

**STUDIES ON ETHANOL PRODUCTION
FROM AGRO-RESIDUES**

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**STUDIES ON ETHANOL PRODUCTION
FROM AGRO-RESIDUES**

*Thesis submitted to the
University of Agricultural Sciences, Dharwad
in partial fulfilment of the requirements for the*

degree of

MASTER OF SCIENCE (AGRICULTURE)

in

AGRICULTURAL MICROBIOLOGY

By

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C E R T I F I C A T E

This is to certify that the thesis entitled "STUDIES ON ETHANOL PRODUCTION FROM AGRO-RESIDUES" submitted by Mr. MANJUNATH S. GURAV for the degree of MASTER OF SCIENCE (AGRICULTURE) in AGRICULTURAL MICROBIOLOGY to the University of Agricultural Sciences, Dharwad is a record of research work done by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any other degree, diploma, associateship, fellowship or other similar titles.

Place : DHARWAD

Date : 15-7-04


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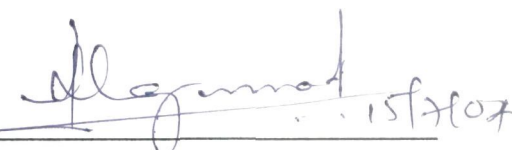
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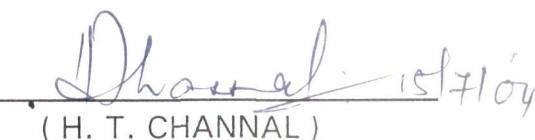
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(MANJUNATH S. GURAV)

CONTENTS

Chapter No.	Title	Page No.
I.	INTRODUCTION	01
II.	REVIEW OF LITERATURE	04
III.	MATERIAL AND METHODS	20
IV.	EXPERIMENTAL RESULTS	34
V.	DISCUSSION	62
VI.	SUMMARY	75
VII.	REFERENCES	79
	APPENDICES	95

LIST OF TABLES

Table No.	Title	Page No.
1.	List of substrates along with source	21
2.	List of cultures along with source	21
3.	List of microbiological treatments	23
4.	Initial chemical constituents in raw substrates of paddy straw, wheat straw and bagasse	35
5a.	Effect of microbiological pretreatment on sugar and other Constituents in paddy straw	37
5b.	Effect of microbiological pretreatment on sugar and other constituents in Wheat straw	40
5c.	Effect of microbiological pretreatment on sugar and other constituents in Bagasse	43
6a.	Effect of crude cellulase enzyme on release of reducing sugar at interval of 5 days and 10 days in paddy straw	46
6b.	Effect of crude cellulase enzyme on release of reducing sugar at interval of 5 days and 10 days in wheat straw	46
6c.	Effect of crude cellulase enzyme on release of reducing sugar at interval of 5 days and 10 days in bagasse	46
7.	Effect of crude cellulase enzyme (from <i>Trichoderma reesei</i> on <i>M&R Medium</i>) on the release of sugar in 3 crop residues	49
8a.	Ethanol production in fungal pre treated, Paddy straw (with substrate)	51
8b.	Ethanol production in fungal pre treated, wheat straw (with substrate)	53
8c.	Ethanol production in fungal pretreated, bagasse (with substrate)	55
9a.	Ethanol production in fungal pre treated Paddy straw (without substrate)	57
9b.	Ethanol production in fungal pre treated wheat straw (without substrate)	59
9c.	Ethanol production in fungal pretreated bagasse (without substrate)	61

LIST OF FIGURES

Figure No.	Title	Between Pages
1.	Effect of microbiological pretreatment on total and reducing sugar (mg g^{-1}) and cellulase (IU) in paddy straw	37-38
2.	Effect of microbiological pre treatment on total sugar, reducing sugar (mg/g) and cellulase (IU) in wheat straw	40-41
3.	Effect of microbiological pre treatment on total sugar, reducing sugar (mg/g) and cellulase (IU) in bagasse	43-44
4.	Effect of crude cellulase enzyme (from <i>Trichoderma reesei</i>) on release of sugars in three substrates	49-50
5.	Ethanol Production in fungal pretreated Paddy, Wheat straw and Bagasse (with substrates) mg L^{-1}	51-52
6.	Ethanol Production in fungal pretreated Paddy, Wheat straw and Bagasse (without substrate) mg L^{-1}	57-58

LIST OF PLATES

Plate No.	Title	Between Pages
1.	Microbiological pre-treatment for paddy straw	37-38
2.	Microbiological pre-treatment for wheat straw	40-41
3.	Microbiological pre-treatment for bagasse	43-44
4.	Cellulase enzyme extracted from MN and R media inoculated with <i>Trichoderma reesei</i>	55-56
5.	Experimental setup for fermentation	55-56

APPENDICES

Appendix No.	Title	Page No.
1.	Potato dextrose agar medium (PDA)	95
2.	Malt extract glucose yeast extract - peptone (MGYP) agar medium	95
3.	Mandels and Reese Medium (M and R medium)	96
4.	<i>Zymomonas mobilis</i> selective media	97

Introduction

I. INTRODUCTION

Environmental pollution is the major challenge that world is facing today. Increase in population and advanced technologies resulted in fast depletion of the naturally occurring conventional fossil fuel reserves such as petrol, diesel, kerosene, coal etc. (Ramanathan, 2000). These non-renewable energy sources are mainly consumed in transport sector. India is a larger consumer of petroleum products. The transport sector accounts for almost 50 percent of the total energy consumed. According to estimates made by oil companies, the demand for motor gasoline and diesel will touch 13.2 and 77.6 million tons respectively by 2006-2007 (Reddy and Venkataraman, 2002). In India the numbers of vehicles registered in 1951 were 3 lakh, which has increased to 531 lakh in 2000-2001 (Uppal, 2001).

India has increased its refining capacity to 106.5 million tones from 61.5 million tones in order to meet such huge quantity of petroleum requirement. But India is still importing nearly 70% of its annual crude petroleum requirement, which is approximately 110 million tons, and the expenditure on crude purchase of these petroleum products is in the range of 800 billion dollars per year. Our sole dependence on petrol or diesel not only increases financial burden but also leads to environmental pollution due to the emission of smoke containing lead, benzene, sulfur dioxide, nitrogen oxide, carbon monoxide etc. These gases contribute to 64 percent of air pollution in our metros and adjoining suburbs, leading to health hazards. Therefore, it is necessary to search for alternate energy source, which are non-petroleum based and renewable, such as solar, wind and biomass.

Among these, biomass (dedicated energy crops) is the most important and excellent energy source from which fuels of solid, liquid and gaseous nature can be produced. Among the liquid fuels, ethanol is used as an

alternative to petroleum by blending with gasohol at the rate of 20 percent (Ramanathan, 2000). Scientific research confirms that biofuel has a less harmful effect on human health than petroleum fuel. Biofuel emission have decreased level of polycyclic aromatic hydrocarbons (PAH) and nitrated PAH compounds that have been identified as cancer causing compounds. Also biofuel are non toxic, biodegradable and improve biomass productivity, create employment opportunities and enhance rural purchasing power. So ethanol has tremendous potential to dominate the alternative fuel scenario in the future.

Thirty-five countries in the world are using 10 percent alcohol with petrol and 15 percent alcohol with diesel. The admixture of petrol-alcohol and diesel-alcohol are known as gasohol and diesohol respectively. The alcohol producing countries include USA, Argentina, Brazil., Zimbabwe and India. Brazil at present saves 8 billion dollars per year by blending 20 per cent of ethanol along with petrol. Presently, 65 per cent of alcohol is produced by America, 18 percent by Asia and 15 percent by Europe (Ahuja, 2001).

Our country has the potential to save nearly 80 million liters of petrol annually if 10 percent alcohol is blended with petrol. Ethanol is an alcohol produced by fermenting sugars present in sugar rich biomass. All over the globe, 60 percent alcohol is produced from sugar crops, 38 percent by other crops and only 7 percent by synthetic means (Ahuja, 2001).

In India, there are 295 distilleries producing 1058 million liters of alcohol as against annual consumption of 1266 million liters (Arbatti, 2001). Nearly 90 percent of alcohol is produced from molasses, a byproduct of sugarcane industry. However, due to the higher cost of molasses it has become necessary to search for an alternate biomass substrate and also technologies for ethanol production. Although the cost of ethanol production from

lignocellulosic biomass has been reduced over the past 14 years. Earlier it was 3.60 dollars per gallon but now it is 1.22 dollars per gallon (Lynd *et al.* 1991). The other alternate biomass sources tried successfully are the pineapple, cannery waste and starch (Nigam, 1999).

According to the available statistics, paddy, wheat and sugarcane are the major crops in India. It is reported that 606 mt of paddy straw, 299 mt of wheat straw and 87 thousand tonne of bagasse were produced per year. But these crop residues are burnt to sterilize the soil as it kills all the weed seeds, insect eggs and larvae and also to meet daily fuel requirement. It is also reported that 39% of agricultural biomass were burnt in Asia, 29% in Latin America, 13% in Africa and 41% in India (Reddy and Venkataraman, 2002). Instead of burning, if these lignocellulosics could be used for biofuel production, it could reduce the cost of production and decrease environmental pollution.

Since these crop residues are made up of complex polymers it is essential to pretreat it microbiologically for conversion of cellulosic biomass into fermentable sugar, for production of ethanol. Considering all these, the present investigation was taken up with different pretreatment methods and different fermentation methods for ethanol production with the following objectives.

1. To maximize the release of reducing sugars by microbiological and enzymatic pretreatment of agro-residues.
2. To standardize the process of ethanol production from above derived pretreated material.

Review of Literature

II. REVIEW OF LITERATURE

Cellulose is the most abundant constituent of organic matter of plant origin in the biosphere. It is predicted that 10^{15} kg of cellulose is synthesized and degraded on the earth every year. Cellulose can be extensively used only when it is broken down to glucose. Presently, molasses is being used in many of the distilleries as its sugar content is suitable for production of ethanol. But, its availability at high cost could increase the cost of production. Apart from molasses, other easily saccharifiable sugar rich substrates such as sugar beet, sweet sorghum, carob pods and rain tree pods have been identified so far as best alternate to molasses. The metabolic pathways adopted by yeast for utilizing various sugars viz., glucose, fructose; sucrose (Gunasekaran *et al.* 1994) and xylose (Kotter and Ciriacy 1993). Several approaches to improve the ethanol production are being employed which include selection of suitable and easily available substrates, ideal pretreatment method for utilizing the substrates including microbiological pretreatment and crude enzyme pretreatment (George *et al.*, 1992) and simultaneous saccharification and fermentation (SSF) (Krishna *et al.* 1998). The literature pertaining to these aspects have been reviewed.

2.1. SUBSTRATES

At present, molasses is the main substrate being used for production of ethanol. Molasses contains high amount of reducing sugar. Similar sugars have been observed in sweet sorghum, sugar beet and rain tree pods etc. which can form alternate substrates for ethanol production. Likewise many other cellulosic substrates also could be potential for ethanol production as

they contain considerable amount of bound sugars. The various biomass substrates having potential for alcohol production are being reviewed here.

An attempt to release maximum reducing sugars from bagasse and rice husk by acid hydrolysis was made by Singh *et al.* (1984). Hermann *et al.* (1986) obtained maximum ethanol of 41-42 g L⁻¹ from concentrated deproteinized whey having a lactose content of 23 percent by using *Z. mobilis*, immobilized with sodium alginate. Co-fermentation of sweet sorghum juice and grains was studied by Gibbons and Westby (1989) for production of fuel ethanol and obtained 3.5 percent (v/v) ethanol from 6.5 – 7.6 percent (w/w) reducing sugar after fermentation. Nimbkar *et al.* (1989) successfully fermented unsterilized juice of sweet sorghum by using *S. cerevisiae* strain 3319 and obtained maximum alcohol of 12.45 percent (v/v).

