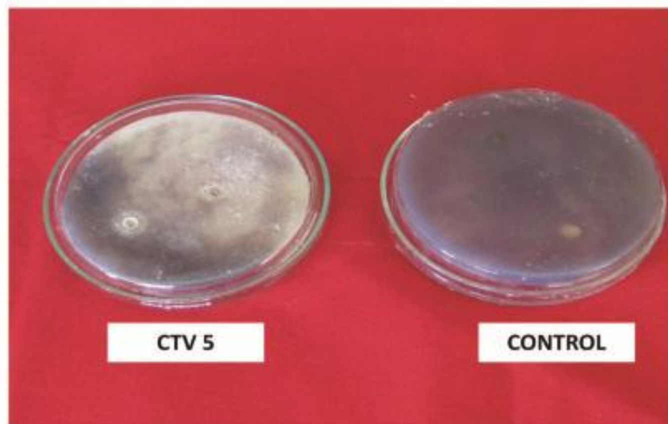


Plate 3 d. *T. viride* showing lignolytic activity activity on lignolytic basal medium

Table 6. Lignocellulolytic activity of selected fungal strains

Lignocellulosic Strain	Area of clearance (mm)			Esculin agar test	Bavendamm test
	Cellulose (1%)	Carboxy Methyl Cellulose(1%)	Xylan (1%)		
CTV 1	12.5	7.4	28.5	+	+
CTV2	11.6	6.3	25.4	+	+
CTV 3	9.8	8.7	23.3	+	+
CTV 4	11.5	7.1	34.5	+	+
CTV 5	25.2	10.1	38.1	+	+
CTV 6	16.4	9.2	13.2	+	+
CTV 7	21.3	9.2	25.4	+	+
CTV 8	19.7	7.2	15.3	+	+
CTV 9	16.5	4.7	30.9	+	+
CTV 10	13.2	7.3	25.5	+	+
CTV 11	12.4	7.4	22.9	+	+
CTV 12	12.5	3.2	17.8	+	+
CTV 13	7.9	2.3	19.8	+	+
CTV 14	5.2	2.3	12.7	+	+
CTV 15	14.3	7.4	25.8	+	+
NCIM 1195	22.2	8.7	34.6	+	+
CAN 1	4.7	2.7	12.4	+	+
CAN 2	6.5	1.9	10.1	+	+
CAN 3	6.7	9.2	15.9	+	+
CAN 4	5.9	2.1	13.8	+	+
CAN 5	4.4	4.2	12.7	+	+
CAN 6	4.1	4.3	9.8	+	+
CAN 7	4.2	3.4	8.0	+	+
CAN 8	3.4	3.0	8.0	+	+
CAN 9	3.6	3.0	12.0	+	+
CAN 10	3.7	2.7	13.0	+	+

Screening of *Phanerochaete chrysosporium* for enzymatic activity revealed CPC 3 with CMCase, xylanase and FPU activity of 0.70, 11.76 and 1.36 U/ml, respectively, which was on par with reference strain NCIM 1197 which recorded maximum CMCase, xylanase and FPU activity of 0.71, 14.86 and 1.37 U/ml respectively (Table 9).

Among all the isolates of *Aspergillus awamori*, CAA 1 recorded maximum CMCase xylanase and FPU activity of 0.64, 0.84 and 1.78 U / ml, respectively, which was significantly higher than all the isolates, including the reference strain, PSF which recorded a CMCase, xylanase and FPU activity of 0.57, 0.64 and 1.63 U/ml respectively (Table 10).

4.5 Isolation and identification of *Zymomonas mobilis*

According to the different morphology of the colony and fermentation of different carbohydrates, ten strains of bacteria were isolated from sugarcane juice and three were identified as *Zymomonas mobilis.* From the Gram staining examination, 9 isolates (SCB 1 -9) were identified as gram-negative and 1 isolate (SCB 10) was identified as gram positive. 7 out of 10 isolates were identifies as bacilli and the remaining as cocci. All the isolates were shown to develope characteristic cream to white colony on RM media (Table 11).

Motility tests revealed 6 out of 10 isolates positive for the test. The biochemical tests revealed all the isolates positive for catalase and vogs prosskaeur test and negative for methyl red and oxidase test. The ability of the bacterial isolates to ferment various carbohydrates is presented in table 15. All the bacterial isolates were able to ferment glucose fructose and sucrose while only 7 isolates could ferment lactose. The isolates which particularly showed positive result to motility test and negative to lactose utilization (SCB1, SCB 5 and SCB 6) were identified as *Zymomonas mobilis* in comparison with the reference strain NCIM 2915 and were tentatively coded as ZMI (1-3) (Plate 4).

Table 7. Secondary screening of *Trichoderma viride* isolates for lignocellulosic enzyme activities

Isolate	CMCase activity (U/ml)	Xylanase (U/ml)	FPU (U/ml)
CTV 1	0.60	2.33	1.97
CTV2	0.61	2.51	1.93
CTV 3	0.58	2.07	1.76
CTV 4	0.64	3.24	1.90
CTV 5	0.72	5.52	2.16
CTV 6	0.59	3.19	1.76
CTV 7	0.62	4.19	1.74
CTV 8	0.50	4.27	1.54
CTV 9	0.64	3.83	2.14
CTV 10	0.64	4.00	1.98
CTV 11	0.63	4.12	1.84
CTV 12	0.55	4.32	1.66
CTV 13	0.60	3.19	1.72
CTV 14	0.59	4.74	1.83
CTV 15	0.66	4.71	2.08
NCIM 1195	0.64	5.32	2.06
S.Em±	0.008	0.15	0.06
CD at 1%	0.03	0.57	0.25

Table 8. Secondary screening of *Aspergillus niger* isolates for lignocellulosic enzyme activities

Isolate	CMCase activity (U/ml)	Xylanase (U/ml)	FPU (U/ml)
CAN 1	0.60	3.05	1.57
CAN 2	0.60	3.28	1.67
CAN 3	0.62	3.29	1.78
CAN 4	0.62	2.12	1.54
CAN 5	0.62	2.19	1.47
CAN 6	0.62	2.44	1.60
CAN 7	0.63	2.62	1.42
CAN 8	0.59	2.03	1.44
CAN 9	0.55	2.06	1.52
CAN 10	0.56	2.01	1.54
CAN 11	0.60	2.18	1.63
CAN 12	0.52	1.91	1.57
CAN 13	0.51	1.86	1.41
CAN 14	0.49	1.88	1.64
CAN 15	0.51	3.16	1.56
NCIM 793	0.63	3.05	1.66
S.Em±	0.01	0.04	0.03
CD at 1%	0.04	0.17	0.14

Table 9. Secondary screening of *Phanerochaete chrysosporium* isolates for lignocellulosic enzyme activities

Isolate	CMCase activity (U/ml)	Xylanase(U/ml)	FPU(U/ml)
CPC 1	0.63	11.76	1.31
CPC 2	0.63	10.06	1.31
CPC 3	0.70	12.18	1.36
CPC 4	0.68	9.25	1.11
CPC 5	0.63	7.27	1.22
CPC 6	0.55	8.23	1.13
CPC 7	0.67	8.11	1.13
NCIM 1197	0.71	14.86	1.37
S.Em±	0.01	0.50	0.02
CD at 1%	0.05	1.06	0.08

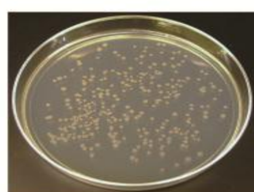
Table 10. Secondary screening of *Aspergillus awamori* isolates for lignocellulosic enzyme activities

Isolate	CMCase activity (U/ml)	Xylanase (U/ml)	FPU (U/ml)
CAA 1	0.64	0.84	1.78
CAA 2	0.58	0.67	1.67
CAA 3	0.51	0.74	1.57
CAA 4	0.51	0.76	1.54
CAA 5	0.56	0.64	1.47
CAA 6	0.52	0.65	1.60
CAA 7	0.53	0.63	1.42
CAA 8	0.57	0.72	1.44
CAA 9	0.53	0.67	1.52
CAA 10	0.44	0.76	1.54
PSF 10	0.57	0.64	1.63
S.Em±	0.02	0.02	0.02
CD at 1%	0.06	0.06	0.06

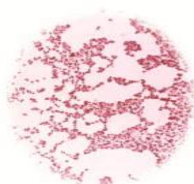
4.6 Isolation and identification of yeast

A total of 15 yeasts were isolated from grape juice were identified based on the colony and cell morphology, including the growth of isolates in liquid medium as well as the assimilation and fermentation of carbohydrates. All the yeast isolates fermented glucose, fructose and sucrose, but not lactose. Further all the isolates on Sabouraud's dextrose agar developed either a smooth glabrous white to cream coloured colony or a cream mucoid slimy colony (Table 12).

The biochemical tests revealed 5 isolates positive for flocculation test and three isolates for cyclohexamide test. The isolates which particularly showed negative result to flocculation test, cyclohexamide test and lactose utilization test (YI 1, YI 3, YI 3, YI 8, YI 9, YI 10, YI 12 and YI 13) were identified as *Saccharomyces sp.* in comparison with the reference strain NCIM 3095 and were coded as SCI (1-8) (Plate 4).



Zymomonas mobilis on Rich medium



Cells of *Z. mobilis* at 100 X

Saccharomyces cerevisiae on MGYP agar

Cells of *S. cerevisiae* at 100 X

Plate 4. Ethanological bacteria and yeast isolated for fermentation

Table 11. Morphological and biochemical characterization of bacterial isolates

Isolate code	Morphological characteristics			Biochemical Characteristics						Carbon source utilization			
	Colour of colony	Shape	Gram Reaction	C	MR	VP	H_2S	M	O	Sucrose	fructose	glucose	lactose
SCB 1	Cream/white	Rod	-	+	-	+	-	+	-	+	+	+	-
SCB 2	Cream/white	Cocci	-	+	-	+	-	-	-	+	+	+	+
SCB 3	Cream/white	Cocci	-	+	-	+	-	-	-	+	+	+	+
SCB 4	Cream/white	Rod	-	+	-	+	-	-	-	+	+	+	+
SCB 5	Cream/white	Rod	-	+	-	+	-	+	-	+	+	+	-
SCB 6	Cream/white	Rod	-	+	-	+	-	+	-	+	+	+	-
SCB 7	Cream/white	Rod	-	+	-	+	-	+	-	+	+	+	+
SCB 8	Cream/white	Rod	-	+	-	+	-	+	-	+	+	+	+
SCB 9	Cream/white	Rod	+	+	-	+	-	+	-	+	+	+	-
SCB 10	Cream/white	Cocci	+	+	-	+	-	-	-	+	+	+	+
NCIM 2915	Cream/white	Rod	-	+	-	+	-	+	-	+	+	+	-

C = Catalase, MR: = Methyl Red, VP = Vogs Prosskaeur, M = Motility, O = oxidase

Table 12. Morphological and Biochemical characterization of *Saccharomyces cerevisiae*

Isolatecode	Colony character on SDA	Flocculation	Cyclohexamide test	Fermentative capacity test			
				Glucose	Fructose	Sucrose	Lactose
YI 1	Smooth glabrous white to cream colored colony	-	-	+	+	+	-
YI 2	Cream mucoid slimy colony	+	-	+	+	+	+
YI 3	Smooth glabrous white to cream colored colony	-	-	+	+	-	-
YI 4	Cream mucoid slimy colony	+	-	+	+	+	+
YI 5	Smooth glabrous white to cream coloured colony	-	-	+	+	+	-
YI 6	Smooth glabrous white to cream coloured colony	-	+	+	+	+	+
YI 7	Smooth glabrous white to cream colored colony	+	+	+	+	+	+
YI 8	Smooth glabrous white to cream coloured colony	-	-	+	+	+	-
YI 9	Smooth glabrous white to cream coloured colony	-	-	+	+	+	-
YI 10	Smooth glabrous white to cream coloured colony	-	-	+	+	+	-
YI 11	Smooth glabrous white to cream colored colony	-	+	+	+	+	+
YI2	Smooth glabrous white to cream coloured colony	-	-	+	+	+	-
YI 13	Smooth glabrous white to cream coloured colony	-	-	+	+	+	-
YI 14	Cream mucoid slimy colony	+	-	+	+	+	+
YI 15	Cream mucoid slimy colony	+	-	+	+	+	+
NCIM 3095	Smooth glabrous white to cream colored colony	-	-	+	+	-	-

4.7 Evaluation of selected yeast and bacteria strains for ethanol production

The *Saccharomyces* and *Zymomonas* isolated were selected to evaluate the ethanol fermentation capacity in RM medium containing 5% of glucose. There was a significant difference in ethanol produced by the different microorganisms. *Z. mobilis* reference strain NCIM 2915 produced 0.13 mg/g of ethanol with a conversion efficiency of 50.98% which was on par with isolate ZMI 3, which produced the highest concentration of ethanol (0.16 mg/g), with a conversion efficiency of 62.74%, while *S cerevisiae* SCI 7 produced 0.10 mg/ g of ethanol with a conversion efficiency of which was comparable to its reference strain NCIM 3095 (0.11 mg/g) where as *Pichia stipitis* reference strain NCIM 3498 produced 0.12 mg/g with a conversion efficiency of 50.98% compared to *S cerevisiae* (Table 13).

4.8 Effect of fungal pre treatment on proximate composition of Sunflower Seed Hull (SSH)

The data pertaining to the release of reducing sugar, total sugar non-reducing sugar and protein content in fungal pretreated SSH are presented in Table 14.

4.8.1 Effect of fungal pretreatment on SSH

Trichoderma viride (CTV 5) and *Aspergillus niger* (CAN 3) treated SSH showed significantly higher reducing sugar of 13.67 mg g-1 among all the different consortia, when compared to control (2.01 mg/g) and the rest of the treatments. The next best treatment observed in releasing reducing sugar was consortia of *P. chrysosporium* (CPC 3) and *T. viride* (12.83 mg/g).Similar pattern of result was observed in total sugar and non reducing sugars with fungal consortia of *T. viride* (CTV 5) and *A. niger* (CAN 3) recording maximum total and non reducing sugar of 22.48 mg/g and 8.81 mg/g, respectively, which was significantly higher than all other treatments and control (0.94 mg/g; 2.96 mg/g). Highest protein content was recorded in *T. viride* (CTV 5) and *A. niger* (CAN 3) treated SSH of 4.69 mg/g which was significantly higher than all treatments and uninoculated control (2.42 mg/g) (Table 14).

Table 13. Evaluation of yeast and bacterial strains for ethanol production

Isolate	Ethanol produced (g/l)	Ethanol yield (g/g)	Conversion Efficiency (%)
SCI 1	0.06	0.12	23.52
SCI 2	0.08	0.16	31.37
SCI 3	0.08	0.16	32.0
SCI 4	0.07	0.14	27.45
SCI 5	0.07	0.14	27.45
SCI 6	0.09	0.18	35.29
SCI 7	0.10	0.20	39.21
SCI 8	0.05	0.10	19.6
NCIM 3095	0.11	0.22	44.0
ZM1 1	0.13	0.26	50.98
ZMI 2	0.12	0.24	50.98
ZMI 3	0.16	0.32	62.74
NCIM 2915	0.12	0.24	47.05
NCIM 3498	0.12	0.24	50.98
S.Em±	0.01	0.01	0.21
CD at 1%	0.03	0.03	0.65

Table 14. Effect of fungal pre treatment on proximate composition of Sunflower Seed Hull (SSH)

Treatment	Reducing sugar (mg/g)	Non reducing sugar (mg/g)	Total sugar (mg/g)	Protein (mg/g)
Control	2.01	0.94	2.96	2.42
SSH treated with *Aspergillus awamori* (CAA 1)	4.81	1.25	6.06	2.58
SSH treated with *Aspergillus niger* (CAN 3)	6.08	2.83	8.92	2.62
SSH treated with *Phanerochaete chrysosporium* (CPC 3)	6.31	3.61	9.93	2.97
SSH treated *with Trichoderma viride* (CTV 5)	8.53	5.38	13.92	3.05
SSH treated with *Aspergillus awamori* (CAA 1) and *Aspergillus niger* (CAN 3)	10.02	6.72	16.73	3.20
SSH treated with *Aspergillus awamori* (CAA1) and *Phanerochaete chrysosporium* (CPC 3)	9.69	6.22	15.91	2.44
SSH treated with *Aspergillus awamori* (CAA 1) and *Trichoderma viride* (CTV 5)	12.27	7.15	19.42	3.42
SSH treated with *Aspergillus niger* (CAN 3) and *Phanerochaete chrysosporium* (CPC 3)	11.09	6.85	17.94	3.33
SSH treated with *Aspergillus niger* (CAN 3) and *Trichoderma viride* (CTV 5)	13.67	8.81	22.48	4.69
SSH treated with *Phanerochaete chrysosporium* (CPC 3) and *Trichoderma viride* (CTV 5)	12.83	7.60	20.44	3.67
S.Em±	0.07	0.09	0.10	0.07
CD at 1%	0.25	0.35	0.41	0.27

4.8.2 Effect of crude enzyme extract on proximate composition of fungal pretreated SSH

Crude enzyme extract treatment was carried out on fungal pretreated for hydrolysis to yield maximum reducing sugars (Table 15). All the treatments were significantly higher than control (2.32 mg/g) with maximum reducing sugar recorded in fungal pretreated SSH treated with crude enzyme extract of *Trichoderma viride* (62.34 mg/g) followed by fungal pretreated SSH treated with crude enzyme extract of *Aspergillus niger* (58.25 mg/g). SSH treated with crude enzyme extract of *T. viride* (32.20 mg/g and 94.88 mg/g) recorded maximum non-reducing and total sugar, which was found significantly superior over other treatments and control (1.24 mg/g and 3.56 mg/g). The next best treatment was found to be SSH treated with crude enzyme extract of *Aspergillus niger* for the production of non-reducing (26.27 mg/g) and total sugars (84.47 mg/g). SSH treated with crude enzyme extract of *T. viride* (7.87 mg/g) recorded highest protein content, followed by SSH treated with crude enzyme extract of *A. niger* (6.13 mg/g) which was significantly higher than all treatments and uninoculated control (3.50 mg/g).

4.9 Effect of alkali pretreatment on proximate composition of SSH

4.9.1 Effect of microbial consortia on proximate composition of alkali pretreated SSH

For comparison, alkali pretreatment of SSH followed by saccharification with lignocellulosic fungi was carried out wherein, alkali pretreated SSH treated with *Aspergillus niger* and *Trichoderma viride* showed maximum release of reducing sugars (37.16 mg/g), non reducing sugars (78.38 mg/g) and total sugar (115.53 mg/g) which was significantly higher than all other treatments including control (2.42 mg/g; 3.17 mg/g; 5.59 mg/g). Protein content estimated in the same treatment (11.34 mg/g) also showed a significant difference from all other treatments including control (3.36 mg/g) (Table 16).

4.9.2 Effect of crude enzyme on proximate composition of alkali pretreated SSH

Pretreatment by alkali followed by saccharification by crude enzyme extract showed highest release of maximum reducing, non reducing and total sugars with maximum protein content among all the pretreatments and treatment combinations. Among all the treatments, SSH treated with the crude enzyme of *Trichoderma viride* released maximum reducing sugar (174.35 mg/g) and total sugar (217.56 mg/g) with a

minimum non reducing sugar (46.29 mg/g) estimated in the same. All the values estimated for this treatment were found to be significantly higher than the control which released 2.50, 2.63 and 5.13 mg/g of reducing, non-reducing and total sugars, respectively. The effectiveness of this treatment can be traced back to the high protein content estimated in the same treatment (39.12 mg/g) which showed a significant difference from all other treatments including control (3.14 mg/g) (Table 17).

4.10 Ethanol production

Ethanol production was estimated for each of the above enlisted pretreatment and treatment combinations by taking only the best treatment for fermentation (Plate 5).

4.10.1 Ethanol production by yeasts and bacterial strains from fungal pretreated SSH and fungal treated SSH hydrolysed with crude enzyme

Following hydrolysis, ethanol production was carried out by local isolates and reference strains of *S. cerevisiae, Z. mobilis* and *P. stipitis.* The data obtained are presented in Table 18, 19 and 20.

4.10.1.1 Ethanol production by *Saccharomyces cerevisiae* SCI 7 (local isolate) from SSH treated with fungal consortia

Fungal treated hydrolysate of SSH fermented by *S. cerevisiae* (SCI 7) revealed an ethanol production, ranging from 0.14 mg/g to 2.79 mg/g over a period of 48 h, with maximal production at 24 h of incubation (3.06 mg/g), significantly higher than ethanol produced at all other time of incubation, including control (0.14 g/l), recording a maximum fermentation efficiency of 43.8% (Table 18). Further, a slight decrease in ethanol production and fermentation efficiency was noted until 48 h. A prominent drastic decrease in residual reducing sugar was observed from 0 (13.40 mg/g) to 24 h (7.78 mg/g) followed by a decrease up to 48 h recording 6.98 mg/g of residual reducing sugar, which showed a significant difference compared to the actual reducing sugar present in the hydrolysate (13.67 mg/g).

Plate 5. Experimental set up for ethanol production

Table 15. Effect of crude enzyme on proximate composition of fungal pretreated SSH

Treatment	Reducing sugar (mg/g)	Non reducing sugar (mg/g)	Total sugar (mg/g)	Protein (mg/g)
Control	2.32	1.24	3.56	3.50
Fungal pretreated SSH treated with crude enzyme of *Trichoderma viride* (CTV 5)	62.34	32.20	94.88	7.87
Fungal pretreated SSH treated with crude enzyme of *Phanerochaete chrysosporium* (CPC 3)	54.27	23.56	77.84	5.40
Fungal pretreated SSH treated with crude enzyme of *Aspergillus niger* (CAN 3)	58.25	26.27	84.47	6.13
Fungal pretreated SSH treated with crude enzyme of *Aspergillus awamori* (CAA 1)	54.05	22.10	76.14	5.07
S.Em±	0.33	0.46	0.15	0.04
CD at 1%	1.47	2.07	0.70	0.18

Table 16. Effect of microbial consortia on proximate composition of alkali pretreated SSH

Treatment	Non reducing sugar (mg/g)	Reducing sugar (mg/g)	Total reducing sugar (mg/g)	Protein (mg/g)
Control	2.42	3.17	5.59	3.36
Alkali pretreated SSH treated with *Aspergillus awamori* (CAA 1)	15.03	28.61	43.65	8.36
Alkali pretreated SSH treated with *Aspergillus niger* (CAN 3)	17.71	32.26	49.97	8.36
Alkali pretreated SSH treated with *Phanerochaete chrysosporium* (CPC 3)	16.48	29.65	46.13	8.73
Alkali pretreated SSH treated *with Trichoderma viride* (CTV 5)	17.83	36.57	54.56	9.63
Alkali pretreated SSH treated with *Aspergillus awamori* (CAA 1) and *Aspergillus niger* (CAN 3)	25.24	60.23	85.47	9.84
Alkali pretreated SSH treated with *Aspergillus awamori* (CAA1) and *Phanerochaete chrysosporium* (CPC 3)	23.49	59.77	83.26	9.89
Alkali pretreated SSH treated with *Aspergillus awamori* (CAA 1) and *Trichoderma viride* (CTV 5)	29.62	65.44	95.06	10.80
Alkali pretreated SSH treated with *Aspergillus niger* (CAN 3) and *Phanerochaete chrysosporium* (CPC 3)	28.34	63.14	91.50	10.60
Alkali pretreated SSH treated with *Aspergillus niger* (CAN 3) and *Trichoderma viride* (CTV 5)	37.16	78.38	115.53	11.34
Alkali pretreated SSH treated with *Phanerochaete chrysosporium* (CPC 3) and *Trichoderma viride* (CTV 5)	31.83	68.80	100.63	10.69
S.Em±	0.67	0.07	0.13	0.14
CD at 1%	0.39	0.43	0.53	0.55

Table 17. Effect of crude enzyme on proximate composition of alkali pretreated SSH

Treatment	Reducing sugars (mg/g)	Non reducing sugars (mg/g)	Total sugars (mg/g)	Protein (mg/g)
Control	2.50	2.63	5.13	3.14
SSH treated with crude enzyme of *Trichoderma viride* (CTV 5)	174.35	46.29	217.56	39.12
SSH treated with crude enzyme of *Phanerochaete chrysosporium* (CPC 3)	155.03	46.52	201.55	33.45
SSH treated with crude enzyme of *Aspergillus niger* (CAN 3)	164.67	47.34	213.86	31.25
SSH treated with crude enzyme of *Aspergillus awamori* (CAA 1)	132.81	53.45	187.05	29.14
SEm ±	1.04	1.75	1.99	1.23
CD at 1%	4.56	4.68	8.91	3.24

4.10.1.2 Ethanol production by *Saccharomyces cerevisiae* SCI 7 (local isolate) from fungal pretreated SSH hydrolysed by crude enzyme extract of *Trichoderma viride.*

Data furnished in Table 18 revealed an increase in the production of ethanol from crude extract hydrolysed SSH, when treated with *S. cerevisiae* local isolate SCI 7, analysed over a period of 48 h, with maximum ethanol produced at 24 h of incubation (22.32 g/l), showing a significant difference from control (0.35 g/l), recording a maximum fermentation efficiency of 70.36%, thereafter exhibiting a gradual decrease in ethanol production, receding to 19.90 mg/g of ethanol at 48 h of incubation. A drastic decrease in residual reducing sugar was observed with an initial residual reducing sugar content of 61.66 mg/g scaled down to 17.21 mg/g over a time period of 48 h.

4.10.1.3 Ethanol production by *Saccharomyces cerevisiae* NCIM 3095 (reference strain) from SSH treated with fungal consortia

Ethanol production by *S. cerevisiae* reference strain NCIM 3095 revealed the same pattern of ethanol production over a period of 48 h when compared to local isolate SCI 7, with better ethanol recovery (Table 18). Ethanol production was recorded maximum (3.65 g/l) after 24 h of incubation with a fermentation efficiency of 61.67%, which was significantly higher than ethanol produced at all other incubation time, including control (0.14 mg/g), followed by a moderate decrease in ethanol production receding to 3.18 g/l after 48 h of incubation. A significant decrease in residual reducing sugar was observed over a time period of 48 h with an initial residual sugar of 13.38 mg/g reduced drastically to 6.51 mg/g after 24 h, followed by a slight decrease until 48 h resulting in 6.35 mg/ g residual sugars.

4.10.1.4 Ethanol production by *Saccharomyces cerevisiae* NCIM 3095 (reference strain) from fungal pretreated SSH hydrolysed by crude enzyme extract of *Trichoderma viride.*

Compilation of results extracted from Table 18 based on the ethanol production and efficiency of *S. cerevisiae* reference strain NCIM 3095 from SSH hydrolysed by crude extract revealed a varied pattern of ethanol production ranging from 0.35 to 20.05 g/l with its maximum production at 24 h of incubation producing 23.15 g/l of ethanol which was significantly higher than ethanol produced at all other time intervals including

control (0.35 g/l). A high fermentation efficiency of 72.81% was recorded at 24 h, followed by a gradual decline by 63.05% after 48 h of incubation. A substantial decrease in residual reducing sugar was observed with an initial residual reducing sugar content of 61.64 mg/g reduced to 15.74 mg/g over a time period of 48 h.

Table 18. Ethanol production by *Saccharomyces cerevisiae* SCI 7 (local isolate) and NCIM 3095 (reference strain) from fungal consortia treated SSH and crude enzyme extract of *Trichoderma viride* treated SSH

Time (hrs)	SCI 7								NCIM 3095							
	Fungal treated SSH				Fungal treated SSH hydrolysed with crude enzyme				Fungal treated SSH				Fungal treated SSH hydrolysed with crude enzyme			
	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)
0	0.14	0.01	2.00	13.40	0.35	0.01	1.09	61.66	0.14	0.01	2.05	13.38	0.35	0.01	1.1	61.64
12	2.01	0.15	28.80	9.72	12.27	0.19	38.60	38.27	2.10	0.15	30.12	9.55	13.44	0.21	42.27	35.34
18	2.92	0.21	41.9	8.00	19.42	0.31	61.09	24.25	3.60	0.26	51.63	6.61	20.88	0.33	65.66	21.40
24	3.06	0.22	43.8	7.78	22.32	0.35	70.36	18.47	3.65	0.27	52.35	6.51	23.15	0.37	72.81	16.84
30	2.87	0.21	41.2	7.50	21.23	0.34	66.78	18.39	3.45	0.25	49.48	6.49	22.01	0.35	69.22	16.51
36	2.82	0.21	40.4	7.17	20.24	0.32	63.67	17.94	3.31	0.24	47.47	6.41	20.47	0.33	64.37	16.12
42	2.80	0.20	40.2	7.02	20.17	0.32	63.49	17.51	3.29	0.24	49.19	6.38	20.14	0.32	63.35	15.91
48	2.79	0.20	40.01	6.98	19.90	0.31	62.60	17.21	3.18	0.23	45.19	6.35	20.05	0.32	63.05	15.74
S.Em±	0.02	0.01	0.41	0.02	0.06	0.01	0.22	0.03	0.04	0.01	0.17	0.02	0.15	0.01	0.19	0.12
CD at 1%	0.07	0.02	1.22	0.09	0.24	0.02	0.8	0.14	0.19	0.03	0.51	0.07	0.63	0.03	0.63	0.5

E = Ethanol, EP = Ethanol Productivity, E = Fermentation Efficiency, RS = Residual Reducing Sugar

4.10.1.5 Ethanol production by *Zymomonas mobilis* NCIM 2915 (reference strain) from SSH treated with fungal consortia

Fungal treated hydrolysate of SSH fermented by *Z. mobilis* reference strain NCIM 2915 revealed a progressive increase in ethanol production, ranging from 0.14 to 4.17 mg/g over a period of 48 h, with its maximum production at 48 h of incubation, with a significant ethanol yield recorded from 24 to 48 h ranging from 4.14 to 4.17 g/l when compared to control (0.14 g/l) recording a maximum fermentation efficiency of 59.81% after 48 h. A constant noteworthy decrease in residual reducing sugar was noticed over a period of 48 h, with an initial residual reducing sugar content of 13.39 mg/g curtailed down to 5.49 mg /g at the end of 48 h (Table 19).

4.10.1.6 Ethanol production by *Zymomonas mobilis* NCIM 2915 (reference strain) from fungal pretreated SSH hydrolysed by crude enzyme extract of *Trichoderma viride*

Results pertaining to Table 19 revealed a consistent increase in the production of ethanol from crude extract hydrolysed SSH, when treated with *Z. mobilis* reference strain NCIM 2915, over a period of 48 h, with significant ethanol production at 48 h of incubation (23.10 mg/g), recording a maximum fermentation efficiency of 72.65%, when compared to control (0.37 g/l). An appreciable decrease in residual reducing sugar was observed with an initial residual reducing sugar content of 61.61 mg/g reduced to 17.04 mg/g over a time period of 48 h.

4.10.1.7. Ethanol production by *Zymomonas mobilis* ZMI 3 (local isolate) from SSH treated with fungal consortia

Local isolate of *Zymomonas mobilis,* ZMI 3 produced ethanol from fungal consortia treated SSH, conceding the same manner as that of NCIM 2095, but with better ethanol recovery. Ethanol production was recorded maximum (4.36 g/l) after 48 h of incubation with a fermentation efficiency of 62.53%, which was significantly higher than ethanol produced at all other incubation time including control (0.15 g/l) A consistent decrease in residual reducing sugar was noticed over a period of 48 h, with an initial residual reducing sugar content of 13.37 mg/g curtailed to 5.12 mg /g at the end of 48 h.

4.10.1.8 Ethanol production by *Zymomonas mobilis* ZMI 3 (local isolate) from fungal pretreated SSH hydrolysed by crude enzyme extract of *Trichoderma viride.*

Analysis of the results obtained from Table 19 on ethanol production from crude extract treated SSH inoculated with *Zymomonas mobilis* local strain, ZMI 3, indicated high ethanol recovery of 23.17 g/l with a high fermentation efficiency of 72.87% after 48 h of incubation, with drastic reduction in residual reducing sugar observed over a time period of 48 h with initial sugar content of 61.61mg/g significantly reduced to 16.90 mg/g after 48 h.