Pineapple wastes (containing 11.7% soluble sugars) was fermented for ethanol production by Bankoffi and Han (1990) and they obtained 0.8 percent ethanol in 48 hours. Lynd *et al.* (1991) obtained 400 billion liters of ethanol by microbial conversion of the sugar residues present in waste and yard trash and from U.S. landfills. Corn derived wastes alone contributed nearly 10 times of ethanol. Waste paper was treated with steam in a pressure vessel at 170 to 220°C, and then enzymatically saccharified and fermented for ethanol production by Capek *et al.* (1992) and obtained 460 liters/tonne of ethanol that is increase of 29 percent over control.

Starch crops such as corn and lignocellulosic biomass like grass, wood, agricultural wastes and municipal wastes were used for ethanol production from simultaneous saccharification and fermentation (SSF) by Wyamn and Goodman (1993) The technical possibilities of the microbial production of

ethanol from potato waste using *Clostridium acetobutylicum* DSM 1731 was studied by Grobber *et al.* (1993). A solid state fermentation process was developed by Pandey (1994) for efficient utilization of agro industrial residues such as cassava, bagasse, apple pomace, coffee pulp and husk etc for the production of ethanol, single cell protein, enzymes and organic acid etc. Takahashi *et al* (1994) assessed the potentiality of sugars *viz*, glucose (5 g L^{-1}) for biofuel production. It was reported that 42.5 g L^{-1} of ethanol was obtained in 96 hours yielding 0.49 g of alcohol per g of sugar and they noticed that first sugar to be consumed was glucose, followed by arabinose and xylose.

Premi Devi and Singh (1995) used acid hydrolysate of water hyacinth with 13 percent total reducing sugar for conversion to ethanol by using *C. shehate*. Olsson and Hahn (1996) carried out experiment to produce ethanol with a high yield from all sugars present (hexose as well as pentoses) in lignocellulosic hydrolysates. Duff and Murray (1996) used forest products, industrial waste such as pulp and paper industry waste for the production of biofuel. Hammond *et al.* (1996) constructed laboratory studies to assess the ethanol production potential from waste banana *viz*, whole fruit, pulp, peel and obtained ethanol yield of 0.91, 0.082 and 0.006 kg respectively. Marakis and Marakis (1996) used ripe deseeded carob fruit (*Ceratonia siliqua*) rich in water soluble sugars (54.7%) for production of ethanol and fructose syrup of 4.75 percent (v/v) and 10.49 percent (w/w) respectively.

Miyamoto (1997) obtained 150-200 liters of alcohol from 720 kg of raw material like sugarcane bagasse, rice straw, forest wastes in pilot scale unit. Dominguez *et al.* (1997) tested potentiality of corn cobs for biofuel production, which produced 5 g L^{-1} ethanol. Suchi Srivastava *et al* (1997)

obtained ethanol from guava pulp by using three different strains of *S. cerevisiae*.

Shirai *et al.* (1998) cultivated micro algae in the solution from the desalting process of soy sauce waste treatment and utilized the algal biomass for ethanol production of 11 mg g⁻¹ (dry cell weight) of *Dunaliella* cells. Bioethanol from wet oxidised wheat straw was observed by Ahring *et al.* (1999) using *Thermoanaerobacter mathranii*. An attempt to convert spruce and birch to ethanol by using *S. cerevisiae* was made by Mohammad *et al.* (1999). Ethanol yield of 2.90 percent (v/v) from damaged sorghum and 2.09 percent (v/v) was obtained by Suresh *et al.* (1999) from damaged rice grains by simultaneous saccharification and fermentation process.

Bvochora *et al.* (2000) obtained maximum ethanol of 16.8 percent (v/v) from mixture of sweet sorghum juice and sorghum grains. Fermentation was carried out using *S. cerevisiae* strain N96. The economics of fuel ethanol production from dry milled corn starch using *Z. mobilis* was studied by Krishna *et al.* (2000) and obtained ethanol yield of 24 g L⁻¹ from a 15 million gal yr⁻¹ capacity ethanol plant. The fermentation of root crops for ethanol namely cassava, potato, yam, sweet potato was studied by Ramanathan (2000).

Nakamura *et al.* (2001) reported that rice straw is a potential feed stock for production of power and fuel. The potentiality of pods of *Samanea saman* for ethanol production was revealed by Geeta *et al.* (2001). Shiva *et al.* (2001) conducted experiment for fermentation of agricultural wastes such as Jowar stalks and left over corn stalks to ethanol.

2.2. PRETREATMENT METHOD

Lignocelluloses (biomass) are composed of cellulose, hemicelluloses, lignin, protein and various extraneous matter of which cellulose component is viable source for sugars. The physical and chemical properties of cellulose depend on its tertiary structure and the degree of polymerization is inversely related to solubility (Cowling and Brown, 1969). Since it is in bound form the polymer needs external force to break down and release the sugars. Therefore, pretreatment methods are employed which may be physical, chemical or biological. Swelling in crystalline regions is obtained by suitable mechanical or physical chemical treatments such as steam treatment milling, mineral acids and alkaline treatment (Goksoyar and Erickson 1980). Ball milling and compression milling decrease the degree of crystallinity and also molecular weight of cellulose (Tassinari *et al.*, 1980). Adding small amount of mineral acid is useful in softening certain wood species with a high lignin content and low nitric acid content as they are less responsive to steam pretreatment alone (Noble and Nguyen, 1980).

2.2.1. Microbiological pretreatment

Mark *et al.* (1984) studied fungal activities involved in lignocellulose degradation by *Pleurotus*. They reported that after 4 days of treatment with *Pleurotus*, the activity of laccase, catechol oxidase, peroxidase and cellulase were detected and their activity declined rapidly after 8-10 days of growth. They also reported that lignin degradation began after 10 days and reached maximum after 21 days. Manuel *et al.* (1990) studied substrate dependent degradation patterns in the decay of wheat straw and beech wood by lignolytic fungi and degraded pattern defined in terms of chemical evolution of

substrates and changes in lignin and polysaccharides. They reported that *Trametes versicolor* caused an important degradation of lignin and increased substrate digestibility.

Lignin degradation and activities related to lignin degradation was studied in the solid state fermentation with two white rot fungi *Pleurotus ostreatus* and *P. chrysosporium* by Zohar *et al.* (1992). They concluded that *P. chrysosporium* grew vigorously, resulting in rapid, nonselective degradation of 55 percent of the organic component of cotton stalk within 15 days where as *Pleurotus ostreatus* grew slowly and resulted in 20 percent degradation after 30 days.

Masaaki and Takashi (1995) obtained maximum reducing sugars from wood meal, using lignin degrading fungus *P. chrysosporium* followed by steam explosion. The amount of sugar obtained was 76 percent at 28th day of incubation period and steam explosion at a steam temperature of 215°C for 6.5 minutes. Ammonia treated bagasse with 80 percent moisture content was subjected to mixed culture solid substrate fermentation with *Trichoderma reesei* LM: UC4 and *Aspergillus phoenicis* QM329, in flask for cellulase production. Maximum release of cellulase obtained on the 4th day of incubation that is 38.6 IU /g (Duenas *et al.* 1995).

Anne *et al.* (1996) obtained 65 percent w/w yield of glucose when wheat straw was subjected to steam pretreatment of temperature 170°C for 5-10 minutes. Zayed and Meyer (1996) examined *T. viride* and *A. niger* for their ability to produce fermentable sugars from cellulosic waste and achieved reducing sugar extraction of 27 g from 50 g delignified wheat straw at 25 to 35°C within 3 days. Stenberg *et al.* (1998) obtained maximum reducing sugar

of 42.1 g per 100 g of soft wood when treated with 2–6 per cent of SO₂ and subjected to steam pretreatment of 2100°C for 5 minutes. Ethanol yield of 0.83 g per l obtained from wheat straw when subjected to 370°C following single step process (Alfani *et al.*, 2000).

Geeta *et al.* (2002) studied the relative efficiency of different fungal cultures (*Aspergillus foetidus*, *A. niger*, *P. chrysosporium* and *T.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⁻¹ and accounts to 53.40 percent, followed, by *T.viride* and *A. niger*. Similarly, percent 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 per cent loss in total solids as compared to uninoculated, control and that inoculated with *Aspergillus foetidus*.

Vidyavathi (2003) reported that pods of *Samanea saman* treated with different fungal cultures, released maximum reducing sugars. Among them, *Aspergillus foetidus* NCIM 515 was efficient in release of maximum reducing sugar (24.26 g L⁻¹) and also loss in weight (48.32%) followed by *A. niger* NCIM616, which recorded 20.66 g L⁻¹ and loss in weight of 44 percent.

2.2.2. Enzymatic pretreatment

Fan *et al.* (1980) reported that lignocellulose materials, which are water insoluble, are recalcitrant to acid and enzymatic hydrolysis. The major structural features leading to such resistance are the degree of crystallinity of cellulose, the capillary structure in cellulose and lignin sheath associated with cellulose.

When *T. reesei* strain (L-84) was grown on different carbon source viz., whatman CC41, 2 percent wheat bran, paper pulp, rayon pulp, starch and lactose. The highest enzyme production was obtained with whatman CC41 Warzywoda *et al.* (1983). Gould (1984) recognized that delignification greatly enhances the susceptibility of cellulose to enzymatic hydrolysis and complete elimination of lignin is not necessary to solubilize most of the holocellulose in lignocellulosic material.

William *et al.* (1986) reported that steam pretreatment was found to be effective followed by enzymatic hydrolysis using enzymes from *T. reesei* and *A. niger* on hybrid poplarwood. They concluded that the effect of pretreatment was believed to be primarily due to the increase in pore surface area accessible to enzyme molecule.

The results presented by Turker and Mavituna (1987) indicates that immobilization of *T. reesei* with in polyurethane foam matrices has no detrimental effect on the growth pattern and enzyme production but the absence of nitrogen source in the production medium increases production up to 1.5×10^3 FPAUI⁻¹. Kahlon and Choudhary (1988) have compared the rate of hydrolysis of water hyacinth with acid (H₂SO₄ at 15 lb for 15 min) and enzyme (cellulase) and obtained maximum hydrolysis of 25.13 percent with enzyme and also correlated enzymatic saccharification for maximum ethanol yield than the acid treatment.

Azzam (1989) studied various process, conditions to optimize the enzymatic effectiveness. He obtained 95 percent of glucose when sugarcane bagasse treated with 2 percent alkaline hydrogen peroxide for 8 hours followed by cellulase enzyme treatment obtained by *T. viride*.

Robert *et al.* (1990) studied the optimization of temperature and enzyme concentration in the enzymatic saccharification of willow. They reported that maximum conversion of cellulose to glucose was obtained using a mixture of 15 percent celluclast and 3 percent Novozyme commercial enzyme at temperature of 40°C and they also reported that yield decreased with increasing temperature due to enzyme deactivation at higher temperatures. Inhibition of *T. reesei* cellulase by sugars and solvents was studied by Mark *et al.* (1990). They reported that glucose, cellobiose, ethanol and butanol were no competitive inhibitors, -gluconolactone was a mixed inhibitor and acetone was non-competitive activator of cellulase enzyme. Lars and Karl (1990) reported that monomeric sugar yield was only 12 percent when wheat straw was subjected to steam pretreatment. The yield of sugars increased to 70 percent when steam pretreated wheat straw, was subjected to enzyme treatment.

George *et al.* (1992) studied enzymatic hydrolysis of cellulose for production of fuel ethanol by simultaneous saccharification and fermentation (SSF) process and kinetic of cellulose hydrolysis by cellulase and β -glucosidase. They also found out that these two enzymes were inhibited by hydrolysis and fermentation products. According to them cellobiose and glucose were the strong inhibitors of cellulase and β -glucosidase respectively. Parminder *et al.* (1998) reported that release of reducing sugar was 268.5 mg g⁻¹ when rice straw was treated with 4 percent sodium hydroxide in combination with 60-minute steam pressure followed by enzyme treatment of concentration of 25 FPU g⁻¹ substrates.