4.10.1.9 Ethanol production by *Pichia stipitis* NCIM 3498 (reference strain) from SSH treated with fungal consortia

P. stipitis reference strain NCIM 3498 produced ethanol from fungal consortia treated SSH with ethanol production consistently increasing over a period of time. Ethanol production was recorded maximum (3.83 g/l) after 48 h of incubation with a fermentation efficiency of 54.93%, which was significantly higher than ethanol produced at all other incubation time along with control (0.15 g/l) An appreciable decrease in residual reducing sugar was observed with an initial residual reducing sugar content of 13.37 mg/g reduced to 6.16 mg/g over a time period of 48 h.

4.10.1.10. Ethanol production by *Pichia stipitis* NCIM 3498 (reference strain) from fungal pretreated SSH hydrolysed by crude enzyme extract of *Trichoderma viride*

Results pertaining to Table 20 revealed a consistent increase in the production of ethanol from crude extract hydrolysed SSH, inoculated with *P. stipitis* reference strain; NCIM 3498, over a period of 48 h, with maximum ethanol production at 48 h of incubation (22.97 mg/g), recording a maximum fermentation efficiency of 72.24%, with drastic reduction in residual reducing sugar observed over a time period of 48 h with initial sugar content of 61.65 mg/g significantly reduced to 17.30 mg/g after 48 h.

Table 19. Ethanol production by *Zymomonas mobilis* ZMI 3 (local isolate) and NCIM 2915 (reference strain) from fungal consortia treated SSH and crude enzyme extract of *Trichoderma viride* treated SSH

Time (hrs)	NCIM 2915								ZMI 3							
	Fungal treated SSH				Fungal treated SSH hydrolysed with crude enzyme				Fungal treated SSH				Fungal treated SSH hydrolysed with crude enzyme			
	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)
0	0.14	0.01	2.00	13.39	0.37	0.01	1.16	61.61	0.15	0.01	2.15	13.37	0.37	0.01	1.16	61.61
12	2.27	0.16	32.56	9.21	11.44	0.18	35.98	39.90	2.32	0.16	33.27	9.12	11.95	0.19	37.58	38.90
18	4.07	0.29	58.37	5.68	17.45	0.27	54.88	28.12	4.28	0.31	61.39	5.27	18.01	0.28	56.64	27.02
24	4.14	0.30	59.38	5.52	19.85	0.31	62.43	28.41	4.30	0.31	61.67	5.23	20.12	0.32	63.28	22.88
30	4.15	0.30	59.52	5.53	20.30	0.32	64.47	22.14	4.32	0.32	61.96	5.19	21.78	0.34	68.50	19.63
36	4.16	0.30	59.66	5.51	21.37	0.34	67.21	20.43	4.35	0.32	62.39	5.14	22.13	0.35	69.60	18.94
42	4.16	0.30	59.66	5.51	22.09	0.35	69.57	18.96	4.35	0.32	62.39	5.14	22.27	0.36	70.04	18.67
48	4.17	0.30	59.81	5.49	23.10	0.37	72.65	17.04	4.36	0.32	62.53	5.12	23.17	0.37	72.87	16.90
S.Em±	0.02	0.01	0.2	0.06	0.06	0.01	0.45	0.25	0.02	0.01	0.06	0.05	0.03	0.01	0.39	0.49
CD at 1%	0.06	0.03	0.63	1.53	0.27	0.04	1.43	1.01	0.07	0.03	0.2	0.23	0.13	0.03	1.19	2.02

E = Ethanol, EP = Ethanol Productivity, E = Fermentation Efficiency, RS = Residual Reducing Sugar

Table 20. Ethanol production by *Pichia stipitis* NCIM 3498 (reference strain) from fungal consortia treated SSH and crude enzyme extract of *Trichoderma viride* treated SSH

Time (hrs)	NCIM 3498							
	Fungal treated SSH				Fungal treated SSH hydrolysed with crude enzyme			
	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)
0	0.15	0.01	2.15	13.37	0.35	0.01	1.10	61.65
12	2.14	0.15	30.69	9.47	12.40	0.19	39.00	38.02
18	3.52	0.25	50.48	6.76	18.24	0.29	57.37	26.57
24	3.64	0.26	52.21	6.53	21.02	0.33	66.11	21.12
30	3.67	0.27	52.64	6.47	21.70	0.34	68.25	19.79
36	3.70	0.27	53.07	6.41	22.07	0.35	69.41	19.06
42	3.81	0.28	54.64	6.19	22.14	0.35	69.63	18.92
48	3.83	0.28	54.93	6.16	22.97	0.36	72.24	17.30
S.Em±	0.02	0.01	0.09	0.03	0.14	0.01	0.12	0.40
CD at 1%	0.09	0.03	0.3	0.15	0.57	0.03	0.4	1.67

E = Ethanol, EP = Ethanol Productivity, E = Fermentation Efficiency, RS = Residual Reducing Sugar

4.10.2 Ethanol production by yeasts and bacterial strains from alkali pretreated SSH hydrolysed by fungal and crude enzyme extract treatment

Following hydrolysis, ethanol production was carried out by local isolates and reference strains of *S. cerevisiae, Z. mobilis and P. stipitis.* The data obtained are presented in Table 21, 22 and 23.

4.10.2.1 Ethanol production by *Saccharomyces cerevisiae* SCI 7 (local isolate) from alkali pretreated SSH hydrolysed by fungal consortia.

Ethanol production by *S. cerevisiae* local strain SCI 7 presented in Table 21 revealed a varied pattern of ethanol production from zero to 48 h with its maximum production at 24 h (21.04 g/l) which was significantly higher than ethanol produced at all other time intervals including control (0.32 g/l) recording a maximum fermentation efficiency of 52.63%. However, after 24 h a mild decline in ethanol production was noted curtailing it to 18.32 g/l. A noticeable rapid decrease in residual reducing sugar was observed from 0 (77.75 mg/g) to 24 h (37.12 mg/g) followed by a decelerated decrease up to 48 h recording 32.45 mg/g of residual reducing sugar.

4.10.2.2 Ethanol production by *Saccharomyces cerevisiae* SCI 7 (local isolate) from alkali pretreated SSH hydrolysed by crude enzyme extract

Analysis of the results obtained from Table 21 on ethanol production from crude extract treated SSH inoculated with local strain of *S. cerevisiae* (SCI 7) indicated high ethanol recovery of 62.12 g/l with a high fermentation efficiency of 69.86% after 24 h of incubation, significantly higher than control (1.08 g/l) followed by a mild reduction of ethanol curtailing the efficiency to 56.26% at the end of 48 h. A significant decrease in residual reducing sugar was observed over a time period of 48 h with an initial residual sugar of 56.60 mg/g reduced drastically to 17.28 mg/g after 24 h, followed by a mild decrease until 48 h resulting in 16.12 mg/g residual sugars at the end of the experiment.

4.10.2.3 Ethanol production by *Saccharomyces cerevisiae* NCIM 3095 (reference strain) from alkali pretreated SSH hydrolysed by fungal consortia

Ethanol production by *S. cerevisiae* reference strain NCIM 3095 revealed the same pattern of ethanol production over a period of 48 h when compared to local isolate SCI 7, with better ethanol recovery, as depicted in Table 21. Ethanol production was

recorded maximum (24.45 g/l) after 24 h of incubation with a fermentation efficiency of 61.16%, which was significantly higher than ethanol produced at all other incubation time, including control (0.34 g/l), followed by a moderate decrease in ethanol production receding to 20.12 g/l after 48 h of incubation. A significant decrease in residual reducing sugar was observed over a time period of 48 h with an initial residual sugar of 77.80 mg/g reduced drastically to 34.94 mg/g after 24 h, followed by a mild decrease until 48 h resulting in 28.24 mg/ g residual sugars at the end if the experiment.

4.10.2.4 Ethanol production by *Saccharomyces cerevisiae* NCIM 3095 (reference strain) from alkali pretreated SSH hydrolysed by crude enzyme extract treatment

A perusal of data extracted from Table 20 based on the ethanol production and efficiency of *S. cerevisiae* reference strain NCIM 3095 from SSH hydrolysed by crude extract revealed a varied pattern of ethanol production ranging from 1.08 to 62.08 g/l with its maximum production at 24 h of incubation producing 67 g/l of ethanol which was significantly higher than ethanol produced at all other time intervals. A high fermentation efficiency of 75.34% was recorded at 24 h, followed by a mild decline by 62.08% after 48 h of incubation. A drastic decrease in residual reducing sugar was observed with an initial residual reducing sugar content of 56.65 mg/g drastically reduced to 14.13 mg/g over a time period of 24h followed by slight decrease till 48 h.

Table 21. Ethanol production by *Saccharomyces cerevisiae* SCI 7 (local isolate) and NCIM 3095 (reference strain) from alkali pretreated SSH hydrolysed by fungal and crude enzyme extract treatment

Time (hrs)	SCI 7								NCIM 3095							
	Alkali pretreated SSH hydrolysed with fungal consortia				Alkali pretreated SSH hydrolysed with crude enzyme				Alkali pretreated SSH hydrolysed with fungal consortia				Alkali pretreated SSH hydrolysed with crude enzyme			
	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)
0	0.32	0.05	0.80	77.75	1.08	0.01	1.21	56.60	0.34	0.005	0.800	77.80	1.08	0.01	1.215	56.65
12	14.42	0.18	36.07	50.10	31.25	0.17	35.14	37.19	14.45	0.18	36.14	77.75	32.69	0.18	36.76	36.26
18	20.09	0.25	50.25	38.98	58.39	0.33	65.66	19.69	22.15	0.28	55.41	50.04	64.55	0.37	72.59	15.71
24	21.04	0.26	52.63	37.12	62.12	0.35	69.86	17.28	24.45	0.31	61.16	34.94	67.00	0.38	75.34	14.13
30	20.05	0.25	50.15	36.32	54.15	0.31	60.89	17.17	23.12	0.29	57.83	30.43	65.73	0.377	73.92	14.95
36	19.21	0.24	48.05	35.12	52.08	0.29	58.57	16.39	22.10	0.28	55.28	30.04	64.88	0.37	72.96	15.50
42	18.45	0.23	46.15	34.99	50.11	0.28	56.35	16.24	20.52	0.26	51.20	29.04	63.91	0.36	71.87	16.13
48	18.32	0.23	45.83	32.45	50.03	0.28	56.26	16.12	20.12	0.25	50.33	28.24	62.08	0.35	69.81	17.31
S.Em ±	0.18	0.02	0.08	0.07	0.28	0.01	0.02	0.63	0.03	0.02	0.3	0.08	0.30	0.01	0.02	0.05
CD at 1%	0.76	0.06	0.25	0.29	1.19	0.03	0.06	1.98	0.10	0.06	1.02	0.29	1.24	0.03	0.07	0.17

E = Ethanol, EP = Ethanol Productivity, E = Fermentation Efficiency, RS = Residual Reducing Sugar

4.10.2.5 Ethanol production by *Zymomonas mobilis* NCIM 2915 (reference strain) from alkali pretreated SSH hydrolysed by fungal consortia

Results pertaining to Table 22 revealed a consistent increase in the production of ethanol from fungal treated SSH, when treated with *Z. mobilis* reference strain NCIM 2915, over a period of 48 h, with maximum ethanol production at 48 h of incubation (26.01 mg/g), recording a maximum fermentation efficiency of 65.06%, with drastic reduction in residual reducing sugar observed over a time period of 48 h with initial sugar content of 73.53 mg/g significantly reduced to 27.38 mg/g after 48 h.

4.10.2.6 Ethanol production by *Zymomonas mobilis* NCIM 2915 (reference strain) from alkali pretreated SSH hydrolysed by crude enzyme extract treatment

Analysis of results obtained from Table 22 on ethanol production from crude extract treated SSH indicated an ethanol recovery of 71.25 g/l with a high fermentation efficiency of 72.87% after 48 h of incubation, when treated with *Z. mobilis* reference strain, which was significantly higher than ethanol produced at all other incubation time, along with control (2.5 g/l), with drastic reduction in residual reducing sugar observed over a time period of 48 h with initial sugar content of 55.74 mg/g significantly reduced to 11.39 mg/g after 48 h.

4.10.2.7 Ethanol production by *Zymomonas mobilis* ZMI 3 (local isolate) from alkali pretreated SSH hydrolysed by fungal consortia

Local isolate of *Z. mobilis*, ZMI 3 produced ethanol from fungal consortia treated SSH, conceding the same manner as that of NCIM 2095, but with better ethanol recovery. Ethanol production was recorded maximum (26.97 g/l) after 48 h of incubation with a fermentation efficiency of 66.66%, which was significantly higher than ethanol produced at all other incubation time. A consistent decrease in residual reducing sugar was noticed over a period of 48 h, with an initial residual reducing sugar content of 73.67 mg/g curtailed to 25.49 mg /g at the end of 48 h.

4.10.2.8 Ethanol production by *Zymomonas mobilis* ZMI 3 (local isolate) from alkali pretreated SSH hydrolysed by crude enzyme extract treatment

Ethanol production from crude extract treated SSH, inoculated with *Z. mobilis* local isolate ZMI 3, indicated high ethanol recovery of 73.24 g/l with a high fermentation efficiency of 82.36% after 48 h of incubation, with drastic reduction in

residual reducing sugar observed over a time period of 48 h with initial sugar content of 55.82 mg/g significantly reduced to 10.11 mg/g after 48 h (Table 22).

4.10.2.9 Ethanol production by *Pichia stipitis* NCIM 3498 (reference strain) alkali pretreated SSH hydrolysed by fungal consortia

Reference strain of *P. stipitis*, NCIM 3498 produced ethanol from fungal consortia treated SSH with ethanol production consistently increasing over a period of time. Ethanol production was recorded maximum (24.39 g/l) after 48 h of incubation with a fermentation efficiency of 61.01%, which was significantly higher than ethanol produced at all other incubation time. An appreciable decrease in residual reducing sugar was observed with an initial residual reducing sugar content of 73.47 mg/g reduced to 30.55 mg/g over a time period of 48 h.

4.10.2.10. Ethanol production by *Pichia stipitis* NCIM 3498 (reference strain) from alkali pretreated SSH hydrolysed by crude enzyme extract treatment

Results pertaining to Table 23 revealed a consistent increase in the production of ethanol from crude extract hydrolysed SSH, when treated with *P. stipitis,* NCIM 3095, over a period of 48 h, with maximum ethanol production at 48 h of incubation (67.12 g/l), recording a maximum fermentation efficiency of 75.48%, with drastic reduction in residual reducing sugar observed over a time period of 48 h with initial sugar content of 55.84 mg/g significantly reduced to 14.06 mg/g after 48 h.