Aspergillus and *Bacillus sp* were used as source of enzyme at 5 percent for hydrolysis of yam by Ramanathan (2000) and was able to obtain maximum

reducing sugar content of 84 g L⁻¹. An effective method was developed by Nikolov *et al.* (2000) for production of glucose, using enzymatic hydrolysis of waste cellulose fibers by the cellulase complex from *T. reesei* after pretreatment with H₃PO₄ (0.25%) which resulted 80 percent degradation of substrate.

Sugarcane bagasse was pretreated by Mark *et al.* (2002) along with hot water at temperature range of 170 to 230°C for 1 to 46 minutes and achieved 78.0 percent xylan recovery with 71.80 percent conversion by simultaneous saccharification and fermentation (SSF).

2.3. FERMENTATION (without substrate)

Zossi *et al.* (1990) performed ethanol fermentation of sugarcane juice for 7 days at pH 6.0 with supplementation of SO₂ and achieved fermentation efficiency of 96.9 percent. Mendoza and Raymundo (1990) optimized the fermentation conditions for batch alcohol production from molasses. The parameters selected were inoculum size, temperature, initial sugars and pH which resulted as 10 percent (v/v), 30°C, 10 percent and 5.5 respectively and observed that fermentation efficiency and ethanol yield of 83.3 to 92.6 and 4.6 percent respectively. Cane molasses (18% total sugar) at pH 4.7 was fermented for 40 hour by *S. cerevisiae* with ammonium sulphate (1.5 g L⁻¹) and other N source viz., yeast extract and difco betek. Efficient sugar utilization was with ammonium sulphate (Huertas 1990).

Ferrari *et al.* (1992) evaluated *P.stipitis* NPRLY 71224 for production of ethanol. A maximum ethanol concentration of 70.1 ml L⁻¹ from cane molasses at operating variables of pH 4.2 temperature 32°C in 30 days of operation was obtained by Garcia and Suarez (1992). Gu (1992) studied possibility of

using *Z. mobilis* instead of yeast for industrial alcohol production. They reported that *Z. mobilis* is efficient over *S. cerevisiae* sp ATCC 31821 regarding starch hydrolysis, fermentation rate and ethanol yield.

Kotter and Ciriacy (1993) studied xylose utilization of *S. cerevisiae* transformant that expressed two key enzymes (xylose reductase and xylitol dehydrogenase) derived from *P.stipitis* in xylose metabolism. The thermotolerant alcohol producing yeast strain *Kluyveromyces marxianus* IMB 3 was studied by Fleming *et al.* (1993). The culture grew on media containing 10 percent sucrose (W/V) at 45°C and was able to produce ethanol concentration of 33 g L⁻¹.

The residual sugar concentration of 13.47 g L⁻¹ was observed by Singh and Jain (1994) after fermentation of 100 g L⁻¹ sucrose medium yielding 38.54 g L⁻¹ of ethanol by *Z. mobilis* NRRLB-4286. Ryn *et al.* (1994) obtained 64.3 g L⁻¹ soluble starch with a mixed culture and the starch content in the damaged grains used was lower by 30 percent and 40 percent when compared with the fresh grain of sorghum. Sohn and Seu (1994) achieved maximum ethanol concentration of 86.6 g L⁻¹ by strain of *Kluyveromyces marxianus* from 20 percent (w/v) glucose medium at 40°C in 96 hours of fermentation period. Gunasekaran *et al.* (1994) investigated the fermentation of wheat starch hydrolysate by *Z.mobilis*. They reported that in batch fermentation sugar concentration as high as 223 g L⁻¹ could be fermented to 105 g L⁻¹ ethanol in 70 hours.

Brooks and Ingram (1995) obtained ethanol yield of 40 g L⁻¹ from mixed waste office paper by using recombinant strain of *Klebsiella oxytoca*, which ferment cellobiose and cellotriose to ethanol and also reduces the requirement

of fungal cellulase. Saucedo and Karim (1996) carried out fed batch mode of fermentation with 17 per cent (w/v) Xylose medium at temperature and pH of 34°C and 6.4 and obtained ethanol productivity of 1.8 g L⁻¹ after 40 hours of fermentation. Abate *et al.* (1996) tested pure and mixed cultures of *Z. mobilis* and *S. cerevisiae* for the production of ethanol by fermentation medium containing sucrose (2000 g L⁻¹) at 30°C and obtained ethanol yield of 1.5 g L⁻¹ of sugar consumed in 63 hours fermentation by mixed culture. Marakis and Marakis (1996) studied the effect of six different fermentation periods *viz.*, 0, 24, 48, 72, 90 and 100 hours on ethanol production from aqueous carob extract and achieved maximum alcohol concentration of 4.75 per cent (v/v) at 100 hours of fermentation period.

Scory and Bothast (1997) screened nineteen *Aspergillus* and ten *Rhizopus* strain for their ability to ferment simple sugars (glucose, xylose and arabinose) as well as complex substrates (Cellulose Xylan, corn fiber and corn germ pressing) of which three *Rhizopus* strain, were producing more than 31 g of ethanol by 72 hours and glucose, xylose, cellobiose and corn fiber were fermented with perspective yields of 100, 47, 80 and 40 percent of theoretical yield. Suchi Srivastava *et al.* (1997) used three isolates of *S. cerevisiae* for ethanol production from guava pulp and obtained maximum ethanol yield of 5.8 per cent (w/v) by isolate -2 compared to other two isolates. Co-immobilized cultures of *S. cerevisiae* and *C. shehate* which could convert glucose and xylose simultaneously to ethanol yield of 0.48 g g⁻¹ total sugars was studied by Lebeau *et al.* (1997). Ethanol fermentation from Dunaliella micro algae at 25°C was within 5 days and was able to obtain ethanol yield of 11 mg g⁻¹ of substrate (Shirai *et al.*, 1998)

The fermentation characteristics of *S. cerevisiae* strain, which over expresses a constitutive OLE1 gene, which is necessary for enhancing the ethanol productivity was studied by Kajiwara *et al.* (1999). An experiment was conducted for conversion of corn starch to fuel ethanol, which was 72.2 g L⁻¹ ethanol, produced in 120 minutes residence time (Krishna *et al.* 1999). Batch and fed batch fermentation were examined in a lab scale (3.3L) anaerobic bioreactor. Fed batch technique with a suitably adjusted feed rate was possible to completely ferment the glucose and mannose sugar (Mohammad *et al.* 1999). The fermentation of hydrolysate using *Thermoanaerobacter mathranii* strain A3M1 which can grow on undiluted wood hydrolysate and simultaneously production of ethanol was observed by Ahring *et al.* (1999).

A maximum alcohol yield in 3 days during fermentation of yam to ethanol by *S. cerevisiae* was observed by Ramanathan (2000). Ethanol production on a pilot scale for the conversion of high solids saccharified corn mash to ethanol by continuous fermentation and carbon dioxide stripping was demonstrated by Taylor *et al.* (2000).

Ethanol production by co-culture was studied by Verma *et al.* (2000) using *Saccharomyces diastaticus* and *S. cerevisiae*-21 in raw unhydrolysed starch, which yielded ethanol of 48 percent higher (24.8 g L⁻¹) than that obtained with the monoculture of *Saccharomyces diastaticus* (16.8 g L⁻¹). Gunasekaran and Amutha (2000) used mixed culture of an amylolytic yeast strain *Saccharomyces diastaticus* and *Z. mobilis* for improved ethanol production from cassava starch. The ethanol yield in mixed culture was 36.5 g L⁻¹ which was higher than that of monoculture (24.1 g L⁻¹).

Lopez *et al.* (2000) studied *Clostridium acetobutylicum* ATCC 824 for production of acetone, butanol and ethanol (ABE) from saccharides of domestic organic waste. More than 40 yeast strains were screened by Sreenath and Jeffries (2000) of *P.stipitis* and *C.shehate* to determine their fermentation rates on mixed sugars. The tested strains fermented both glucose and xylose and attained ethanol concentration of $34.8 \pm 2.42 \text{ g L}^{-1}$ with *P.stipitis* and $34.0 \pm 1.67 \text{ g L}^{-1}$ with *C.shehate*.

Geeta *et al.* (2001) screened, standard and native yeasts strain for ethanol production from pods of *Samanea saman* and obtained maximum ethanol concentration of 40.43 g L^{-1} with inoculation local isolate BCY-107 followed by BCY-108 (36.06 g L^{-1}). Four strains of *Z. mobilis* were screened by Panesar *et al.* (2001) for their ability to produce ethanol from molasses medium at pH6. Ramakrishna *et al.* (2001) screened five fructose negative mutants of *Z. mobilis* NCIM 2915 for production of fructose syrup and ethanol from water soluble carob sugars and obtained maximum ethanol and fructose yield by KLR4 mutant (120 g L^{-1} and 4 % w/v) at 120 hours of fermentation.

Two *Erwinia* endoglucanase genes were cloned by Zhou *et al.* (2001) into an ethanol producing *Klebsiella* species resulted in an organism with a capacity to produce 22 percent more ethanol when fermenting crystalline cellulose synergistically with added fungal cellulases. Nakamura *et al.* (2001) studied alcohol fermentation of an enzymatic hydrolysate of steam exploded rice straw in a membrane bioreactor coupled with a pre evaporation system. They reported an ethanol yield of 8.6 percent (w/w), reaching 50 g dm^{-3} in a bioreactor.

Sharma et al. (2002) used *S. cerevisiae* strain SJ-31, which exhibit potential for ethanol production from starch substrate because of its ability to produce α -amylase and glucoamylases. The ethanol produced was 3.4 percent with fermentation efficiency of 91 percent.

2.3.1. Simultaneous saccharification and fermentation (with substrate)

Schwanniomyces occidentalis was used for the release of sugars from heat pretreated sorghum grains by Horn et al. (1992) and the released sugars was fermented by *S. cerevisiae* in simultaneous saccharification and fermentation (SSF). They also reported that nearly 390 liters of ethanol could be produced per tonne grain of sorghum. Lezinou et al. (1994) reported that they achieved ethanol yield of 29.7 g/100 g of dry sorghum stalk in simultaneous saccharification and fermentation (SSF) using *Fusarium oxysporum* (F3) and *Z. mobilis* (CP4).

Krishna et al. (1998) carried out ethanol production by simultaneous saccharification and fermentation (SSF) of pretreated (with alkaline hydrogen peroxide) sugar cane leaves, using cellulolytic enzyme complex from *T. reesei* (QM 9414) and *S. cerevisiae* (NRRL-Y-132). They observed 92 percent conversion of 2.5 percent substrate to sugar and increase in ethanol yield with addition of extra β -glucosidase. Suresh et al. (1999) utilized *A. niger* (NCIM 1248) and *S. cerevisiae* VSJI, for simultaneous saccharification and fermentation (SSF) of grains and obtained ethanol yield of 2.90 percent (v/v). Maximum ethanol production of 10 g/100 g of rice starch and 8.2 g/100 g of sweet sorghum by using thermotolerant yeast isolate (VS3) by simultaneous saccharification and fermentation (SSF) (Sree et al. 1999). Chandrakant and

Bisaria (2000) carried out simultaneous isomerisation and fermentation (SIF) of xylose and simultaneous isomerisation and co-fermentation (SICF) of a glucose/xylose mixture by *S. cerevisiae* in the presence of xylose isomerase by addition of boxate to the medium, which resulted in ethanol concentration of 29.8 g L⁻¹.

Material and Methods

III. MATERIAL AND METHODS

Paddy, wheat and sugar cane are the major crops of Karnataka and are especially grown widely in north Karnataka. The residues of these crops are generally used as fuel or as animal feed. Such agro-residues were collected and investigations were carried out in the Department of Agricultural Microbiology, UAS, Dharwad for the possible production of bioethanol.

The details of materials used and methods employed during the course of investigations are being explained in this chapter.

3.1. SUBSTRATES

Substrates used for investigation are listed in Table-1.

3.2. MICROBIAL CULTURES

The microorganisms in this study along with their sources are described in Table-2.

3.3. MAINTENANCE OF CULTURES

The fungal strains were maintained on Potato Dextrose Agar (PDA), the yeast strains on MGYM medium (Wickerham's medium) and *Z.mobilis* on *Z.mobilis* selective medium (Appendix-1).

3.4. PREPARATION OF THE SAMPLE

The dried straw of paddy, wheat and bagasse of sugarcane were mechanically powdered to 1mm size and used for analysis of total solids, total sugar, reducing sugar, non reducing sugar, carbon and nitrogen.

Table 1: List of substrates along with source

SL.NO	Substrate	Source
1	Paddy straw	Kyarkop village near college campus.
2	Wheat straw	Wheat scheme, MARS, UAS Dharwad.
3	Bagasse	Malprabha co-operative sugar factory.M.K.Hubli.

Table 2: List of cultures along with source

Sl.No.	Strains	Source
1	<i>Trichoderma viride</i>	NCIM, Pune
2	<i>Trichoderma reesei</i>	NCIM, Pune
3	<i>Aspergillus niger</i> (NCIM 616)	NCIM, Pune
4	<i>Aspergillus awamori</i>	IARI, New Delhi
5	<i>Phanerochaete chrysosporium</i>	Bioconversion Lab UAS, Dharwad
6	<i>Pleurotus spp</i>	Bioconversion Lab UAS, Dharwad
7	<i>Saccharomyces cerevisiae</i> CFTRI 101	CFTRI, Mysore.
8	<i>Candida shehate</i> NCIM 3500	NCIM, Pune
9	<i>Pachysolen tannophilus</i> NCIM 3445	NCIM, Pune
10	<i>Pichia sipitis</i> CIM 3498	NCIM, Pune
11	BCY 108 Wheat scheme, MRS, UAS, Dharwad yeast strain	Bioconversion Lab UAS, Dharwad
12	<i>Zymomonas mobilis</i> NCIM 2915	NCIM, Pune

3.5. PRETREATMENT METHODS

Various pretreatment methods employed for the hydrolysis of the substrates are explained below.

3.5.1. Microbiological pretreatment (Geeta *et al.* 2002).

The straw of paddy, wheat and bagasse of sugarcane were dried at 45°C in an oven and powdered in a grinder. The powdered samples were passed through 1mm sieve. Ten grams of finely powdered substrates were transferred to 250 ml Erlenmeyer flasks. The moisture was adjusted to 60% using M and R medium (Mandle and Reese 1965) (Appendix-I). The flasks containing substrates were sterilized under 15 lb pressure for 15 minutes and were cooled. The substrates were inoculated with different fungal cultures individually and also in combinations (table 3). The fungal cultures grown on a plate were inoculated @ 4 – 5 disks per flask. The inoculated flasks were incubated for seven days at ambient temperature. The released sugar was extracted by adding distilled water at the ratio of 1:10 (Substrate: distilled water). The amount of reducing sugar released and cellulase was estimated by Nelson and Somogyis method (1944) and DNSA method (Miller 1959) respectively.

3.5.1.1. Treatments in microbiological pretreatment

The different treatments used in microbiological pretreatment are listed in Table-3.

3.5.2. Enzymatic pretreatment to release maximum reducing sugar

(Nikolov *et al.*, 2000).

In microbiological pretreatment, best treatment and best substrate to release maximum cellulase was selected for extraction of the enzyme. The

Table 3: List of microbiological treatments

SI. NO.	TREATMENTS
1	<i>Trichoderma viride</i> (T1)
2	<i>Trichoderma reesei</i> (T2)
3	<i>Aspergillus niger</i> (T3)
4	<i>Aspergillus awamori</i> (T4)
5	<i>Phanaerochaete chrysosporium</i> (T5)
6	<i>Pleurotus spp</i> (T6)
7	<i>T. viride</i> + <i>T. reesei</i> (T7)
8	<i>T. viride</i> + <i>A. niger</i> (T8)
9	<i>T. viride</i> + <i>A. awamori</i> (T9)
10	<i>T. viride</i> + <i>P. chrysosporium</i> (T10)
11	<i>T. viride</i> + <i>Pleurotus spp</i> (T11)
12	<i>T. reesei</i> + <i>A. niger</i> (T12)
13	<i>T. reesei</i> + <i>A. awamori</i> (T13)
14	<i>T. reesei</i> + <i>P. chrysosporium</i> (T14)
15	<i>T. reesei</i> + <i>Pleurotus spp</i> (T15)
16	<i>A. niger</i> + <i>A. awamori</i> (T16)
17	<i>A. niger</i> + <i>P. chrysosporium</i> (T17)
18	<i>A. niger</i> + <i>Pleurotus spp</i> (T18)
19	<i>A. awamori</i> + <i>P. chrysosporium</i> (T19)
20	<i>A. awamori</i> + <i>Pleurotus spp</i> (T20)
21	<i>P. chrysosporium</i> + <i>Pleurotus spp</i> (T21)

combination of *A. niger* and *T. viride* along with bagasse was used for large scale extraction of cellulase for further studies.

One hundred grams of finely powdered bagasse was transferred to two, five liter bottles and 60% moisture was maintained with M and R medium. The bottles were sterilized for 15 minutes under 15 lb pressure. The substrates were allowed to cool and inoculated with *T. viride* and *A. niger*. The inoculated samples were kept at ambient temperature and analysed at intervals of 5 and 10 days. The cellulase released after 5th and 10th day of incubation was extracted using citrate buffer (pH 4.7) at the ratio of 1:10 (Substrate: buffer). The amount of enzyme released was assayed using FPA method (Mandel *et al.*, 1976).

The enzyme extracts of 5th and 10th day of incubation were inoculated to the flasks containing 10 grams of powdered, sterilized substrates at the ratio of 1:10 (Substrate: Enzyme extract). The flasks were incubated for 7 days under shaking condition (180 rev/min) on rotary shaker, at ambient temperature. After incubation, the reducing sugar released was estimated.

3.5.3. Crude enzyme extraction (Schaffner and Toledo, 1991).

T. reesei was inoculated at the rate of 10-15 disks of agar grown cultures per liter of sterilized M and R medium supplemented with one percent Carboxymethyl cellulose (CMC) powder. The flask was incubated for five days at ambient temperature under continuous shaking condition (180 rev/min) on a rotary shaker. The culture filtrate (crude enzyme) was prepared by passing the culture broth through sintered glass filter under suction. The filtrate was stored at 4°C until further use.

3.5.3.1. Crude enzyme pretreatment (Lars and Karl, 1990).

Ten grams of finely grounded substrates were taken in 250 ml flasks and sterilized under 15 lb pressure for 90 minutes. The above mentioned enzyme extract was inoculated to each flask at the ratio of 1:10 (Substrates: enzyme extract). The flasks were incubated for seven days at ambient temperature under shaking condition. The reducing sugar released after incubation period was estimated.

3.6. FERMENTATION PROCESS FOR ETHANOL PRODUCTION.

The best pretreatment method obtained was the combination of *P. chrysosporium* and *Pleurotus spp*, which released maximum reducing sugar with paddy straw and bagasse respectively. The combination of *T.viride* and *P. chrysosporium* with wheat straw released maximum reducing sugar. Further these combinations along with their substrates were used for next experiments.

One bacterial strain and five yeast strains were used for fermentation of the hydrolysate. The over night grown yeast cultures in MGYB broth and bacteria in *Z.mobilis* selective broth containing, 30×10^6 cells per ml were used as inoculum.

3.6.1. Fermentation of extractant along with substrate

Ten grams of finely powdered substrates were taken in 250 ml flasks and sterilized for 90 minutes at 15 lb pressure. The flasks were inoculated with the respective combinations of fungal strains at the rate of 5 disks in each flask. After seven days of incubation, 100 ml of sterile distilled water was added to each flask under aseptic condition. The growth of fungi was

stopped by application of heat (autoclaving at 121 C for 15 minutes) and flasks were inoculated with bacteria and the yeast strains at the rate of 2% inoculum. The flasks were incubated under aerobic condition for 24 hours. After 24 hours of incubation, anaerobic condition was created by plugging the flasks with cork making a provision for trapping carbon dioxide and incubated at room temperature for 7 days. The amount of alcohol produced was estimated by colorimetric method as described by Caputi *et al.* (1968). The residual sugar was also estimated by Nelson and Somogyis method (Nelson, 1944).

3.6.2. Fermentation of extractant (without substrate)

The filtered extractants of different substrates after microbial pretreatment were fermented using bacteria and the yeast strains, similar to the procedure mentioned in Table-2. The amount of alcohol produced and the residual sugars were estimated as mentioned in 3.6.1.

3.7. CHEMICAL ANALYSIS

3.7.1. Estimation of reducing sugars

The reducing sugars were estimated by following Nelson Somogyis method (Nelson, 1944).

3.7.1.1. Preparation of reagents

3.7.1.1.1. Alkaline Copper Reagent

Solution a: 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartarate, 20 g of sodium bicarbonate and 200 g of sodium sulphate

were dissolved in 800 ml distilled water. Finally it was diluted to 1000 ml with distilled water.

Solution b: 15 g of copper sulfate was dissolved in distilled water. One to two drops of concentrated sulphuric acid was added and final volume was made up to 100 ml with distilled water. Just before use, solution A and B were mixed in the ratio of 24:1.

3.7.1.1.2. Arsenomolybdate reagent

25 grams of ammonium molybdate was dissolved in 40 ml of distilled water, to which 21 ml of concentrated sulphuric acid was added and mixed thoroughly. Three grams of sodium orthoarsenate was dissolved in 25 ml distilled water separately. Finally, both the solutions were mixed and placed in an incubator at 37°C for 24 – 48 hours.

3.7.1.1.3. Standard stock solution of glucose

One hundred mg of D-glucose was dissolved in 50ml of distilled water and final volume made up to 100 ml in volumetric flask. Ten ml of this standard stock solution was diluted to 100 ml in volumetric flask. This working standard contained 100 mg of glucose per ml.

3.7.1.2. Procedure

The representative samples of 0.1 ml from each treatment with replications were taken into thin walled boiling test tubes and 0.9 ml of distilled water was added. A test tube containing one ml of distilled water was taken as blank. Similarly, the standards were also prepared ranging from 100 mg to 1000 mg concentration of glucose. One ml of alkaline copper

reagent was added to each tube including reagent blank and placed them on boiling water bath for 20 minutes. Tubes were cooled under tap and 1 ml of arsenomolybdate reagent was added till effervescence stop. Finally, the volume was made up to 20 ml with distilled water. Absorbance of the standards and the samples were read at 510 nm using Systronics UV Spectrophotometer – 117.

3.7.2. Estimation of total sugar

The amount of total sugar was estimated as mentioned in 3.7.1.

3.7.2.1. Preparation of reagents

3.7.2.1.1. 1N Hydrochloride

The actual normality of HCl in the bottle was calculated and 8.8 ml was diluted with distilled water. The final volume was made up to 100 ml in volumetric flask.

3.7.2.1.2. 0.1N Hydrochloride

0.88 ml of HCl was diluted and volume made up to 100 ml with distilled water in volumetric flask.

3.7.2.1.3. 1N Sodium hydroxide

4 g of NaOH was dissolved in distilled water and the final volume was made up to 100 ml in volumetric flask.