Table 22. Ethanol production by *Zymomonas mobilis* ZMI 3 (local isolate) and NCIM 2915 (reference strain) from from alkali pretreated SSH hydrolysed by fungal and crude enzyme extract treatment

Time (hrs)	NCIM 2915								ZMI 3							
	Alkali pretreated SSH hydrolysed with fungal consortia				Alkali pretreated SSH hydrolysed with crude enzyme				Alkali pretreated SSH hydrolysed with fungal consortia				Alkali pretreated SSH hydrolysed with crude enzyme			
	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)
0	2.47	0.03	6.179	73.53	2.5	0.01	2.811	55.74	2.4	0.03	0.05	73.67	2.37	0.01	2.66	55.82
12	14.91	0.19	37.29	49.14	29.17	0.16	32.80	38.54	15.12	0.19	37.25	48.73	30.02	0.17	33.76	37.99
18	19.45	0.24	48.65	40.24	55.35	0.31	62.24	21.65	20.12	0.25	49.01	38.92	56.39	0.32	63.41	20.98
24	23.12	0.29	57.83	33.04	62.17	0.35	69.91	17.25	24.35	0.31	60.78	30.63	63.15	0.36	71.02	16.62
30	24.04	0.30	60.13	31.24	66.98	0.38	75.32	14.15	25.65	0.32	62.74	28.08	67.18	0.38	75.55	14.02
36	25.67	0.32	64.21	28.04	68.37	0.39	76.89	13.25	26.12	0.33	64.70	27.16	70.04	0.40	78.76	12.17
42	25.92	0.33	64.84	27.55	70.19	0.40	78.93	12.08	26.37	0.33	64.70	26.67	72.14	0.41	81.13	10.82
48	26.01	0.33	65.06	27.38	71.25	0.41	80.12	11.39	26.97	0.34	66.66	25.49	73.24	0.42	82.36	10.11
S.Em±	0.23	0.01	0.3	0.10	0.23	0.01	0.02	0.35	0.02	0.01	0.38	0.12	0.27	0.01	0.03	0.24
CD at 1%	0.76	0.03	1.1	0.45	0.98	0.03	0.07	1.50	0.07	0.03	1.2	0.4	1.15	0.03	0.1	0.74

Table 23. Ethanol production by *Pichia stipitis* NCIM 3498 (reference strain) from alkali pretreated SSH hydrolysed by fungal and crude enzyme extract treatment

Time (hrs)	NCIM 3498							
	Alkali pretreated SSH hydrolysed with fungal consortia				Alkali pretreated SSH hydrolysed with crude enzyme			
	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)	E (g/l)	EP (g/g)	FE (%)	RRS (mg/g)
0	2.5	0.03	6.25	73.47	2.35	0.01	2.64	55.84
12	14.41	0.18	36.04	50.12	31.22	0.17	35.11	37.21
18	19.82	0.25	49.58	39.51	58.99	0.33	66.34	19.30
24	20.05	0.25	50.15	39.06	60.32	0.34	67.83	18.44
30	21.05	0.26	52.65	37.10	62.39	0.35	70.16	17.11
36	21.45	0.27	53.66	36.32	63.53	0.36	71.44	16.37
42	22.45	0.28	56.16	34.36	65.47	0.37	73.62	15.12
48	24.39	0.31	61.01	30.55	67.12	0.38	75.48	14.06
S.Em ±	0.08	0.01	0.05	0.11	0.63	0.01	0.2	0.05
CD at 1%	0.33	0.03	0.17	0.46	2.60	0.03	0.7	0.17

E= Ethanol
EP = Ethanol Productivity
E = Fermentation Efficiency
RS = ResidualReducing Sugar

DISCUSSION

V. DISCUSSION

5.1 Sunflower seed hull

Sunflower Seed Hulls (SSH) are byproducts obtained by dehulling of sunflower seeds. Sunflower seeds contain about 20-30% hulls that are often removed before oil extraction due to deleterious effects on oil press, as they reduce the quality of oil. The hulls obtained as a waste product after de-hulling process have little commercial value and become a disposal problem because of their low bulk density (Sharma, 2004). Therefore, it is essential to develop efficient pretreatment methods to degrade this waste. Contemplating these factors, different pretreatment methods such as chemical and biological methods have been employed to convert SSH to fermentable sugars for fermentation to ethanol.

A thorough understanding of proximate composition of lignocellulosic substance is essential in ethanol quantification studies. Owing to its high cellulose (34.50%) content, SSH acts as an apt source that can be utilized as efficient biomass for ethanol production. (Dorrel and Vick. 1997). In the present study, proximate composition of hulls were estimated, where in, the hulls were analyzed with 1.26 mg/g total sugar, 0.27 mg/g reducing sugar, 4.50% protein content and 8.5% moisture content on dry weight basis, the date for which is furnished in Table 3.

5.2 Isolation, morphological characterization and screening of white rot fungi from compost pits for lignocellulosic activity

A total of fifty lignocellulolytic fungi were isolated from compost pits, at various stages of development. Most of the isolates common to all the samples belonged to the genera of *Trichoderma, Aspergillus, Phanerochaete, Fusarium* and *Penicillium*, which are regarded as the most common in composting materials, due to their thermo tolerance and/or capacity to degrade a wide range of organic waste (Miller 1996). A large majority of the total number of isolates were members of the genus *Aspergillus*. These associations of *Aspergillus* with different composts have been reported (Anastasi *et al.*, 2005). Data on the occurrence of *Aspergillus* corresponds with the figures of Anastasi e*t al.* (2005) who has reported the highest load and the number of species of *Aspergillus* in composts study.

The results obtained also indicate that compost, in its mesophilic stage, harbored more lignocellulolytic fungi than that in thermophilic stage. This is due to the fact that most of the white rot fungi excluding a few, flourish in a mesophilic habitat than thermophilic. However, isolates of genera *Phanerochaete* were recovered from thermophilic stage than mesophilic stage owing to the fact that it is a thermophilic fungus that completes composting process.

Isolation was followed by microscopic and cultural characterization of the fungi. Morphological features are important in microbial taxonomy for many reasons. Therefore isolates obtained were cultured in Czapox Dox agar for tabulating their tentative identity. Isolates which developed light green and yellowish colonies without concentric rings were identified as *Trichoderma viride* compared to similar observations drawn by Sha *et al.* (2012). Microscopic examination revealed globose septate hyphae which confirmed the genera. Isolates which developed white colony with dusty yellow sporulating area were identified as *Aspergillus niger* and those with brownish sporulating area as *Aspergillus awamori* according to conclusions drawn by Sharma and Pandey (2010) and Perrone *et al.* (2011) respectively. Compost at its thermophilic stage was which harbored white colonies with loose cottony texture identified as *Phanerochaete chrysosporium* (Gilman 1944).

Following morphological characterization, all the isolates belonging to *Trichoderma viride, Aspergillus niger, A. awamori* and *Phanerochaete chrysosporium* were selected for qualitative and quantitative analysis of lignocellulolytic activity. Qualitative assays are powerful tools used in screening fungi for lignocellulose degrading enzyme production. Such tests give a positive or negative indication of enzyme production. They are particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data are not required. Plate screening method has previously been used for screening hydrolytic enzymes producing fungi and was reported to be suitable (Bhallal and Joshi, 1993; Abdel- Sater *et al.*, 2000). In the present study, a clear zone of hydrolysis was noticed when the culture filtrate was dropped in wells on cellulose, carboxy methyl cellulose (CMC) and birch wood xylan plates stained with Congo red. Out of 48 isolates, 38 produced clearance zone. Among all the isolates, isolates of *Trichodema viride*, CTV 5 recorded maximum clearance zone (26 mm) significantly higher than all strains including its reference strain, NCIM 1195 (22 mm). The same was observed in CMCase activity also, which shows that

Trichoderma is a better source of cellulolytic enzyme than all other strains used in the study. The results obtained were similar to findings of Devi and Kumar (2012). Observing the area of clear zone produced, differentiated the potent xylanase and cellulase producers. However, halo zone around the isolates on solid agar medium may sometimes be due to the presence of membrane bound hydrolases which cause the formation of the clearing when the substrates are being hydrolysed. This can also be due to the regional presence of enzyme secreted near the growing colony in the agar plates. This however will not occur in liquid medium. Therefore isolates with relatively high enzyme activities were selected for liquid fermentation using birch wood xylan and carboxyl methyl cellulose followed by xylanase and cellulase assays, respectively to get a clear picture of hydrolytic enzyme produced as well as a quantitative estimation of the enzymes. . Among all the isolates, *Trichoderma viride*, isolate, CTV 5 developed maximum CMCase activity (0.72 U ml^{-1}) which was significantly higher to all the isolates, including the reference strain, NCIM1195 (2.16 U ml^{-1})., while maximum xylanolytic activity was recorded in *Phanerochaete chrysosporium* with CMCase, Xylanase and FPU activity of 0.70, 11.76 and 1.36 U ml^{-1}, respectively, which was on par with reference strain NCIM 1197 which recorded maximum CMCase, xylanase and FPU activity of 0.71, 14.86 and 1.37 U ml^{-1}respectively. These findings are in accordance with the findings of Shankarappa (2013) where in, he conducted an experiment on Optimization of growth conditions for production of saccharolytic enzymes by cellulolytic fungi where, five cellulolytic fungi were screened for saccharolytic enzyme production. He observed that *T. reesei* showed highest FPU activity (2.23 U/ml), CMCase activity (3.68 U/ml), glucosidase activity (1.82 U/ml) on cellulose and xylanase activity (3.65 U/ml) using sugarcane bagasse as carbon source followed by *T. viridae* with 2.21 U/ml of FPU activity, 2.90 U/ml CMCase activity, 1.56 IU ml^{-1} glucosidase activity and 2.75 U/ml xylanase activity on cellulose as carbon source when compared to *Aspergillus sydowii, A. awamorii* and *P. chrysosporium.*

In nature, hydrolysis of cellulose occurs as a result of the action of a cellulase complex produced by cellulolytic microorganisms. This complex consists with different actions, *i.e.,* endoglucanases (endo-1, 4- ß -glucanases), cellobiohydrolases (exo-1,4-ß-glucanases), and ß -glucosidases. Endoglucanases (endo-1, 4- ß -glucanases) can hydrolyze internal bonds (preferentiallyably in cellulose amorphous cellulose regions) and release new terminal ends, whereas cellobiohydrolases act on the existing or

endoglucanase-generated chain ends. Both enzymes can degrade amorphous cellulose but, with some exceptions, cellobiohydrolases are the only enzymes that efficiently degrade crystalline cellulose. White rot fungi are said to be great arsenals of these enzymes, especially *Trichoderma reesei, T. viride* and *Aspergillus niger* and *Phanerochaete* (Deshpande *et al.*, 1978). This property has been utilized for saccharification of SSH in the present study.

5.3 Isolation and biochemical characterization of *Zymomonas mobilis*

The isolation of *Zymomonas mobilis* from Sugarcane juice agrees with the work done by Raymundo *et al*, (2005). Morphological examination of the isolates revealed Gram negative, plump rod cells with distinct round ends, and positive to motility test. The same characteristics were observed in reference strain NCIM 2905. Such characters were also reported by earlier workers (Buchanan and Gibbons, 1974; Swings and de Lay, 1981). *Z. mobilis* was found to be facultative anaerobic since it fermented glucose, fructose and sucrose but not lactose. Physiological and biochemical characterization revealed all the isolates positive for catalase while negative for H_2S. Based on the results mentioned, local isolates were identified as *Z. mobilis* (Kluyver and van Niel, 1936). Similar biochemical characters were observed in *Z. mobilis* by Akponah *et al.* (2013) which confirms its identity.

5.4 Isolation and biochemical characterization of *Saccharomyces cerivesiae*

A total of 15 yeast isolates were isolated from grape juice and identified based on the procedures outlined by Asyikeen *et al.* (2012). All the isolates on Sabouraud's dextrose agar developed either a smooth glabrous white to cream coloured colony or a cream mucoid slimy colony. Similar finding was also observed by Kevin (2005) who reported that typical *S. cerevisiae* colonies were creamy and regular colony shape. The biochemical tests revealed 5 isolates positive for flocculation test and three isolates positive for cyclohexamide test. Cyclohexamide test was conducted to differentiate Saccharomyces from *Candida sp.* due to the fact that *Candida* is resistant to cyclohexamide, while *Saccharomyces* is not. The isolates which particularly showed negative result to flocculation test, cyclohexamide test and lactose utilization test (YI 1, YI 3, YI 3, YI 8, YI 9, YI 10, YI 12 and YI 13) were identified as *Saccharomyces* sp. (Asyikeen *et al.,* 2012).

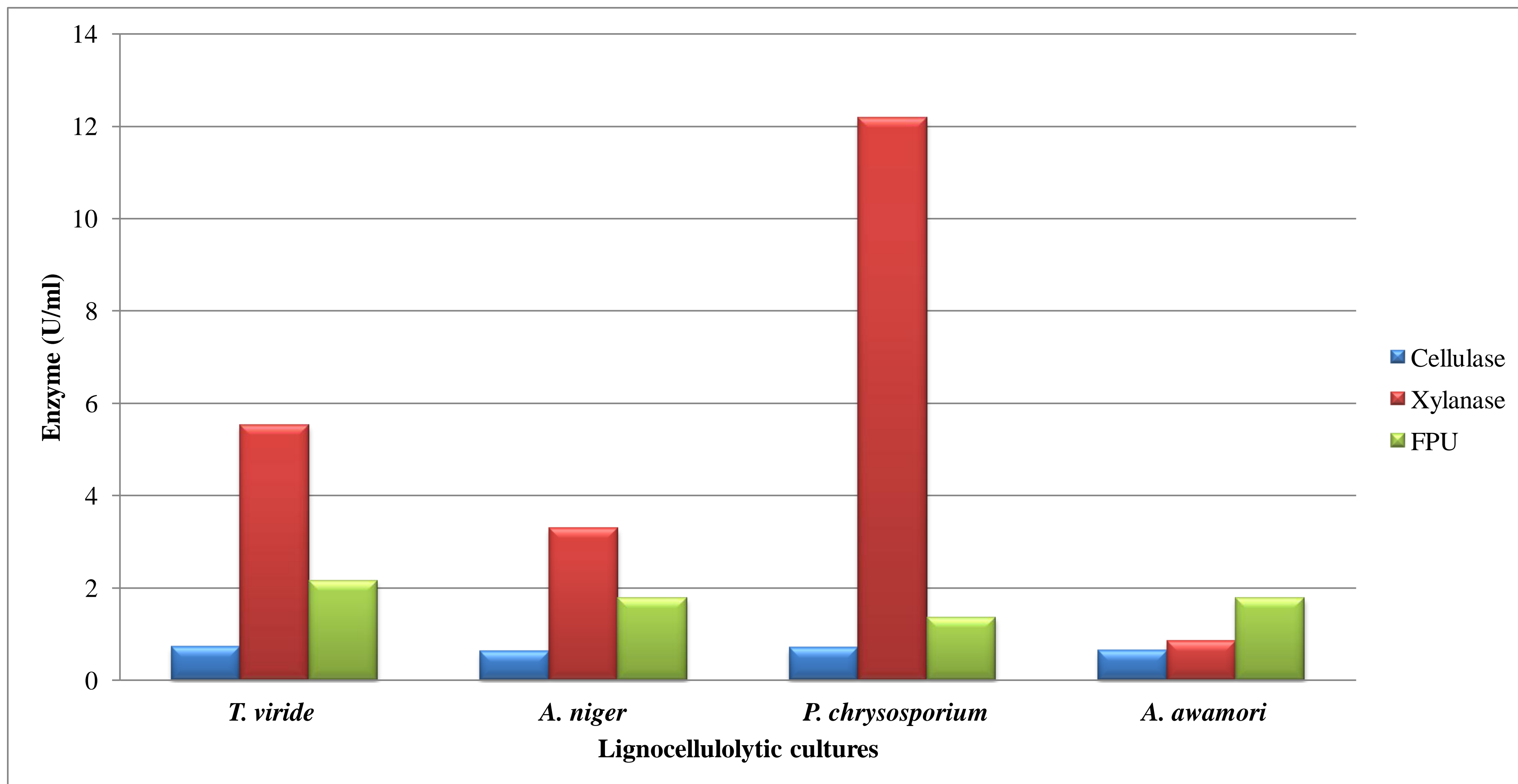


Fig.1. Enzymatic activity of fungal isolates

5.5 Evaluation of selected yeast and bacteria strains for ethanol production

Eight yeast strains and three bacterial isolates identified as *Saccharomyces* and *Zymomonas*, respectively, were selected to evaluate the ethanol fermentation capacity in RM medium containing 5% of glucose. The results pertaining to the above are presented in Fig 2. There was a significant difference in ethanol produced by the different microorganisms. *Z. mobilis* isolate ZMI 3 produced the highest concentration of ethanol (0.16 mg/g), with a conversion efficiency of 62.74% where as reference strain NCIM 2915, produced 0.13 mg/g of ethanol with a conversion efficiency of 50.98% which, while *S cerevisiae* SCI 7 produced 0.10 mg/ g of ethanol with a conversion efficiency of which was comparable to its reference strain NCIM 3095 (0.11 mg/g) where as *Pichia stipitis* reference strain NCIM 3498 produced 0.12 mg/g with a conversion efficiency of 50.98% compared to *S cerevisiae*. In brief these results indicated that, among the isolated bacterial strains, *Z. mobilis* showed maximum conversion efficiency followed by *P. stipitis* and *S cerevisiae.* The studies are in concordance with Rouhollah *et al.* (2007) who showed that *P. stipitis* was able to ferment sugar into ethanol hydrolyzate with highest yield of ethanol (8.18 g/l) compared to *S. cerevisiae* (8.10 g/l) from 20 g/l of initial reducing sugar. The fermentation of acid and enzymatic hydrolysates of *Prosopis juliflora* (Mesquite), containing 18.24 g/l and 37.47 g/l sugars was studied by Gupta *et al*. (2009), using *Pichia stipitis* and *Saccharomyces cerevisiae.* The fermentation produced 7.13 g/l and 18.52 g/l of ethanol with corresponding yield of 0.39 g/g and 0.49 g/g, respectively from *P. stipitis* and *S. cerevisiae*.

5.6 Effect of fungal pre treatment on proximate composition of SSH

Microbial pretreatment involves the use of microorganisms (mainly fungi) to degrade lignin and hemicelluloses but leave the cellulose intact (Kumar and Wyman. 2009; and Sanchez, 2009). Several studies have shown that white-rot fungi are the most effective microorganisms for the pretreatment of lingocellulosic wastes, such as wood chips, wheat straw and softwood (Hatakka, 1983). Therefore local isolates of lignocellulosic fungi were used for pretreatment of SSH.

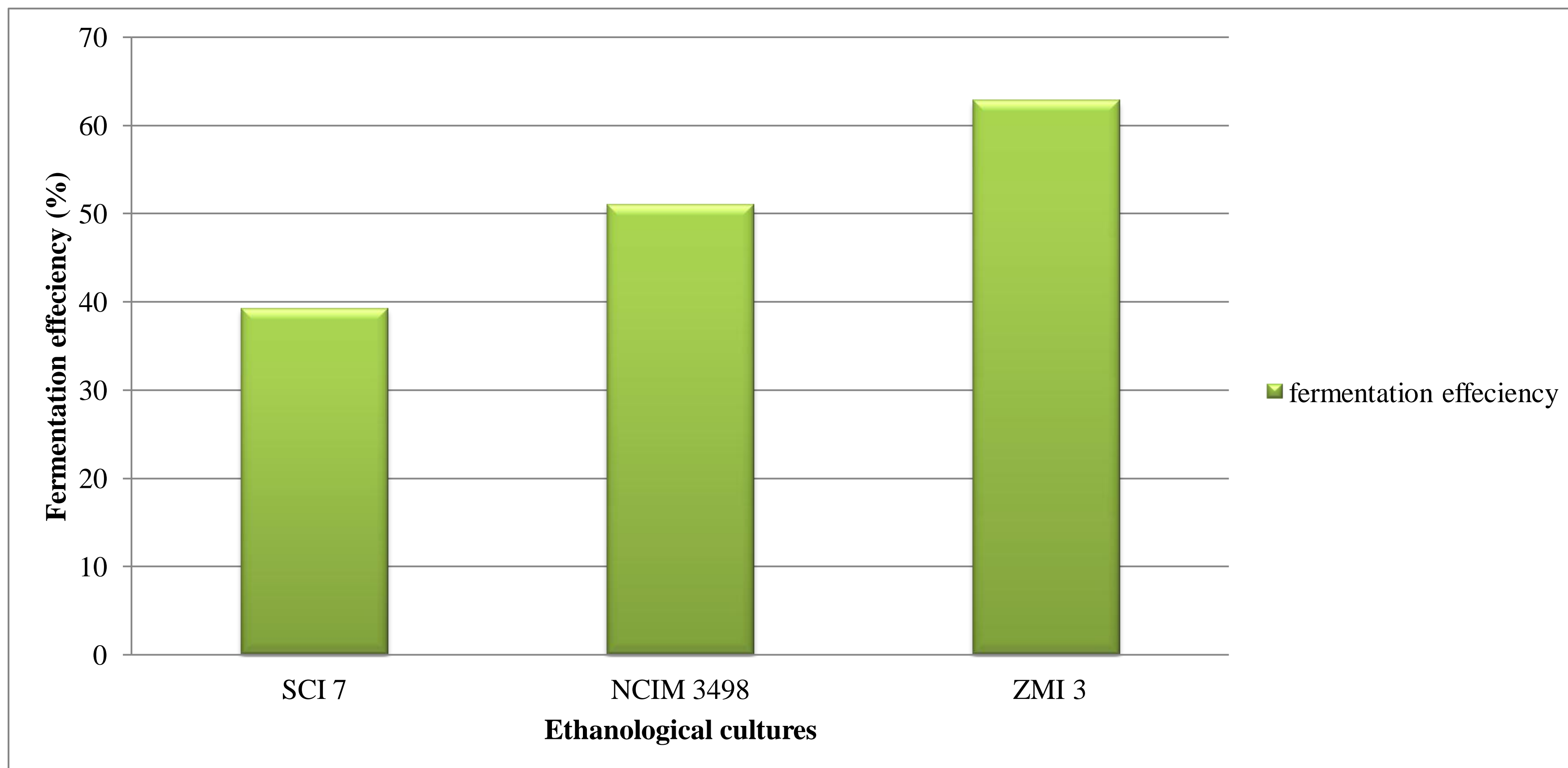


Fig. 2. Fermentation effeciency of ethanlogical yeast and bacteria

5.6.1 Effect of fungal pretreatment on reducing sugars

Microbial pretreatment involves the use of microorganisms (mainly fungi) to degrade lignin and hemicelluloses but leave the cellulose intact (Kumar and Wyman. 2009; and Sanchez, 2009). Several studies have shown that white-rot fungi are the most effective microorganisms for the pretreatment of lingocellulosic wastes, such as wood chips, wheat straw and softwood (Hatakka, 1983). Based on this, SSH was inoculated with fungal consortia where in, inoculated consortia of *Trichoderma viride* (CTV 5) and *Aspergillus niger* (CAN 3) showed significantly higher reducing sugar of 13.67 mg/g among all the isolates. Similar results were obtained by Seema *et al.* (2007) where in, bagasse inoculated with *A. niger* and *T. viride* showed highest reducing sugar yield producing 49 mg per gram of the substrate after 5 days of incubation while rice husk treated with co culture of *Aspergillus awamori* and *Phanerochaete chrysosporium* released maximum reducing sugar (32.5 mg per g) after five days of incubation (Fig 3).

Even though fungal pretreatment alone has the advantage of degrading lignin and hemicelluloses and it has an added advantage of low energy requirements, this pretreatment recorded the lowest reducing sugars when compared to all other pretreatment combinations. This may be due to several reasons which include long periods of time for bio-delignification and utilization of reducing sugar by microorganisms for their growth, which limits its application (Kumar et al., 2008; Zhang *et al*., 2009).

5.6.2 Effect of crude enzyme extract on proximate composition of fungal pretreated SSH

To increase the efficiency of biological pretreatment, the fungal pretreated SSH was saccharified using crude extracts of different white rot fungi. Enzymatic hydrolysis of cellulose is generally carried out by the cellulose-hydrolyzing enzyme cellulases, a mixture of several enzymes that hydrolyze crystalline/amophorous cellulose to fermentable sugars (Duff and Murray, 1996). The conversion of lignocellulosic biomass to fermentable sugars through biocatalyst cellulase derived from cellulolytic organisms has been suggested as an economically feasible process and offers potential to reduce the use of fossil fuels and reduce environmental pollution relative to physicochemical

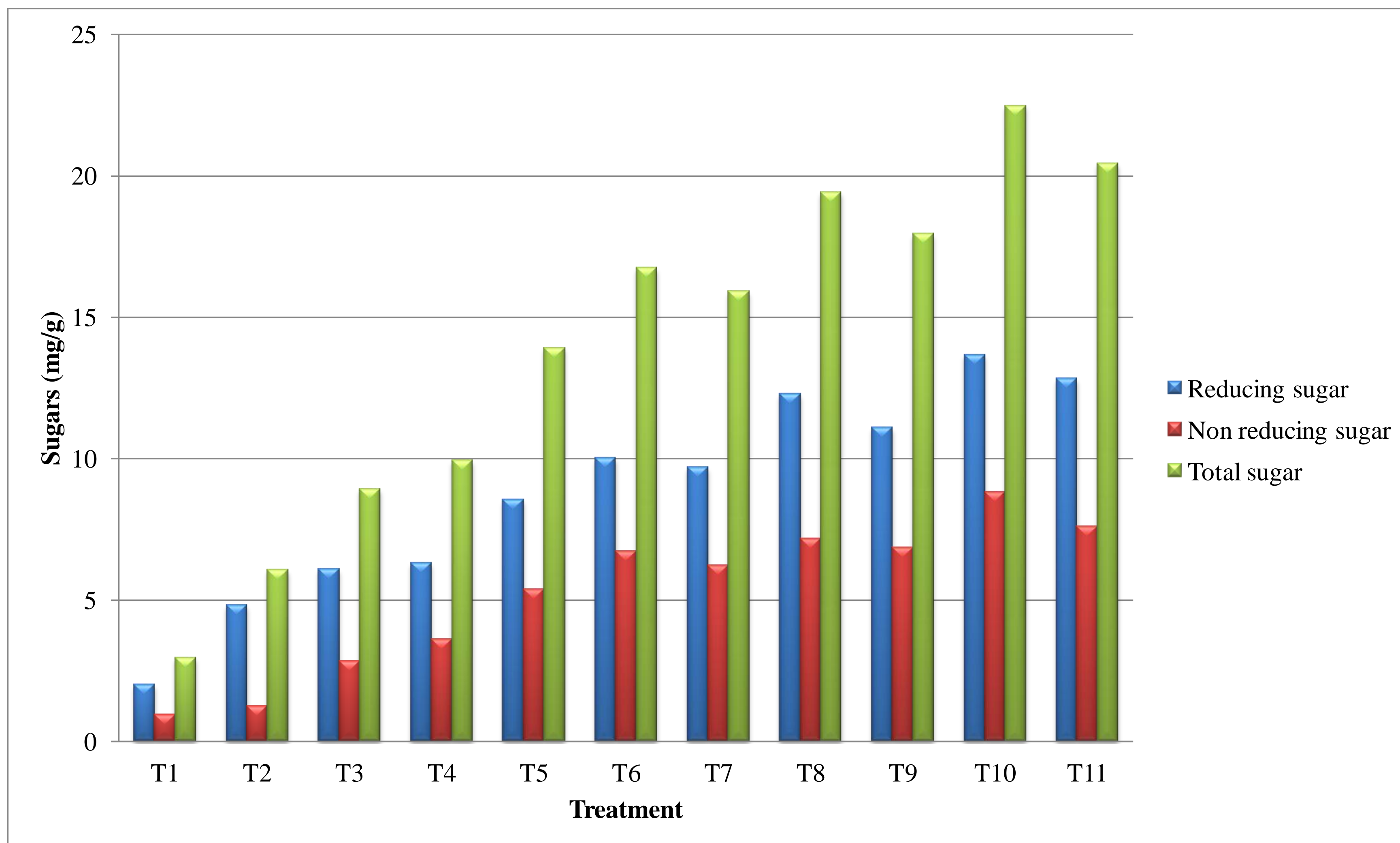


Fig. 3. Effect of fungal pretreatment on proximate composition of SSH

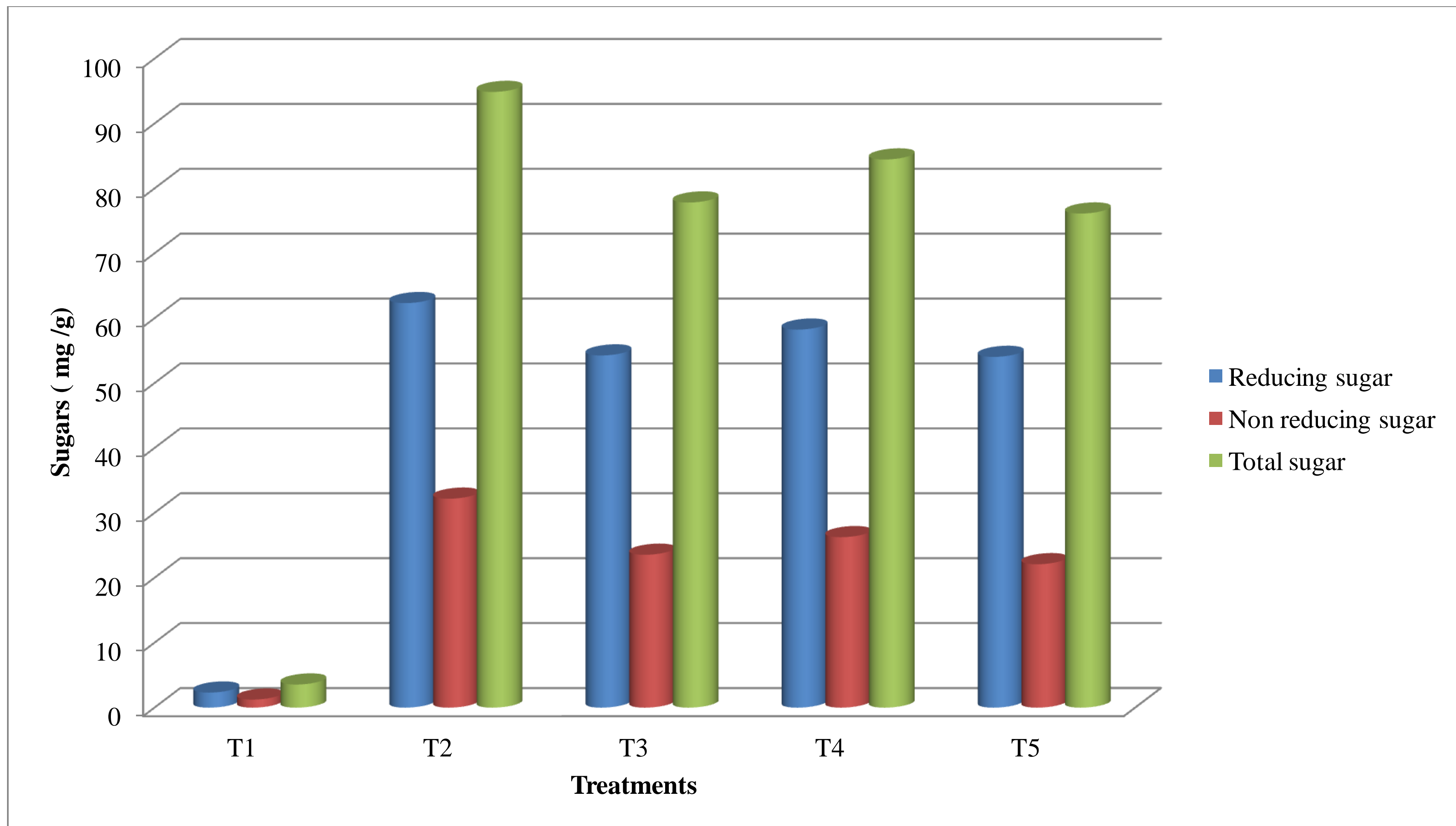


Fig. 4. Effect of crude enzyme extract on proximate composition of fungal pretreated SSH

processes. Figure 4 depicts the effect of crude enzyme extract on proximate composition of fungal pretreated SSH. All the treatments were significantly higher than control (2.32 mg/g) with maximum reducing sugar recorded in fungal pretreated SSH treated with crude enzyme extract of *Trichoderma viride* (62.34 mg/g) followed by fungal pretreated SSH treated with crude enzyme extract of *Aspergillus niger* (58.25 mg/g).Similar results were obtained by Song et al. (2013) who showed that enzymatic saccharification of *Irpex lacteus* pretreated corn stover showed 275.3 mg/g of reducing sugar over a period of 42 days. Results are also in accordance with Bradley *et al.* (1989). According to Lee, (1997) pretreatment with white-rot fungi modifies the structure of biomass and enhances the efficiency of enzymatic hydrolysis. As shown in Fig. 4, only with fungal pretreatment ethanol production, was limited to 13.67 mg /g but with the addition of crude extract, reducing sugar in extract increased to 62.34 mg/g.

5.7 Effect of alkali pre treatment on proximate composition of SSH

Alkali pretreatment refers to the application of alkaline solutions such as NaOH, $Ca(OH)_2$ (lime) or ammonia to remove lignin and a part of the hemicellulose, and efficiently increase the accessibility of enzyme to the cellulose. Alkali pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier *et al.*, 2005). Compared with acid processes, alkaline processes cause less sugar degradation, and many of the caustic salts can be recovered and/or regenerated (Mcdonald *et al.,* 1983).