3.7.2.1.4. Phenolphthalein indicator

One g of phenolphthalein powder was dissolved in 50 ml of 70% alcohol. The final volume was made up to 100 ml with distilled water.

3.7.2.2. Procedure

One ml of representative sample from each treatment and replication was taken in thin walled boiling test tubes. 1 ml of 1 N HCl was added to each tube and placed them in boiling water bath for 15 – 20 minutes. Tubes were cooled and one drop of phenolphthalein indicator was added to each tube. 1 N NaOH was added till pink colour appeared followed by addition of 0.1 N HCl till pink colour disappeared. Finally, the samples were mixed well and volume was made up to 5 ml with distilled water. 0.1 ml representative sample were taken from these tubes and total sugar was estimated as indicated earlier (3.7.1)

3.8. ESTIMATION OF CELLULASE

The amount of cellulase released was estimated by dinitrosalicylic acid (DNSA) method as described by Miller (1959).

3.8.1. Preparation of reagents

3.8.1.1. DNSA

1 gram of dinitrosalicylic acid (DNSA), 200 mg of phenol and 500 mg of sodium sulphite were dissolved in 100 ml of 1% NaOH.

3.8.1.2. Rochelle salt solution 40%

Rochelle salt solution was prepared by dissolving 40 g of potassium sodium tartrate in 100 ml distilled water.

3.8.2. Procedure

The enzyme extract of 0.5 ml from each treatment and replication were added separately to thin walled boiling test tubes containing 32 mg of dry

Whatman No.1 filter paper. Tubes were incubated at 50°C for 1 hour. Immediately after removing the enzyme substrate mixture from the water bath, 0.5 ml DNSA reagent was added and mixed thoroughly. Tubes were kept on water bath at 50°C for 5 minutes. While tubes were warm, 1 ml of 40% potassium sodium tartarate solution was added and the final volume was made up to 5 ml with distilled water. The standards were prepared with glucose in the concentration ranging from 100 mg to 1000 mg/ml. Absorbance in terms of optical density of the standard and the samples were read at 540 nm using systronics UV spectrophotometer-117.

3.9. ESTIMATION OF ORGANIC CARBON

The organic carbon in the sample was estimated by the method described by Jackson (1973).

3.10. ESTIMATION OF TOTAL NITROGEN

The total nitrogen in the sample was estimated by the method as described by Jackson (1973).

3.10.1. Preparation of reagents

3.10.1.1. Digestion mixture

Potassium sulphate: Copper sulphate: Selenium were mixed in 10:10:1 ratio.

3.10.1.2. Sodium hydroxide 40%

Sodium hydroxide solution was prepared by dissolving 40 g of NaOH in 100 ml distilled water.

3.10.1.3. Boric acid 2%

2 g of boric acid was dissolved in 100 ml of distilled water.

3.10.1.4. Mixed indicator

0.1 g of bromocresol green and 0.07 g of methyl red were dissolved in 100 ml of 95% ethanol. 15 ml of this mix was dissolved in 1000 ml of 2% boric acid.

3.10.1.5. 0.01N Sulfuric acid

0.54 ml of H_2SO_4 was dissolved in 1000 ml of distilled water.

3.10.2. Procedure

Dried samples of 0.5 g was digested using 10 ml of concentrated H_2SO_4 in presence of 0.2 g of digestion mixture for 2 hours in microkjeldhal digestion unit. The digested sample was diluted with distilled water. To 10 ml of this diluted sample was distilled with addition of 20 ml of 40% NaOH to make the sample alkaline in microkjeldhal distillation unit. The ammonia evolved was trapped in 2% boric acid mixed indicator solution till colour changed to pink. The distilled samples were titrated against 0.01 N H_2SO_4 till colour changed to green. The N content was calculated from the volume of 0.01N H_2SO_4 consumed.

3.11 ESTIMATION OF ETHANOL

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

3.11.1. Preparation of reagents

3.11.1.1. Potassium dichromate ($K_2Cr_2O_7$)

34 g of $K_2Cr_2O_7$ was dissolved in 500 ml of distilled water and 325 ml of sulphuric acid was added and volume was made up to 1000 ml with distilled water to give 0.23N $K_2Cr_2O_7$.

3.11.1.2. Preparation of stock solution

Standard stock solution of 100 percent pure analytical grade (containing 789 mg ml⁻¹) ethanol was prepared by dissolving 12.6 ml of ethanol in 100 ml distilled water, which resulted in 100 mg ml⁻¹ of standard ethanol.

3.11.1.3. Procedure

One ml of the representative samples from each treatment was transferred to 250 ml round bottom distillation flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74 - 75°C. The distillate was collected in 0.23 N $K_2Cr_2O_7$ reagent, which was kept at the receiving end. The distillate containing alcohol was collected till total volume of 45 ml was obtained. Similarly standards (concentration ranging from 20 – 100 mg ml⁻¹ ethanol) were mixed with 25 ml of $K_2Cr_2O_7$ separately. The distillate of samples and the standards were heated in water bath at 60°C for 20 minutes and were cooled. The volume was made up to 50 ml with distilled water and the optical density was measured at 600 nm using systronics Spectrophotometer-117. The standard curve was plotted considering the concentration against absorbance.

3.12. STATISTICAL ANALYSIS

The results obtained were analysed statistically using completely randomized design as described by Panse & Sukhatme (1985).

Experimental Results

IV. EXPERIMENTAL RESULTS

Ethanol being a biosolar fuel has gained importance recently, specially in the transport sector as it can be blended up to 20 percent with diesel or petrol. Ethanol is produced from common crops like sugar cane, sweet sorghum, beet, potato and also from molasses. In the present study different sugar containing agriculture crop residues were evaluated for ethanol production by adopting microbial pretreatment methods followed by fermentation. The results pertaining to this are being presented in this chapter.

4.1 CHEMICAL COMPOSITION OF PADDY, WHEAT STRAW AND BAGASSE

It was essential to know the naturally available constituents of finely powdered straws of paddy, wheat and bagasse. Hence, proximate analysis was carried out. The results are presented in Table-4. The paddy straw contains reducing sugar (0.03 mg g^{-1}), total sugar (0.56 mg g^{-1}), non reducing sugar (0.53 mg g^{-1}) Carbon (52.2%) nitrogen (0.41%) total solids (90%) moisture (10%). Wheat straw contain reducing sugar (0.02 mg g^{-1}), total sugar (0.60 mg g^{-1}), non-reducing sugar (0.58 mg g^{-1}) carbon (54.6%), nitrogen (0.35%), total solids (89%) & moisture (11%). Bagasse contains reducing sugar (0.16 mg g^{-1}) total sugar (0.69 mg g^{-1}), non-reducing sugar (0.53 mg g^{-1}), carbon (55.4%) and nitrogen (0.43%), total solid (92%) & moisture content (8%).

4.2 EFFECT OF MICROBIOLOGICAL PRETREATMENT ON SUGAR AND OTHER CONSTITUENTS IN PADDY STRAW

The data pertaining to influence of different fungal treatments either individually or in combination to the finely powdered paddy straw in releasing

Table 4: Initial chemical constituents in raw substrates of paddy straw, wheat straw and bagasse

Sl.No	Parameters	Substrates		
		Paddy straw	Wheat straw	Bagasse
1	Total Sugar (mg g ⁻¹)	0.56	0.60	0.69
2	Reducing Sugar (mg g ⁻¹)	0.03	0.02	0.16
3	Non-reducing sugar (mg g ⁻¹)	0.53	0.58	0.53
4	Carbon (%)	52.2	54.6	55.4
5	Nitrogen (%)	0.41	0.35	0.43
6	Total Solid (%)	90	89	92
7	Moisture Content (%)	10	11	8

total sugar, reducing sugar, non reducing sugar, cellulase, carbon and nitrogen is presented in Table-5a.

4.2.1 Total sugar

The data regarding release of total sugar is presented in Table-5a. Among the different treatments, paddy straw treated with combination of *P. chrysosporium* with *Pleurotus spp* has given significantly higher total sugar (2.48 mg g^{-1}) as compared to rest of the treatments. The single inoculation of *P. chrysosporium* was found to be next best treatment to release total sugar (2.10 mg g^{-1}) which was found significantly superior over other treatments except paddy straw treated with combination of *T.viride* and *Pleurotus spp* (2.01 mg g^{-1}) and *T. reesei* and *Pleurotus spp* (2.04 mg g^{-1}). All the treatments showed increase in sugar content as compared to uninoculated control (0.71 mg g^{-1}).

4.2.2 Reducing sugar

The inoculation effect of different fungal culture to paddy straw in the release of reducing sugar is given in table -5a. The paddy straw inoculated with combination of *P. chrysosporium* and *Pleurotus spp* recorded maximum release of reducing sugar (1.27 mg g^{-1}) over all other treatments and uninoculated control. However, other treatments *Pleurotus spp* (1.21 mg g^{-1}), combination of *A. niger* and *P. chrysosporium* (1.18 mg g^{-1}) *A. awamori* and *P. chrysosporium* (1.19 mg g^{-1}) were found to be on par with combined inoculation of *P. chrysosporium* and *Pleurotus spp*.

Table 5a: Effect of microbiological pretreatment on sugar and other constituents in paddy straw

Sl. No	Treatments	Total sugar (mg g ⁻¹)	Reducing sugar (mg g ⁻¹)	Non reducing sugar (mg g ⁻¹)	Cellulase (IU)	Carbon (%)	Nitrogen (%)
1	Paddy straw without inoculation	0.71	0.21	0.50	0.00	52.2	0.41
2	<i>Trichoderma viride</i>	1.48	1.04	0.44	1.58	52.2	0.45
3	<i>Trichoderma reesei</i>	1.12	0.59	0.53	1.33	51.2	0.46
4	<i>Aspergillus niger</i>	1.16	0.61	0.55	1.04	51.2	0.52
5	<i>Aspergillus awamori</i>	1.49	0.81	0.68	2.17	50.2	0.46
6	<i>Phanerochaete chrysosporium</i>	2.10	1.12	0.98	4.29	48.3	0.46
7	<i>Pleurotus spp</i>	1.59	1.21	0.38	2.51	50.2	0.48
8	<i>T. viride</i> + <i>T. reesei</i>	1.34	0.75	0.59	1.56	51.2	0.56
9	<i>T. viride</i> + <i>A. niger</i>	1.09	0.65	0.44	1.18	49.3	0.49
10	<i>T. viride</i> + <i>A. awamori</i>	0.99	0.60	0.39	1.01	50.2	0.60
11	<i>T. viride</i> + <i>P. chrysosporium</i>	1.41	0.79	0.62	1.55	50.2	0.57
12	<i>T. viride</i> + <i>Pleurotus spp</i>	2.01	1.05	0.96	1.63	48.3	0.54
13	<i>T. reesei</i> + <i>A. niger</i>	1.81	0.98	0.82	1.92	50.2	0.49
14	<i>T. reesei</i> + <i>A. awamori</i>	1.11	0.75	0.36	1.32	50.2	0.52
15	<i>T. reesei</i> + <i>P. chrysosporium</i>	1.81	1.03	0.78	1.92	50.2	0.53
16	<i>T. reesei</i> + <i>Pleurotus spp</i>	2.04	1.11	0.93	2.59	50.2	0.52
17	<i>A. niger</i> + <i>A. awamori</i>	1.50	1.04	0.46	2.43	50.2	0.56
18	<i>A. niger</i> + <i>P. chrysosporium</i>	1.79	1.18	0.61	3.00	49.3	0.56
19	<i>A. niger</i> + <i>Pleurotus spp</i>	1.28	0.85	0.43	1.54	47.3	0.54
20	<i>A. awamori</i> + <i>P. chrysosporium</i>	1.81	1.19	0.62	1.38	51.2	0.48
21	<i>A. awamori</i> + <i>Pleurotus spp</i>	1.65	1.03	0.62	2.29	49.3	0.55
22	<i>P. chrysosporium</i> + <i>Pleurotus Spp</i>	2.48	1.27	1.21	3.33	52.2	0.54
	SEM±	0.057	0.036	0.025	0.025	1.543	0.025
	CD at 1%	0.148	0.093	0.066	0.066	3.973	0.066

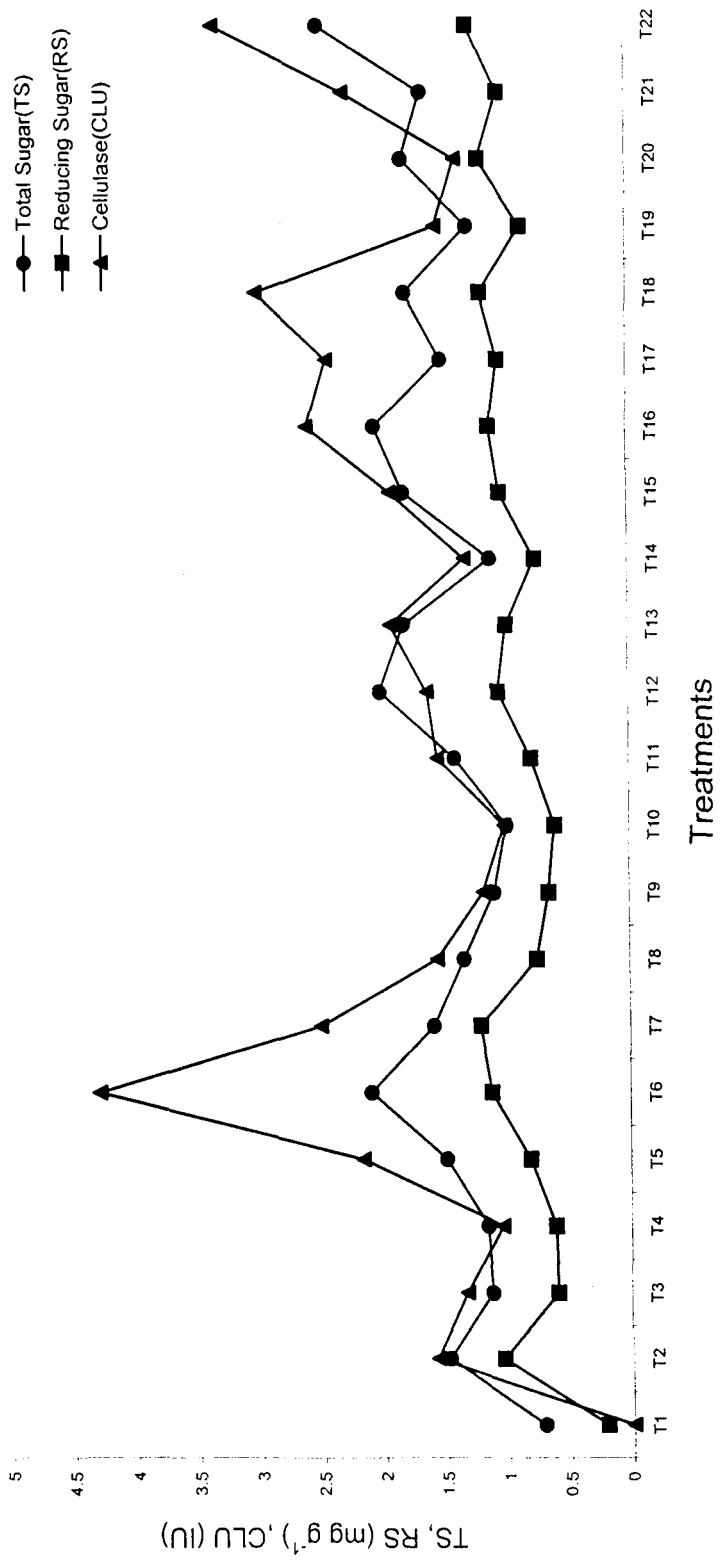


Fig. 1: Effect of microbiological pretreatment on total and reducing sugar (mg g⁻¹) and cellulase (IU) in paddy straw

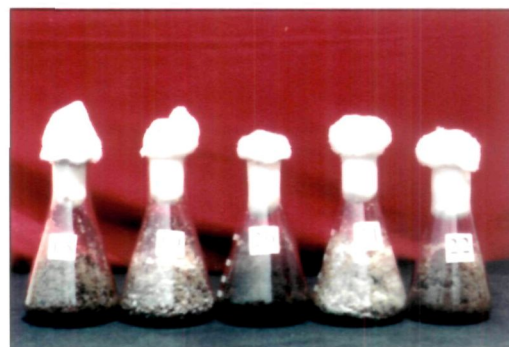
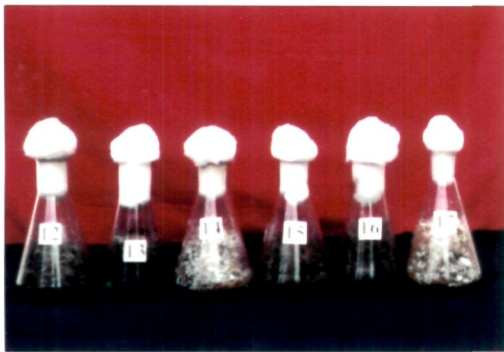
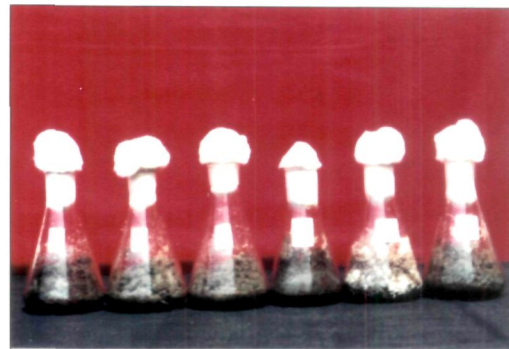


Plate 1: Microbiological pre-treatment for paddy straw

4.2.3 Non reducing sugar

Among fungal cultures, paddy straw inoculated with combination of *P. chrysosporium* and *Pleurotus spp* recorded highest non reducing sugar (1.21 mg g⁻¹) compared to rest of the treatments and uninoculated control. The next best treatment found was single inoculation of *P. chrysosporium* (0.98 mg g⁻¹) followed by combined inoculation of *T.viride* and *Pleurotus spp* (0.96 mg g⁻¹) and combination of *T. reesei* and *Pleurotus spp* (0.93 mg g⁻¹).

4.2.4 Cellulase

The data pertaining to the release of cellulase by fungal treated paddy straw is presented in Table -5a. Among the different treatments paddy straw inoculated with *P. chrysosporium* has shown significantly higher release of cellulase (4.29 IU) as compared to other treatments. The combined inoculation of *P. chrysosporium* and *Pleurotus spp* was found next best treatment to release cellulase (3.33 IU), which is also significantly superior over other treatments. Paddy straw treated with different fungal cultures gave significantly higher cellulase over uninoculated control.

4.2.5 Carbon

The carbon content in the paddy straw was decreased in fungal treated samples. The data pertaining to this is shown in table-5a. But among the treatments, there was no significant decrease in carbon content was observed.

4.2.6 Nitrogen

The nitrogen content in the paddy straw was found to be increased after inoculation of different fungal strains compared to uninoculated control. The data regarding nitrogen content is shown in table -5a.

The nitrogen content in the paddy straw was found to be maximum when inoculated with combination of *T. viride* and *A. awamori* (0.6%). The treatments of *T. viride* in combination with *P. chrysosporium* (0.57%), *T. reesei* (0.56%). *A. niger* in combination with *A. awamori* (0.56%), with *P. chrysosporium* (0.56%) and *Pleurotus spp* (0.54%), *A. awamori* along with *pleurotus spp* (0.55%) and *P. chrysosporium* with *Pleurotus spp* (0.54%) were found to be on par with each other. All these treatments were found to be significantly superior over other treatments and uninoculated control.

4.3 EFFECT OF MICROBIOLOGICAL PRETREATMENT ON SUGAR AND OTHER CONSTITUENTS OF WHEAT STRAW

Wheat straw pretreated with different fungal cultures either individually or in combination had positive influence on total sugar, reducing sugar, non-reducing sugar, cellulase, carbon and nitrogen content as compared to uninoculated control Table-5b.

4.3.1 Total sugar

The total sugar content of wheat straw was found to be increased due to the pretreatment with different fungal cultures. The highest total sugar content was recorded when the substrate was pre treated with combination of *P. chrysosporium* and *Pleurotus spp* (2.54 mg g⁻¹). The next best treatments are combined inoculation of *T. viride* and *P. chrysosporium* and *T. reesei* in combination with *P. chrysosporium* which yielded 2.43 mg g⁻¹ of substrate and were significantly superior than other treatments and over un inoculated control (Table -5b).

Table 5b: Effect of microbiological pretreatment on sugar and other constituents in Wheat straw

Sl. No	Treatments	Total sugar (mg g ⁻¹)	Reducing sugar (mg g ⁻¹)	Non reducing sugar (mg g ⁻¹)	Cellulase (IU)	Carbon (%)	Nitrogen (%)
1	Wheat straw without inoculation	0.64	0.35	0.29	0.00	54.6	0.35
2	<i>Trichoderma viride</i>	1.00	0.54	0.46	2.58	52.9	0.40
3	<i>Trichoderma reesei</i>	1.09	0.77	0.32	2.53	51.3	0.42
4	<i>Aspergillus niger</i>	1.13	0.73	0.40	1.42	53.5	0.37
5	<i>Aspergillus awamori</i>	0.88	0.48	0.40	2.18	50.1	0.48
6	<i>Phanaerochaete chrysosporium</i>	1.37	0.82	0.55	3.20	50.2	0.43
7	<i>Pleurotus spp</i>	1.58	0.93	0.65	4.73	51.3	0.43
8	<i>T. viride</i> + <i>T. reesei</i>	1.27	0.85	0.42	2.35	52.2	0.40
9	<i>T. viride</i> + <i>A. niger</i>	0.69	0.42	0.27	1.02	52.0	0.45
10	<i>T. viride</i> + <i>A. awamori</i>	1.21	0.64	0.57	4.47	50.9	0.39
11	<i>T. viride</i> + <i>P. chrysosporium</i>	2.43	1.47	0.96	2.33	52.0	0.39
12	<i>T. viride</i> + <i>Pleurotus spp</i>	1.47	0.80	0.67	1.36	50.9	0.42
13	<i>T. reesei</i> + <i>A. niger</i>	1.63	1.11	0.52	3.01	52.2	0.42
14	<i>T. reesei</i> + <i>A. awamori</i>	1.23	0.94	0.29	1.85	53.1	0.40
15	<i>T. reesei</i> + <i>P. chrysosporium</i>	2.43	1.39	1.04	2.24	49.8	0.41
16	<i>T. reesei</i> + <i>Pleurotus spp</i>	1.52	0.99	0.53	3.96	52.4	0.45
17	<i>A. niger</i> + <i>A. awamori</i>	1.56	0.82	0.74	2.21	52.3	0.49
18	<i>A. niger</i> + <i>P. chrysosporium</i>	1.47	0.90	0.57	2.94	51.2	0.40
19	<i>A. niger</i> + <i>Pleurotus spp</i>	1.28	1.09	0.19	2.02	52.0	0.40
20	<i>A. awamori</i> + <i>P. chrysosporium</i>	1.11	0.71	0.40	2.94	51.9	0.41
21	<i>A. awamori</i> + <i>Pleurotus spp</i>	1.17	0.94	0.23	3.86	52.3	0.45
22	<i>P. chrysosporium</i> + <i>Pleurotus spp</i>	2.54	1.44	1.10	3.46	52.4	0.43
	SEM±	0.036	0.025	0.025	0.106	0.336	0.016
	CD at 1%	0.093	0.066	0.066	0.273	0.866	0.041