5.7.1 Effect of microbial consortia on proximate composition of alkali pretreated SSH

Alkali pretreated SSH treated with *Aspergillus niger* and *Trichoderma viride* showed maximum release of reducing sugars (37.16 mg/g), non reducing sugars (78.38 mg/g) and total sugar (115.53 mg/g) which was significantly higher than all other treatments including control (2.42 mg/g; 3.17 mg/g; 5.59 mg/g) (Fig.4). Protein content estimated in the same treatment (11.34 mg/g) also showed a significant difference from all other treatments including control (3.36 mg/g). NaOH alters lignin structure hydrolyzing hemicellulose to xylose and other sugars. Sharma *et al,* (2004) conducted

experiments on fermentation of enzymatic hydrolysate of sunflower hulls for ethanol production and made similar conclusions suggesting that alkali treatment with NaOH was the best pretreatment for SSH. 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 hour at 121 °C) by using *Trichoderma viride* from delignified wheat straw within 3 days at 25-30 °C. The variations in release of reducing sugar due to inoculation of different fungi were reported by Narothama Prasad and Geeta (2011). They obtained a maximum reducing sugar of 31.45 mg/g in sugarcane bagasse, 26.41 mg/g 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 (2.56%) and 26.26 mg/g (2.62%), respectively in delignified paddy straw, wheat straw and sugarcane bagasse.

5.7.2 Effect of crude enzyme on proximate composition of SSH pretreated with alkali

Figure 6 depicts the effect of crude enzyme on proximate composition of sunflower seed hull (SSH) pretreated with alkali. Among all the treatment combinations, alkali pretreated SSH treated with the crude enzyme of *Trichoderma viride* released maximum reducing sugar (174.35 mg/g) and total sugar (217.56 mg/g) with a minimum non reducing sugar (46.29 mg/g) estimated in the same. All the values estimated for this treatment were found to be significantly higher than the control which released 2.50, 2.63 and 5.13 mg/g of reducing, non - reducing and total sugars, respectively.

From the above discussed results it is evident that alkali pretreatment followed by enzymatic hydrolysis was the best combination for ethanol production. This may be due to the fact that alkali pretreatment increases accessible surface area for enzymatic action making it the best combination treatment for ethanol production. Enzymatic hydrolysis has an added advantage of not causing formation of inhibitory compounds, little energy requirement, mild reaction conditions, high substrate specificity, high yield of sugars and high hydrolysis efficiency (Saratale *et al.*, 2010). These may be the contributing factors for better saccharification than microbial pretreatment.

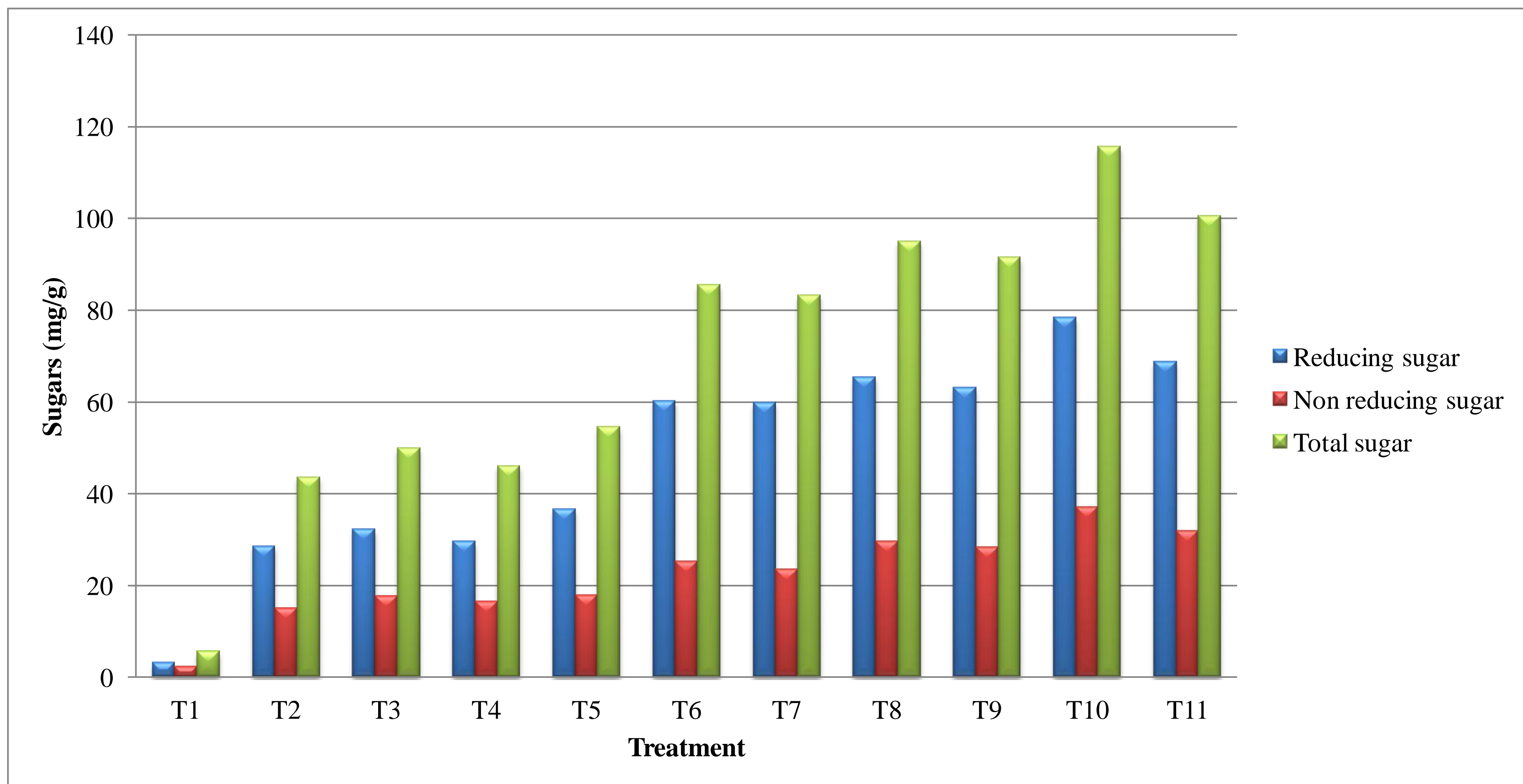


Fig. 5. Effect of alkali pretreated SSH hydrolysed with microbial consortia on proximate composition of SSH

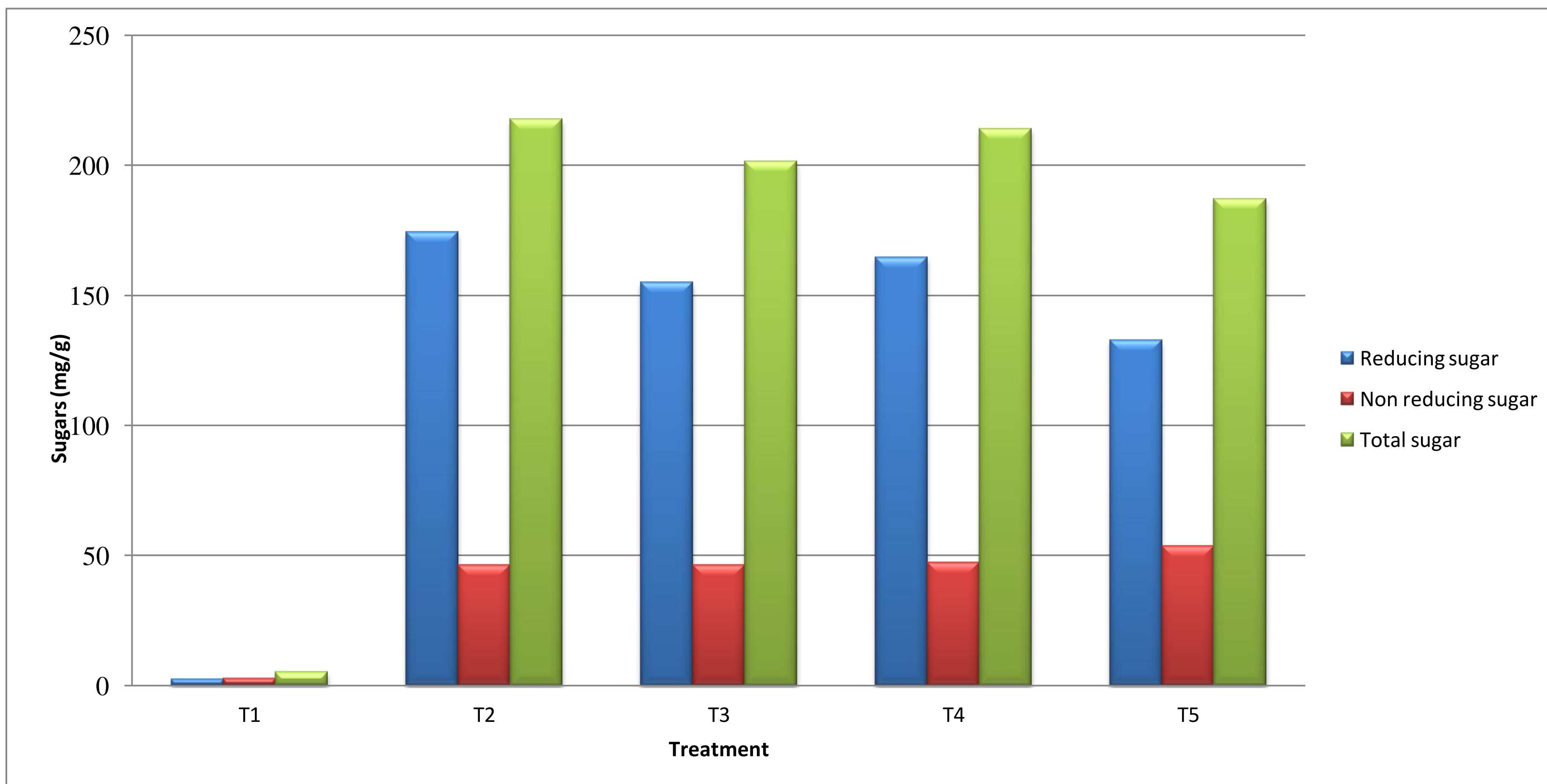


Fig. 6. Effect of crude extract on proximate composition of alkali pretreated SSH

Shankarappa (2013) obtained similar results 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 stating reason that perhaps the higher concentration of substrate had limited enzyme activity resulting in lower release of reducing sugars. Sharma *et al.* (2004) conducted experiments on SSHs wherein he observed that SSH pretreated with alkali, enzymatically hydrolysed by *T. reesei* Rut C- 30 enzyme produced maximum reducing sugar of 201.8 mg/g at 40 °C.

5.8 Ethanol production

The saccharified biomass is used for fermentation by several microorganisms. A wide variety of microorganisms are known to produce ethanol as a product of carbohydrate fermentation. Microorganisms which have received attention in industrial alcohol production include a wide range of yeasts, some molds and a number of specialized bacteria. In the present study, ethanol production was estimated for each of the above enlisted pretreatment and treatment combinations taking only the best treatment.

5.8.1 Ethanol production by yeasts and bacteria from fungal pretreated SSH and Fungal treated SSH hydrolysed with crude enzyme

Observations based on the results pertaining to Figure 7 and 8 revealed that, among the different microorganisms used for ethanol production, *Zymomonas mobilis* produced maximum ethanol when compared *to Pichia stipitis* and *Saccharomyces cerevisiae*. Among *Zymomonas* isolates, local isolate ZMI 3 produced maximum ethanol (4.36 g/l) after 48 h of incubation with a fermentation efficiency of 62.53%, while reference strain NCIM 2915 produced maximum ethanol of 4.17 g/l after 48 h of anaerobic condition. Compared to *Zymomonas mobilis, Pichia stipitis* (NCIM 3498) produced less ethanol after 48 h of incubation with a fermentation efficiency of 54.93%.

The pattern of ethanol production was shown to be similar for these two organisms over a period of time. *Saccharomyces cerevisiae* showed a varying pattern of ethanol production, wherein ethanol production showed an appreciable increase over a period of 24 h (anaerobic condition), following which a gradual decrease in ethanol was noted till 48 h. This result is in accordance with observations recorded by Akponah *et al*, (2013). *S. cerevisiae* (SCI 7) produced ethanol, ranging from 0.14 mg/g to 2.79 mg/g over a period of 48 hrs, with maximal production at 24 hrs of incubation (3.06 mg/g), where as reference strain NCIM 3095 recorded maximum ethanol production (3.65 g/l) after 24 h of incubation with a fermentation efficiency of 61.67%. The variations exhibited among same isolates may be attributed to species variation.

Similar patterns of ethanol production was recorded with fungal pretreated SSH hydrolysed by crude enzyme extract of *Trichoderma viride,* where in local isolate ZMI 3 produced maximum ethanol (23.17 g/l) after 48 h of incubation with a fermentation efficiency of 72.87%. Compared to *Zymomonas mobilis, Pichia stipitis* (NCIM 3498) produced less ethanol of 22.97 g/l after 48 h of incubation with a fermentation efficiency of 72.94%. *S. cerevisiae* (SCI 7) produced maximum ethanol at 24 hrs of incubation (22.32 g/l).

The results are in accordance with studies conducted by Manjunath and Geetha (2007) on the effectiveness of fungal pretreatment of bagasse for ethanol production by *Zymomonas mobilis* and *Saccharomyces cerevisiae* wherein they obtained a maximum ethanol of 614.4 mg/l and 480 mg/l, respectively followed by *Candida shehatae* (398.40 mg/l). Four strains of *Zymomonas mobilis* were screened by Panesar *et al.* (2001) for their ability to produce ethanol from molasses medium at pH 6.

The results of this study in line with more reference stated that *Z. mobilis* can convert mixture of sugar into ethanol 92%-94% is greater compared to the yeast which only reached 88-90% (Silalahi, 1987). This possibility may be due to the fact that in ethanol fermentation of *Z. mobilis*, conversion of glucose into two molecules of ethanol produced one molecule of ATP. The low energy generated ATP resulted in cell mass produced low and high ethanol produced.