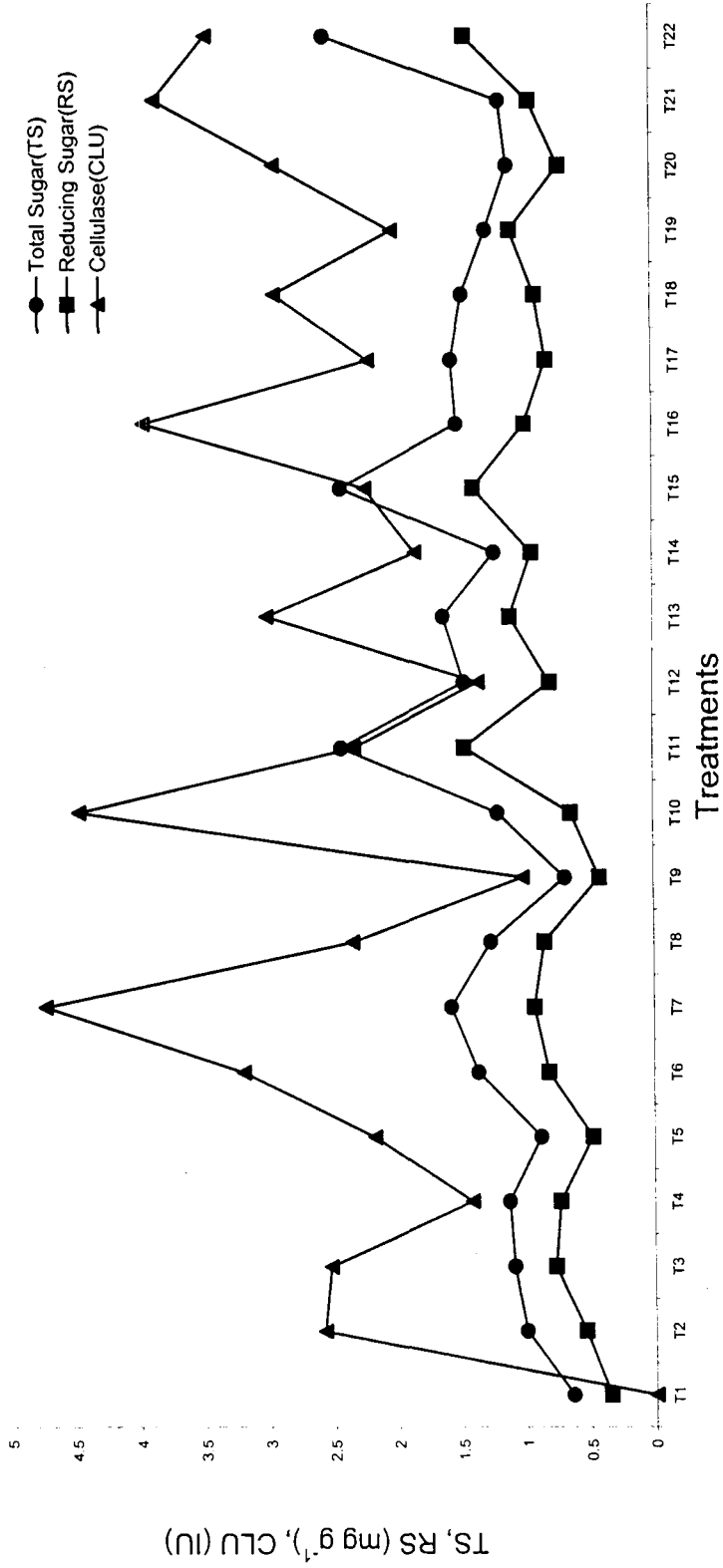


Fig. 2: Effect of microbiological pre treatment on total sugar, reducing sugar (mg/g) and cellulase (IU) in wheat straw

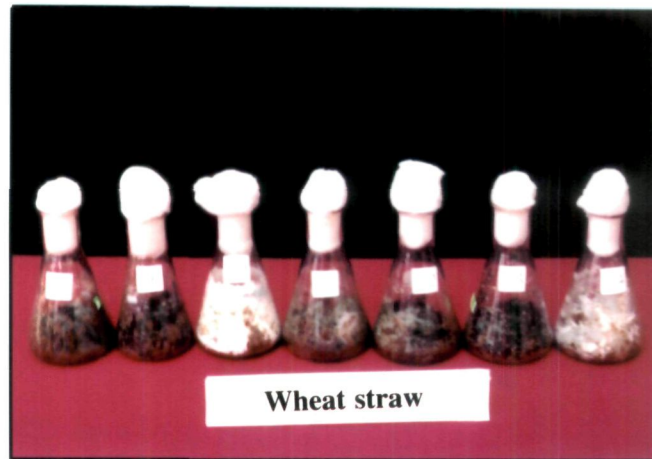
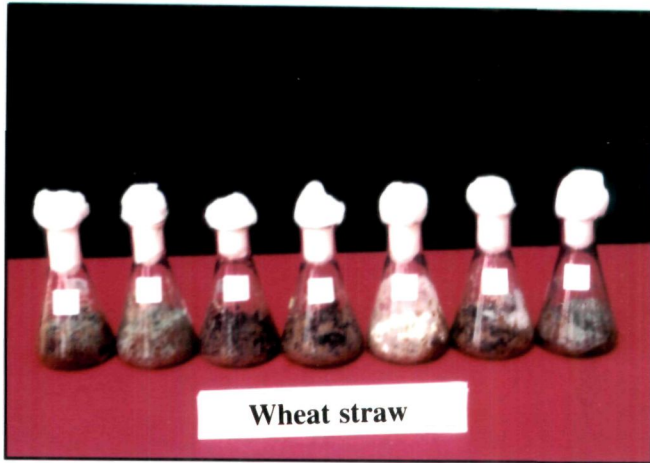


Plate 2: Microbiological pre-treatment for wheat straw

4.3.2 Reducing sugar

The reducing sugar content in wheat straw was increased significantly with inoculation of different fungal cultures, which ranged from 0.35 mg g⁻¹ to 1.47 mg g⁻¹. The highest reducing sugar content in wheat straw recorded was 1.47 mg g⁻¹ when inoculated with *T. viride* along with *P. chrysosporium* which was found to be significantly superior over other treatment and over uninoculated control except wheat straw pretreated with both *P. chrysosporium* and *Pleurotus spp* (1.44 mg g⁻¹) which was found on par with combination of *T. viride* and *P. chrysosporium* (Table -5b).

4.3.3 Non reducing sugar

The data pertaining to release of non reducing sugar is being presented in table-5b. The highest non reducing sugar content was recorded when the wheat straw was pretreated with combination of *P. chrysosporium* and *Pleurotus spp* (1.10 mg g⁻¹) followed by inoculation of *T. reesei* with *P. chrysosporium* 1.04 mg g⁻¹. These two treatments were found to be significantly higher than other treatments and over uninoculated control.

4.3.4 Cellulase

The variation in the release of cellulase from fungal pretreated wheat straw is presented in table-5b. Among the different treatments, wheat straw inoculated with *Pleurotus spp* resulted significantly higher cellulase (4.73 IU) production than rest of the treatment. Followed by the combined inoculation of *T. viride* and *A. awamori* (4.47 IU).

4.3.5 Carbon

The Data pertaining to the carbon content in pretreated wheat straw is presented in Table-5b. The maximum reduction in the carbon content after biological pretreatment was observed in wheat straw inoculated with *A.awamori* (50.1%) alone followed by *P. chrysosporium* (50.2%) when compared with un inoculated control (54.6%).

4.3.6 Nitrogen

The nitrogen content in wheat straw was found to be increased by different fungal treatments table-5b. The highest nitrogen content was recorded when wheat straw was treated with combination of *A. niger* and *A .awamori* (0.49%) followed by single inoculation of *A. awamori* (0.48%) and were significantly superior over other treatments and uninoculated control.

4.4 EFFECT OF MICROBIOLOGICAL PRETREATMENT ON SUGAR AND OTHER CONSTITUENTS IN BAGASSE

Powdered bagasse pretreated with different fungal cultures either individually or in combination had positive influence on total sugar, reducing sugar, non reducing sugar, cellulase, carbon and nitrogen (Table- 5c)

4.4.1 Total sugar

The data regarding the release of total sugar from bagasse, treated biologically with different fungal cultures is shown in Table-5c. Among the different treatments bagasse treated with combination of *P. chrysosporium* and *Pleurotus spp* has given significantly higher total sugar (2.41 mg g⁻¹) than

Table 5c: Effect of microbiological pretreatment on sugar and other constituents in Bagasse

Sl. No	Treatments	Total sugar (mg g ⁻¹)	Reducing sugar (mg g ⁻¹)	Non reducing sugar (mg g ⁻¹)	Cellulase (IU)	Carbon (%)	Nitrogen (%)
1	Bagasse without inoculation	0.65	0.28	0.37	0.00	55.4	0.43
2	<i>Trichoderma viride</i>	1.22	0.63	0.59	0.69	54.2	0.58
3	<i>Trichoderma reesei</i>	1.34	0.59	0.49	0.75	53.5	0.56
4	<i>Aspergillus niger</i>	1.20	0.70	0.50	0.98	53.0	0.55
5	<i>Aspergillus awamori</i>	1.36	0.73	0.63	0.51	54.5	0.51
6	<i>Phanaerochaete chrysosporium</i>	1.69	0.80	0.89	1.02	53.4	0.51
7	<i>Pleurotus spp</i>	1.22	0.64	0.58	3.29	52.8	0.54
8	<i>T. viride</i> + <i>T. reesei</i>	1.99	1.23	0.76	4.79	54.2	0.55
9	<i>T. viride</i> + <i>A. niger</i>	1.13	0.61	0.52	0.80	53.4	0.57
10	<i>T. viride</i> + <i>A. awamori</i>	1.29	0.67	0.62	3.37	53.1	0.57
11	<i>T. viride</i> + <i>P. chrysosporium</i>	1.27	0.553	0.72	2.22	54.6	0.57
12	<i>T. viride</i> + <i>Pleurotus spp</i>	1.41	0.83	0.58	1.66	52.6	0.49
13	<i>T. reesei</i> + <i>A. niger</i>	2.13	1.39	0.74	2.45	53.4	0.50
14	<i>T. reesei</i> + <i>A. awamori</i>	1.08	0.57	0.51	1.05	53.5	0.48
15	<i>T. reesei</i> + <i>P. chrysosporium</i>	1.46	0.84	0.62	3.80	54.2	0.51
16	<i>T. reesei</i> + <i>Pleurotus spp</i>	2.27	1.60	0.67	0.77	53.6	0.55
17	<i>A. niger</i> + <i>A. awamori</i>	0.87	0.46	0.41	1.69	52.6	0.51
18	<i>A. niger</i> + <i>P. chrysosporium</i>	0.92	0.54	0.38	0.86	54.2	0.57
19	<i>A. niger</i> + <i>Pleurotus spp</i>	1.15	0.65	0.50	0.47	54.2	0.54
20	<i>A. awamori</i> + <i>P. chrysosporium</i>	1.34	0.84	0.50	1.07	52.9	0.54
21	<i>A. awamori</i> + <i>Pleurotus spp</i>	1.01	0.67	0.34	0.55	53.4	0.56
22	<i>P. chrysosporium</i> + <i>Pleurotus spp</i>	2.41	1.97	0.44	3.51	53.9	0.55
	SEM±	0.036	0.025	0.025	0.025	0.498	0.025
	CD at1%	0.093	0.066	0.066	0.066	1.284	0.066