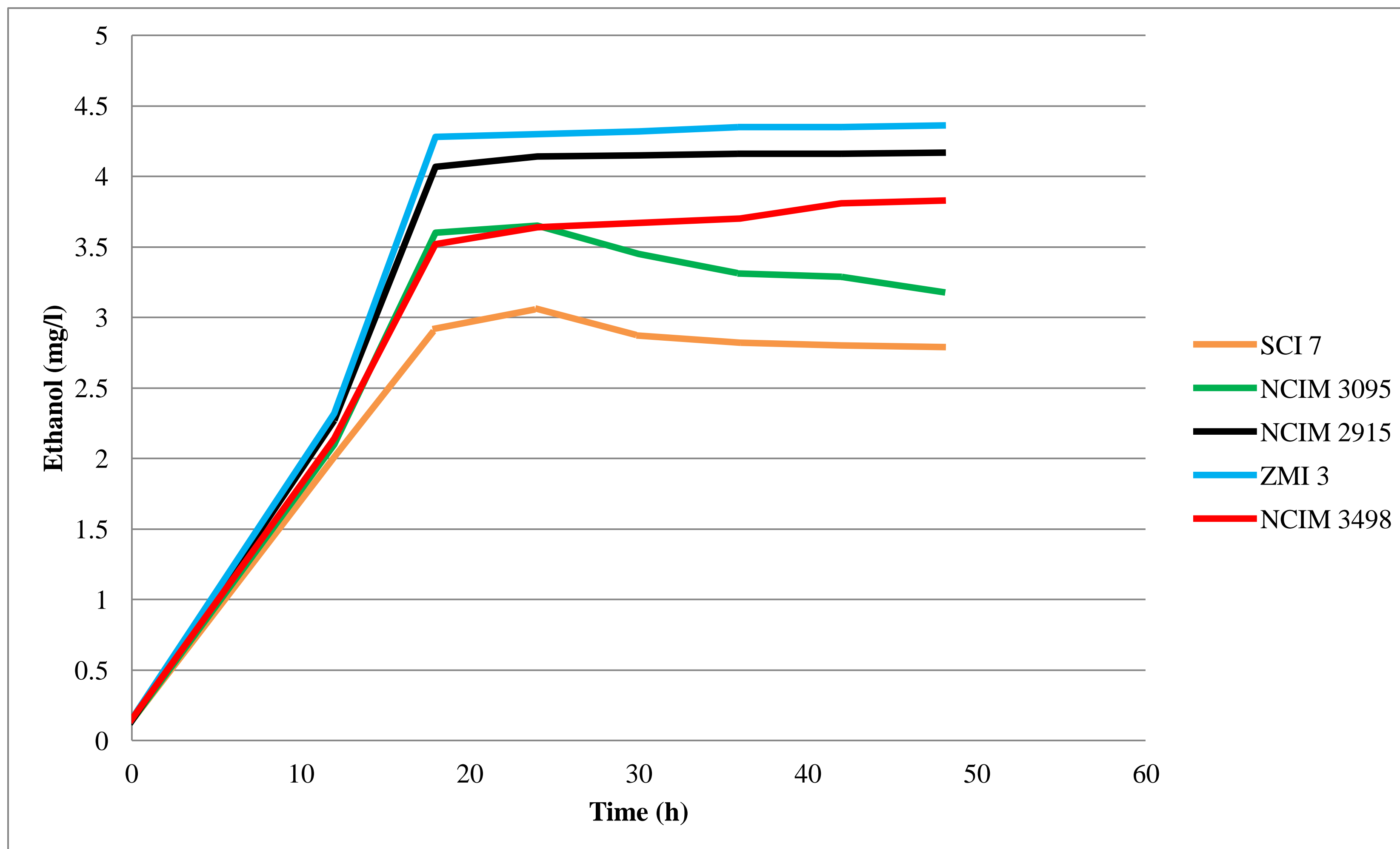


Fig. 7. Ethanol production by yeasts and bacteria from fungal pretreated SSH

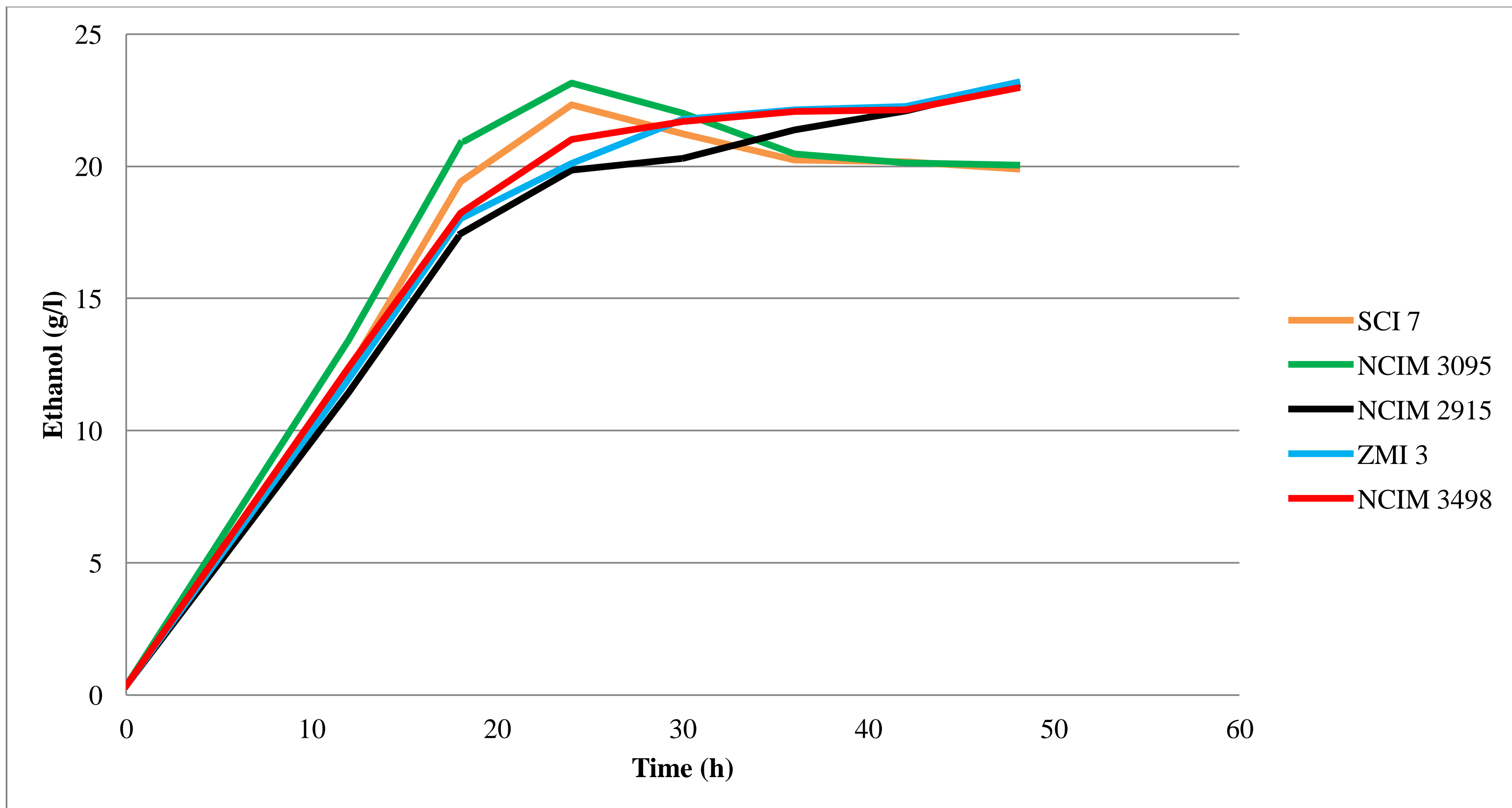


Fig. 8. Ethanol production by bacteria and yeast from fungal pretreated SSH hydrolysed by crude extract

5.8.2 Ethanol production by yeasts and bacterial strains from alkali pretreated SSH hydrolysed by fungal and crude enzyme extract treatment

Ethanol production by yeasts and bacteria from alkali pretreated SSH revealed similar observations as discussed in ethanol production from fungal pretreated SSH, but here an appreciable increase in ethanol recovery was noted even though fermentation efficiency was comparable, due to higher reducing sugar obtained during pretreatment (Fig 9, 10). Among the different microorganisms used for ethanol production, *Zymomonas mobilis* local isolate ZMI 3 produced maximum ethanol (26.97 g/l) after 48 h of incubation with a fermentation efficiency of 65.06% after 48 h of anaerobic condition when compared *to Pichia stipitis* and *Saccharomyces cerevisiae*. Compared to *Z. mobilis, P. stipitis* (NCIM 3498) produced less ethanol of 23.54 g/l after 48 h of incubation with a fermentation efficiency of 58.88%. *S. cerevisiae* (SCI 7) produced maximum ethanol at 24 hrs of incubation (21.04 g/l), where as reference strain NCIM 3095 recorded maximum ethanol production after 24 h of incubation with a fermentation efficiency of 61.16%. Similar patterns of ethanol production was recorded with alkali pretreated SSH hydrolysed by crude enzyme extract of *T. viride, Z. mobilis* local isolate ZMI 3 produced maximum ethanol after 48 h of incubation with a fermentation efficiency of 71.16% after 48 h of anaerobic condition when compared *to P. stipitis* and *S. cerevisiae*. Compared to *Z. mobilis, P. stipitis* (NCIM 3498) produced lesser ethanol of 67.12 g/l after 48 h of incubation with a fermentation efficiency of 60.49%. *S. cerevisiae* (SCI 7) produced maximum ethanol at 24 h of incubation (62.12 g/l). The difference in ethanol production by local and reference strains of same organism may be attributed to species variation. Similar studies conducted by Amrutha and Gunasekaran (2000) showed that liquefied cassava starch fermented by monoculture and co-culture using *Saccharomyces diastaticus* and *Z. 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). Similar results were obtained by Davis *et al.* (2005), wherein, they observed an ethanol production of 28 g per l by *Z. mobilis* ZM 4, when the hydrolysate of wheat stillage was supplemented with 5 g per l yeast extract and

40 g per l glucose with residual xylose of 2.6 g/l. Rani *et al.* (2006) reported the initial reducing sugar to be 3.55 per cent. But, tend to decrease as the fermentation progressed and at the end of the fermentation reaction it was 1.68 per cent in case of *S. cerevisiae* and 0.91 per cent in case of *Z. mobilis* from the medium containing 50 g per l cane sugar, 5 g diammonium phosphate and 1 g yeast extract. Singh *et al.* (1990) observed saccharification of 76 per cent of alkali pretreated sugarcane bagasse yielding a reducing sugar of 4.2 mg per ml from 0.5 per cent substrate and 0.3 U per ml enzyme (cellulase) derived from *Aspergillus niger* in 48 h at 55 °C. Nimbkar *et al.* (1989) studied the sugars utilization by yeast during fermentation of juice of sweet sorghum with 20 per cent fermentation sugars at 48, 72 and 120 h and observed decrease in sugars content to 2, 4 and 1% at 48, 72 and 120 h, respectively.

Based on the results mentioned above, a lot of conclusions have been drawn, by many scientists debating upon the high efficiency of *Zymomonas a*nd *Pichia.sp* over *Saccharomyces*. The results of this study in line with more reference stated that *Z. mobilis* can convert mixture of sugar into ethanol 92%-94% is greater compared to the yeast which only reached 88-90% (Silalahi, 1987). This possibility may be due to the fact that in ethanol fermentation of *Z. mobilis*, conversion of glucose into two molecules of ethanol produced one molecule of ATP. The low energy generated ATP resulted in cell mass produced low and high ethanol produced.

According to Algar and Scopes, (1985), in *Z. mobilis,* the E-D pathway enzymes are more tolerant to ethanol, as the cell-free system of *Z. mobilis* can rapidly consume glucose and produce ethanol more than 15% w/v. The cell membrane of *Z. mobilis* has acquired altered fatty acid content to counteract the adverse effects of ethanol. The major fatty acids occurring in *Z. mobilis* are myristic acid, palmitic acid, and *cis*-vaccenic acid. Among the phospholipids, phosphotidyl ethanolamine is the most abundantly present. The high concentrations of cis-vaccenic acid and unusual hopanoids in the membrane are responsible for the high ethanol tolerance (Buchholz *et al.,* 1987). Adding to this, bacteria are known to multiply faster than yeast thus *Z. mobilis* might have reached the lag phase faster than *S. cerevisiae* and therefore utilized its substrate faster.

Pichia stipitis has the highest known natural ability of any yeast to directly ferment xylose, converting it to ethanol. Research has revealed that engineered strains of *S. stipitis* produced 57 g/l ethanol from pure xylose in under 48 h and adapted strains

produced significant amounts of ethanol from acid hydrolysates of lignocellulose. SSH contains 21% xylan, which can be effectively broken down into ethanol by *Pichia.sp* but the fermentation efficiency of *Zymomonas* is more than *Pichia* due to its high ethanol tolerance, thereby making it the second best fermenting organism in this study. *S. cerevisiae* is preferred for ethanol production from grain and sugar cane, because it ferments hexose sugars very rapidly and is very robust. However, it does not natively metabolize xylose. This limits the usefulness of *S. cerevisiae* in the production of fuels and chemicals from plant cell walls, which contain a large amount of xylose. In response to this problem, *S. cerevisiae* has been engineered to ferment xylose through the addition of the *S. stiptis* genes, XYL1 and XYL2, coding for xylose reductase and xylitol dehydrogenase, respectively. The concerted action of these enzymes converts xylose to xylulose, which is naturally fermented by *S. cerevisiae* (Jeffries, 2007).

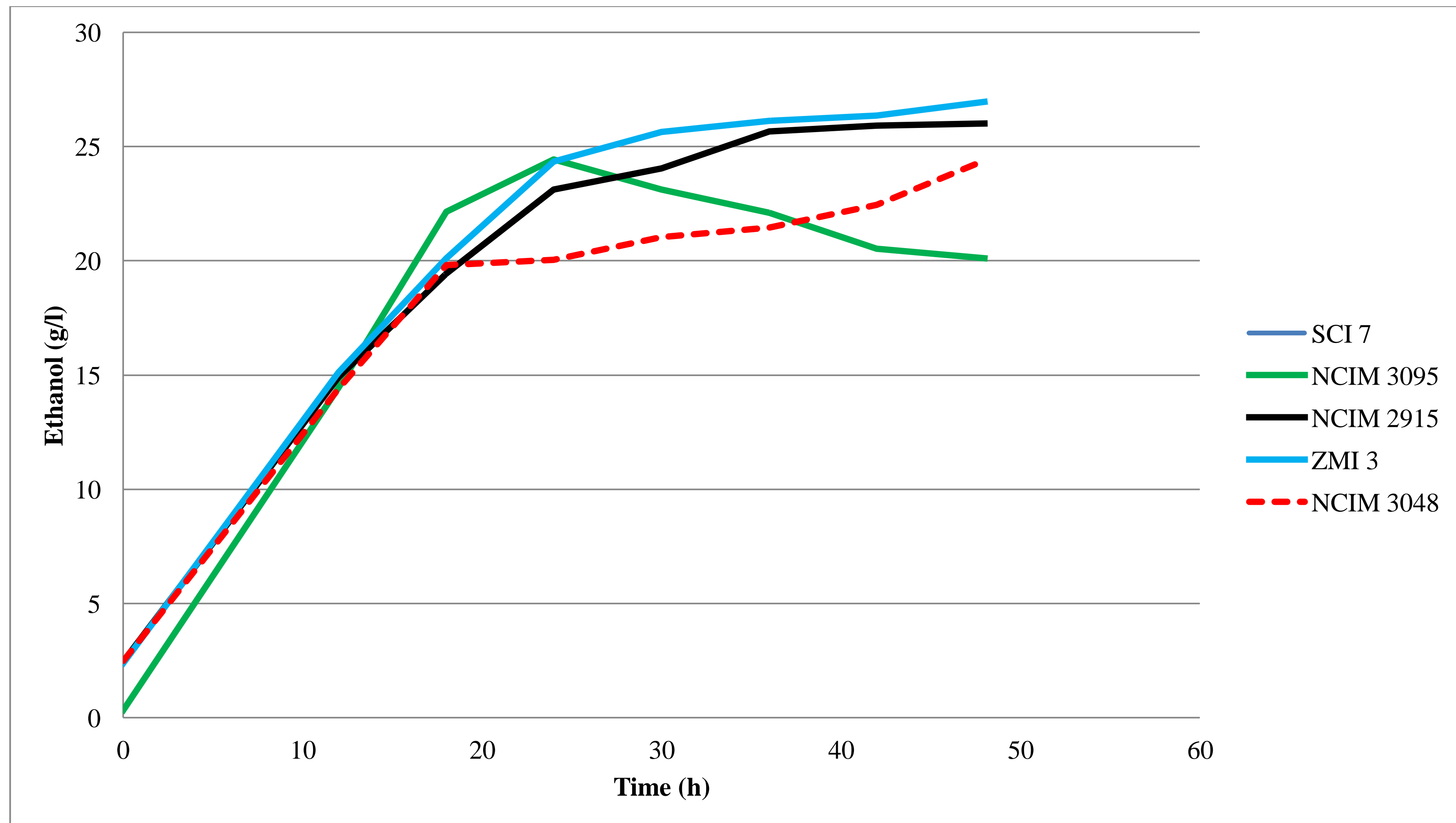


Fig. 9. Ethanol production by yeasts and bacteria from alkali treated SSH hydrolysed by fungi

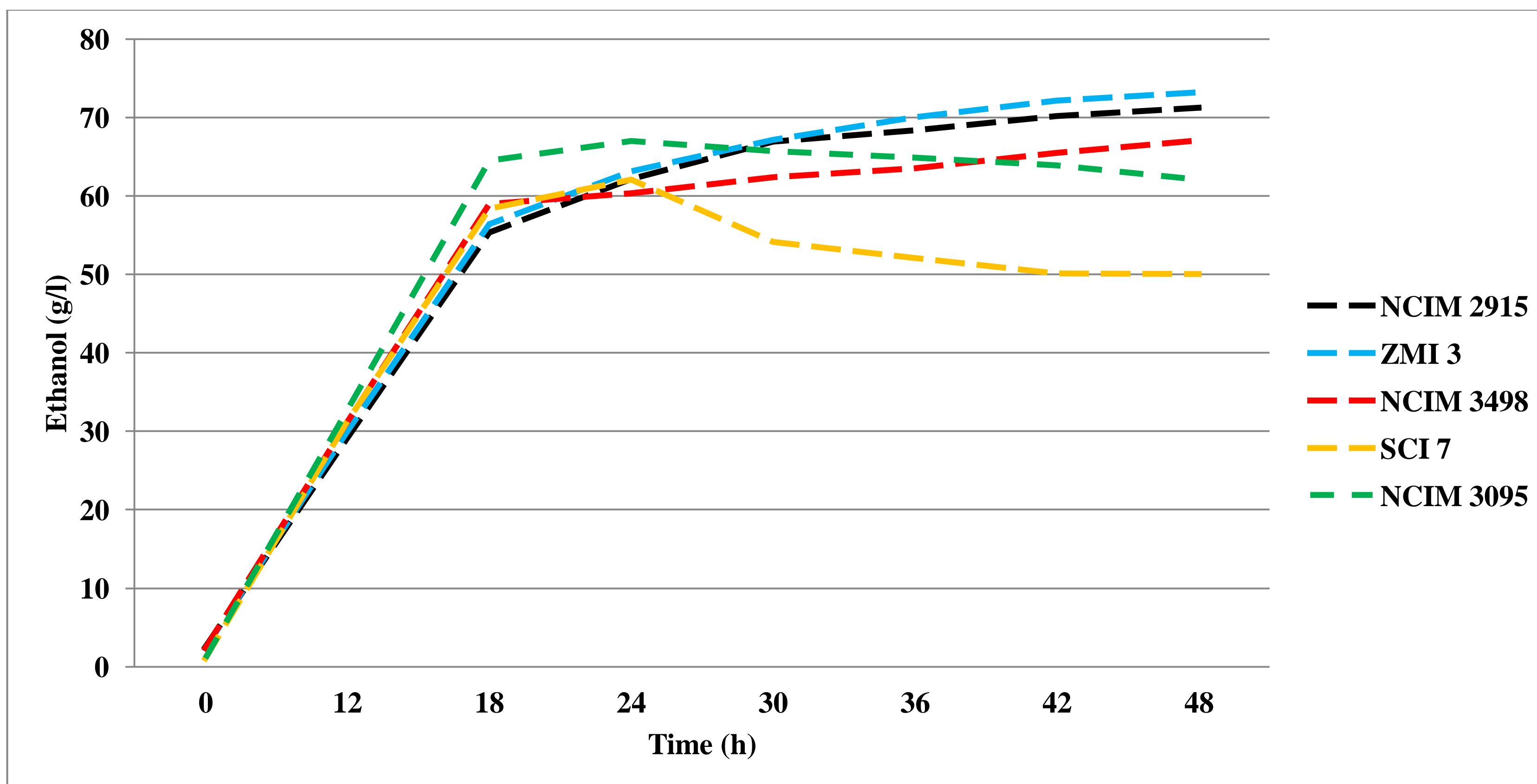


Fig. 10. Ethanol production by bacteria and yeasts on alkali pretreated SSH hydrolysed by crude extract

SUMMARY AND CONCLUSIONS

VI. SUMMARY AND CONCLUSIONS

Present investigation was undertaken at the Department of Agricultural Microbiology, College of Agriculture, Raichur, Karnataka during 2013-14 with the objective of isolating efficient lignocellulosic fungi for effective pretreatment of Sunflower Seed Hull (SSH), followed by its hydrolysis into fermentable sugars using a combination of treatments, there after treating the hydrolysate with ethanological yeasts and bacteria to produce ethanol. The results of the present investigation are summarized as follows.

- A total of 50 lignocellulosic fungi were isolated from compost pits situated in Raichur and Gulbarga districts, out of which morphological characterization revealed 15 isolates as *Trichoderma viride*, 15 as *Aspergillus niger*, 10 as *Aspergillus awamori* and 8 as *Phanerochaete chrysosporium*
- The isolates were further screened qualitatively using plate assays and quantitatively using enzymatic assays with reference strains as control, where in all the isolates were found to be good lignocellulose degraders. Out of all the isolates *Trichoderma viride* CTV 5 isolate expressed highest CMCase and FPU activity, while CPC 3, isolate of *Phanerochaete chrysosporium* exhibited maximum xylanolytic activity.
- Efficient ethanological yeasts and bacteria were isolated from grape and sugarcane juice respectively and were studied for their morphological and biochemical characters where in, 8 isolates were identified as *Saccharomyces cerevisiae* and 3 as *Zymomonas mobilis*. All the ethanological strains, including reference strain *Pichia stipitis* , were screened for ethanol production, from which results obtained show that among the isolated bacterial strains, *Z. mobilis* (ZMI 3) showed maximum conversion efficiency followed by *P. stipitis* and *S cerevisiae* (SCI 7).
- Fungal pretreatment with consortia of *Trichoderma viride* and *Aspergillus niger* alone showed low reducing sugar of 13.67 mg/g, fungal pretreated SSH hydrolysed with fungal crude enzyme extract of *Trichoderma viride* released 62.34 mg / g of reducing sugars.
- SSH pretreated with alkali hydrolysed with fungal consortia of *Trichoderma viride* and *Aspergillus niger* released more reducing sugar of 78.38 mg/g higher than

microbial pretreated SSH, which shows that alkali pretreatment followed by hydrolysis with fungal consortia is more effective for SSH than fungal pretreatment alone, but is less when compared to enzymatic hydrolysis, which produced maximum reducing sugar of 174.35 mg/g when treated with crude enzyme extract of *Trichoderma viride*.

- From each treatment combinations, ethanol production was estimated using efficient strains of locally isolated *Zymomonas mobilis*, *Saccharomyces cerevisiae* and their reference strain including *Pichia stipitis.* Out of all the treatments , local isolate of *Zymomonas mobilis* (ZMI 3) treated with SSH hydrolysed by crude extract and pretreated by alkali produced maximum ethanol of 73.24 g/ l ethanol with a high fermentation efficiency of 82.36% followed by *P. stipitis* (NCIM 3498) and *S. cerevisiae* (NCIM 3095) with an efficiency of 75.48% and 62.03%.

In conclusion, Sunflowe seed hull pretreated with local isolates of lignocellulosic fungi followed by hydrolysis with its crude enzyme extract released more reducing sugar when compared to fungal pretreatment alone, which reveals the importance of biological enzymes in saccharification. In this study, local isolates of both *Z. mobilis* and *S. cerevisiae,* exhibited potential for ethanol production with maximum fermentation efficiency of 82.36% which demonstrates the suitability of indigenous *Z.mobilis* and *S. cerevisiae* for ethanol production from sunflower seed hull. Therefore, harnessing the potentials of these organisms with correct biological pre treatment combinations would greatly help utilize lignocellulosic wastes for ethanol production minimizing the production cost.

Future line of work

- Optimization of growth conditions for lignocellulolytic fungi for effective pretreatment.
- Optimization of the enzyme mixture and hydrolytic reaction in order to improve sugar yield.
- Over expression of ß glucosidase enzymes in lignocellulolytic fungi for improved lignocellulotic activity.
- Genetic engineering of *Saccharomyces* for xylanolytic activity.
- Identification/ development of ethanological bacteria and yeasts which can ferment severely inhibiting hydrolysates.

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APPENDIX

APPENDIX

A. REAGENTS

- **Di Nitro Salicylic Acid (DNSA)**: one gram of 3, 5-dinitrosalycylic acid (DNSA), 200 mg of crystalline phenol and 50 mg of sodium sulphite were dissolved in 100 ml of one per cent NaOH and was stored at 40 $^{\circ}$C.
- **Rochelle salt solution** (40%): It was prepared by dissolving 40 g of potassium sodium tartrate in 100 ml distilled water.
- **One N hydrochloric acid**: About 8.8 ml of concentrated HCl was diluted with distilled water to a final volume of 100 ml in a 100 ml volumetric flask.
- **0.1N hydrochloric acid**: About 0.88 ml of hydrochloric acid was diluted and the volume was made up to 100 ml with distilled water in a volumetric flask.
- **1N sodium hydroxide**: About 4 g of NaOH was dissolved in little amount of distilled water and the final volume was made up to 100 ml in a 100 ml volumetric flask.
- **Phenolphthalein indicator**: Phenolphthalein powder of 1 g was dissolved in 50 ml of70 percent alcohol. The final volume was made up to 100 ml with distilled water.
- **Alkaline copper reagent**

  Solution A- 2% Sodium Carbonate in 0.1 N NaOH

  Solution B- 1% Sodium Potassium Tartarate.

  Solution C- 0.5% Copper Sulfate

  Solutions A, B and C were mixed in 100:1:1 proportion just before use.

- **Citrate Phosphate buffer of pH 4.8 (0.1 M)**

Citrate phosphate buffer was prepared by dissolving 0.3708 g of citric acid and 2.392 g of sodium phosphate in distilled water, pH of the buffer was adjusted to 5.0 and the volume was made to 100 ml with distilled water.

- **Potassium dichromate ($K_2Cr_2O_7$) 0.23 N**: About 34 g of $K_2Cr_2O_7$ was dissolved in 500 ml of distilled water. To this 325 ml of concentrated sulphuric acid was added and the volume was made upto 1000 ml with distilled water.

- **Carboxymethyl cellulose 1% (w/v)**

One g of CMC was dissolved in distilled water and volume made up to 100 ml. It was left for one day for complete dissolution

Xylose 0.1% (w/v)

On hundred ml of xylose was dissolved in distilled water and made up to 100 ml

B. STANDARD SOLUTION

Glucose standard

- Stock solution of glucose: Standard stock solution having the concentration of 1 mg glucose/ml was prepared by dissolving 100 mg of D-glucose in small amount of distilled water and final volume was made up to 100 ml with distilled water.

- Working standard solution: About 10 ml of the stock was diluted to 100 ml with distilled water in a 100 ml volumetric flask to obtain the glucose concentration of 100 µg glucose/ml.

- **Protein standard**

1. Stock Protein Standard Solution: Fifty mg of bovine serum albumin (BSA) was dissolved in distilled water and made up to 50 ml in a volumetric flask to obtain a concentration of 1mg protein per ml.

2. Working Standard Solution: Ten ml of the stock standard solution was diluted to 100 ml of distilled water in a volumetric flask to obtain a concentration of 100 µg of protein per ml.

Ethanol standard

1. Preparation of stock solution : It was prepared by mixing 12.6 ml of analytical grade ethanol (789 mg/ml) with little amount of distilled water and making up the volume to 100 ml using distilled water, this gives 100 mg ethanol/ml.

C. MEDIA

- **Cellulolysis basal medium**

Component	Amount
$C_4H_{12}N_2O_6$	5 g
Yeast Extract	0.1 g
KH_2PO_4	1 g
$CaCl_2.2H_2O$	0.01 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Distilled water	1.0 L

- **Czapeck Dox Medium**

Component	Amount
Glucose	20.0 g
KH_2PO_4	1.0 g
KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	50.0 mg
$ZnSO_4.7H_2O$	50.0 mg
$NaNO_3$	1.0 g
Agar	20.0 g
Distilled water	1L

- **LME basal medium (LBM)**

Component	Amount
KH_2PO_4	1 g
Yeast Extract	0.01 g
$C_4H_{12}N_2O_6$	0.5 g
$CuSO_4.5H_2O$	0.001 g
$MgSO_4 \cdot 7H_2O$	0.5
$Fe(SO_4)_3$	0.001 g
$CaCl_2 \cdot 2H_2O$	0.01 g
$MnSO_4.H_2O$	0.001 g
Distilled water	1.0 L

- **Mandels agar media**

Urea	:	10.0 g
Peptone	:	0.3 g
Yeast extract	:	0.75 g
$(NH_4)_2SO_4$	:	0.25 g
KH_2PO_4	:	1.4 g
$CaCl_2$	:	2.0 g
$MgSO_4.7H_2O$	:	0.3 g
Distilled water	:	1 L

- **MGYP medium (Yeast culture)**

Malt extract	:	0.3 g
Glucose	:	1.0 g
Yeast extract	:	0.3 g
Peptone	:	0.5 g
Distilled water	:	100.0 ml
Agar	:	72.0 g
pH	:	6.4-6.8

- **MR-VP Medium (Glucose Phosphate Broth)**

Buffered peptone N	:	7.0 g
Dextrose	:	5.0 g
Dipotassium phosphate	:	5.0 g
Final pH (at 25°C)	:	6.9 ± 0.2

- **MYG medium**

Malt extract	:	0.5 g
Yeast extract	:	1.0 g
Glucose	:	100.0 ml
Distilled water	:	1 L
Agar	:	2.0 g

- **Potato Dextrose Agar (Fungal cultures)**

Two hundred g of peeled potatoes are cut into small pieces and suspended in 1000 ml of distilled water and steamed for 30 min. Decant the extract or filter through muslin cloth and make the final volume to 1000 ml. Add 20 g of Dextrose,0.1 g of yeast extract and 20.0 g of agar.

- **RM medium**

Glucose	:	20.0 g
Yeast Extract	:	10.0 g
K_2HPO_4	:	2.0 g
Agar (if needed)	:	15.0 g
DI water	:	1000 ml

- **Sabouraud Dextrose Agar**

Dextrose	:	40.0 g
Mycological, peptone	:	10.0 g
Agar	:	15.0 g
pH	:	l5.6±0.2

- **YPD medium for Zymomonas**

Yeast extract	:	0.5 g
Peptone	:	0.5 g
Dextrose	:	2.0 g
Distilled water	:	1 00.0 ml
Agar	:	2.0 g
pH	:	6.2-6.5

- **Xylanolysis basal medium (XBM)**

$C_4H_{12}N_2O$	:	5 g
Yeast Extract	:	0.1 g
KH_2PO_4	:	1 g
$CaCI_2.2H_2O$	:	0.001 g
$MgSO_4.7H_2O$	:	0.5 g
Distilled water	:	1.0 L

BIOLOGICAL PRETREATMENT OF SUNFLOWER SEED HULL FOR ETHANOL PRODUCTION

SNEHA S. NAIR **2014** **Dr. K TAMIL VENDAN**

Major Advisor

ABSTRACT

Over the last few decades, excessive consumption of fossil fuels has resulted in high level of pollution, particularly in urban areas. With the expansion of human population and increase of industrial prosperity, global energy consumption also has increased gradually. In this scenario, renewable sources such as sunflower seed hull, high in cellulose, having disposal problem owing to its low bulk density, can be converted to fermentable sugars for the production of bio ethanol. Therefore, the present study was conducted on bioethanol production from sunflower seed hull with the objective of achieving higher recovery of fermentable sugars through delignification and saccharification through biological and chemical methods followed by fermentation of the derived sugars to bioethanol using efficient ethanological bacteria and yeasts. SSH was initially delignified by pretreatment with different lignocellulolytic fungi *viz., Trichoderma viride*, *Aspergillus niger*, *A.awamori* and *Phanerochaete chrysosporium* isolated from compost pits. The delignified substrates were further saccharified using cellulolytic fungi and their crude enzymes. While fungal pretreatment with consortia of *T. viride* and *A. niger* alone showed low reducing sugar of 13.67 mg/g, fungal pretreated sunflower seed hull hydrolysed with fungal crude enzyme extract of *T. viride* released 62.34 mg / g of reducing sugars. Following saccharification, the pre-treated substrates were fermented to bioethanol using local and reference strains of *Zymomonas mobilis*, *Saccharomyces cerevisiae* and *Pichia stipitis* where, inoculation with local strain of *Z. mobilis* (ZMI 3) , resulted in the highest ethanol yield of 73.24 g/l ethanol recording a high fermentation efficiency of 82.36% followed by *P. stipitis* (NCIM 3498) and *S. cerevisiae* (NCIM 3095) recording an efficiency of 75.48% and 62.03% , respectively which demonstrates the suitability of indigenous *Z. mobilis* and *S. cerevisiae* for ethanol production from sunflower seed hull