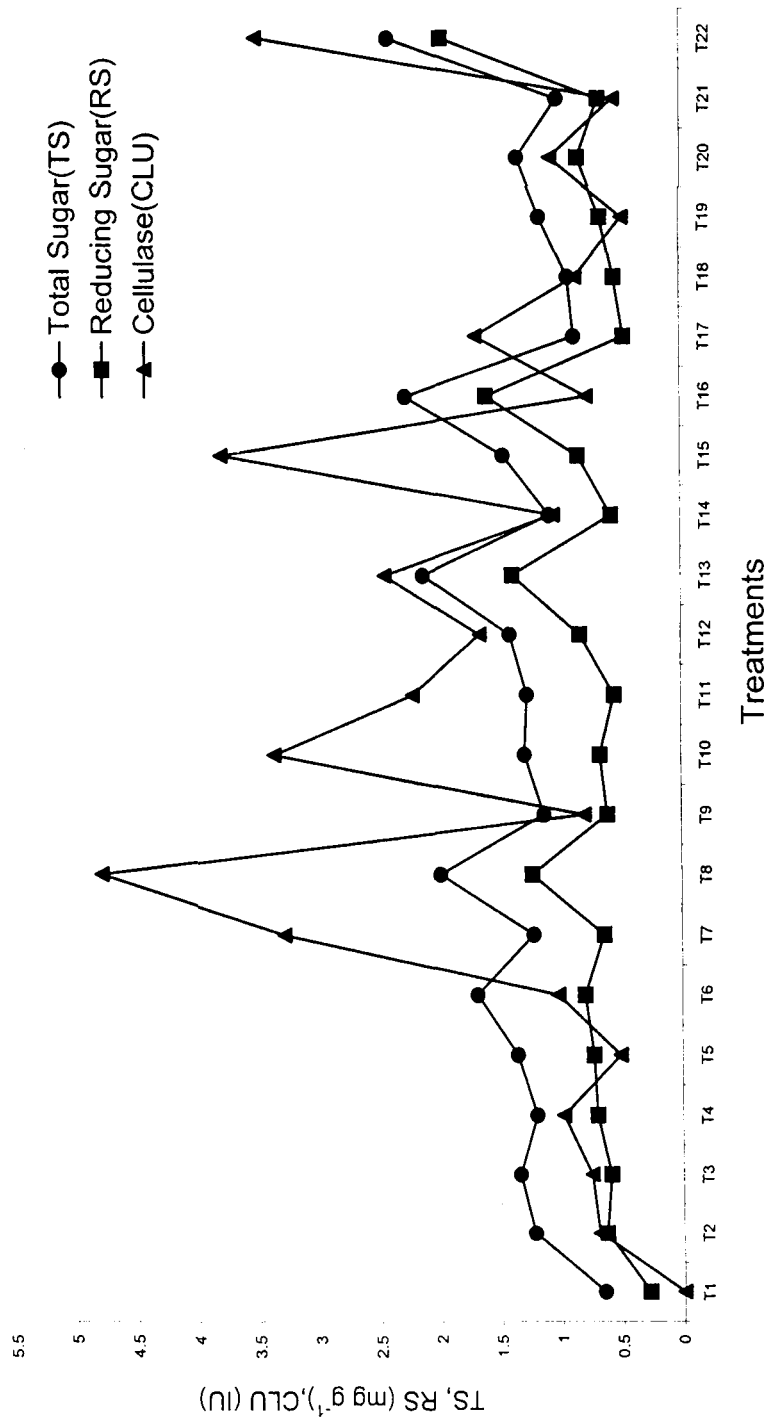


Fig. 3: Effect of microbiological pre treatment on total sugar, reducing sugar (mg/g) and cellulase (IU) in bagasse

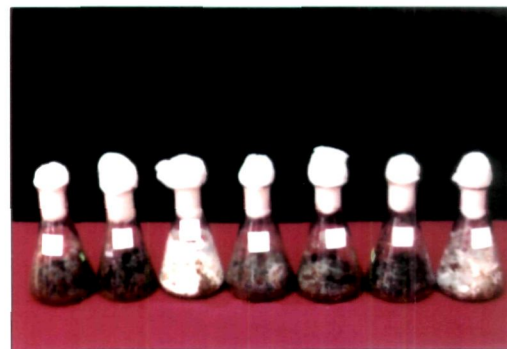


Plate 3: Microbiological pre-treatment for bagasse

other treatments. The bagasse treated with *T. reesei* and *Pleurotus spp* was also found to be good treatment releasing total sugar of 2.27 mg g⁻¹. All the biological treatment in bagasse showed significantly higher total sugar over uninoculated control.

4.4.2 Reducing sugar

The powdered bagasse inoculated with different fungal cultures had positive influence in releasing reducing sugar compared to uninoculated control (Table-5c).

Among fungal cultures, bagasse inoculated with combination of *P. chrysosporium* and *Pleurotus spp* recorded significantly higher reducing sugar (1.97 mg g⁻¹), which was significantly superior over rest of the treatments and uninoculated control followed by combined inoculation of *T. reesei* with *Pleurotus spp* (1.60 mg g⁻¹).

4.4.3 Non reducing sugar

Significant differences with respect to release of cellulase due to inoculation of different fungal strains to bagasse are recorded in Table-5c. Single inoculation treatment with *P. chrysosporium* was found significantly superior over other treatments (0.89 mg g⁻¹). The next best treatment found was combined inoculation of *T. viride* and *T. reesei*, which recorded 0.76 mg g⁻¹. Bagasse inoculated with different fungal cultures gave significantly higher results than uninoculated control (0.37 mg g⁻¹).

4.4.4 Cellulase

The inoculation effect of different fungal cultures to bagasse in the release of cellulase is being presented in Table-5c. Among all the treatments, bagasse inoculated with combination of *T. viride* and *T. reesei* has shown significantly higher cellulase (4.79 IU) followed by bagasse inoculated with both *T. reesei* and *P. chrysosporium*, which recorded 3.80 IU and were significantly superior over other treatments and uninoculated control.

4.4.5 Carbon

Significant difference with respect to decrease in the carbon content in bagasse after biological pretreatment was presented in (Table-5c).

The maximum decrease in the carbon content was observed in the bagasse inoculated with combination of *T.viride* and *P. chrysosporium* (52.6%) and *A. niger* with *A.awamori* (52.6%) compared to uninoculated control (55.4%).

4.4.6 Nitrogen

The nitrogen content in fungal pretreated bagasse is being presented in Table-5c. Bagasse inoculated with *T. viride* alone showed higher nitrogen content (0.58%), which was significantly superior over uninoculated control (0.43%). All other fungal treatments were found on par among themselves.

4.5 EFFECT OF CRUDE CELLULASE ENZYME ON RELEASE OF REDUCING SUGAR IN PADDY STRAW, WHEAT STRAW AND BAGASSE

In general, paddy straw pretreated with crude enzyme extracted at 5th and 10th day of incubation period had positive influence on total sugar, reducing sugar and non reducing sugar (Table-6a).

Table 6a: Effect of crude cellulase enzyme on release of reducing sugar at interval of 5 days and 10 days in paddy straw

Sl.No.	Treatments	5 th Day	10 th Day
1	Total sugar (mg g ⁻¹)	1.45	1.54
2	Reducing sugar (mg g ⁻¹)	0.88	0.98
3	Non Reducing sugar (mg g ⁻¹)	0.57	0.61
	SEM±	0.388	0.239
	CD at 1%	1.302	0.800

Table 6b: Effect of crude cellulase enzyme on release of reducing sugar at interval of 5 days and 10 days in wheat straw

Sl.No.	Treatments	5 th Day	10 th Day
1	Total sugar (mg g ⁻¹)	1.16	1.83
2	Reducing sugar (mg g ⁻¹)	0.84	1.42
3	Non Reducing sugar (mg g ⁻¹)	0.32	0.41
	SEM ±	0.154	0.139
	CD at 1%	0.512	0.462

Table 6c: Effect of crude cellulase enzyme on release of reducing sugar at interval of 5 days and 10 days in bagasse

Sl.No.	Treatments	5 th Day	10 th Day
1	Total sugar (mg g ⁻¹)	1.21	1.60
2	Reducing sugar (mg g ⁻¹)	0.81	1.16
3	Non Reducing sugar (mg g ⁻¹)	0.40	0.44
	SEM±	0.189	0.197
	CD at 1%	0.313	0.652

4.5.1 Paddy straw

The paddy straw inoculated with crude cellulase enzyme extracted at 10th day of incubation period showed maximum release of total sugar, reducing sugar and non reducing sugar, (1.54 mg g⁻¹, 0.98 mg g⁻¹, 0.61 mg g⁻¹ respectively) compared to 5th day extracted cellulase. The release of total sugar (1.45 mg g⁻¹), reducing sugar (0.88 mg g⁻¹), non reducing sugar (0.57 mg g⁻¹) from 5th day extracted cellulase were found to be on par with 10th day extracted cellulase.

4.5.2 Wheat straw

The data pertaining to release of total sugar, reducing sugar and non reducing sugar by 5th and 10th day extracted cellulase to wheat straw is presented in Table-6a.

The total sugar, reducing sugar, non reducing sugar released were found to be maximum when wheat straw was treated with 10th day extracted cellulase (1.83 mg g⁻¹, 1.42 mg g⁻¹, 0.40 mg g⁻¹) compared to 5th day extracted cellulase treatment, which showed total sugar (1.16 mg g⁻¹), reducing sugar (0.84 mg g⁻¹), non reducing sugar (0.32 mg g⁻¹). The total sugar and reducing sugar released from 10th day extracted cellulase were found significantly superior over the 5th day extracted cellulase treatment in the release total sugar and reducing sugar.

4.5.3 Bagasse

The variation observed on release of total sugar, reducing sugar, non reducing sugar from bagasse when pretreated with 5th and 10th day extracted

cellulase enzyme was presented in Table-6c. The crude enzyme extracted on 10th which was pretreated to bagasse had shown maximum release of total sugar (1.60 mg g⁻¹) reducing sugar (1.16 mg g⁻¹) and non reducing sugar (0.44 mg g⁻¹) were compared to 5th day extracted cellulase treatment, which released total sugar (1.21 mg g⁻¹), reducing sugar (0.81mg g⁻¹) non reducing sugar (0.40 mg g⁻¹) .The 5th day extracted cellulase treatment was found to be on par with 10th day extracted cellulase treatment with respect to total sugar, reducing sugar and non reducing sugar.

4.6. EFFECT OF CRUDE CELLULASE ENZYME (FROM *T. reesei* ON M & R MEDIUM) ON THE RELESE OF SUGAR AND OTHER CONSTITUENTS IN THREE CROP RESIDUES

Similar to crude enzyme treatment extracted from bagasse, enzyme from *T.viride* also had positive influence on release of total sugar, reducing sugar, and non reducing sugar from paddy straw, wheat straw and bagasse. The data pertaining to it is being presented in Table-7.

4.6.1. Total sugar

The release of total sugar from paddy straw, wheat straw and bagasse treated with crude enzyme obtained from *T. reesei* is shown in Table-7. Among the substrates, wheat straw inoculated with *T. reesei* enzyme has shown significantly higher total sugar (2.32 mg g⁻¹) compared to other substrates. The next best substrate in release of total sugar was paddy straw (1.67 mg g⁻¹).

Table 7: Effect of crude cellulase enzyme (from *Trichoderma reesei* on *M& R Medium*) on the release of sugar in 3 crop residues

Sl. No	Treatments	Total sugar (mg g ⁻¹)	Reducing sugar (mg g ⁻¹)	Non reducing sugar (mg g ⁻¹)
1	Paddy straw	1.670	0.967	0.74
2	Wheat straw	2.323	1.227	1.10
3	Bagasse	1.390	0.703	0.69
	SEM±	0.051	0.025	0.025
	CD at 1%	0.190	0.045	0.045

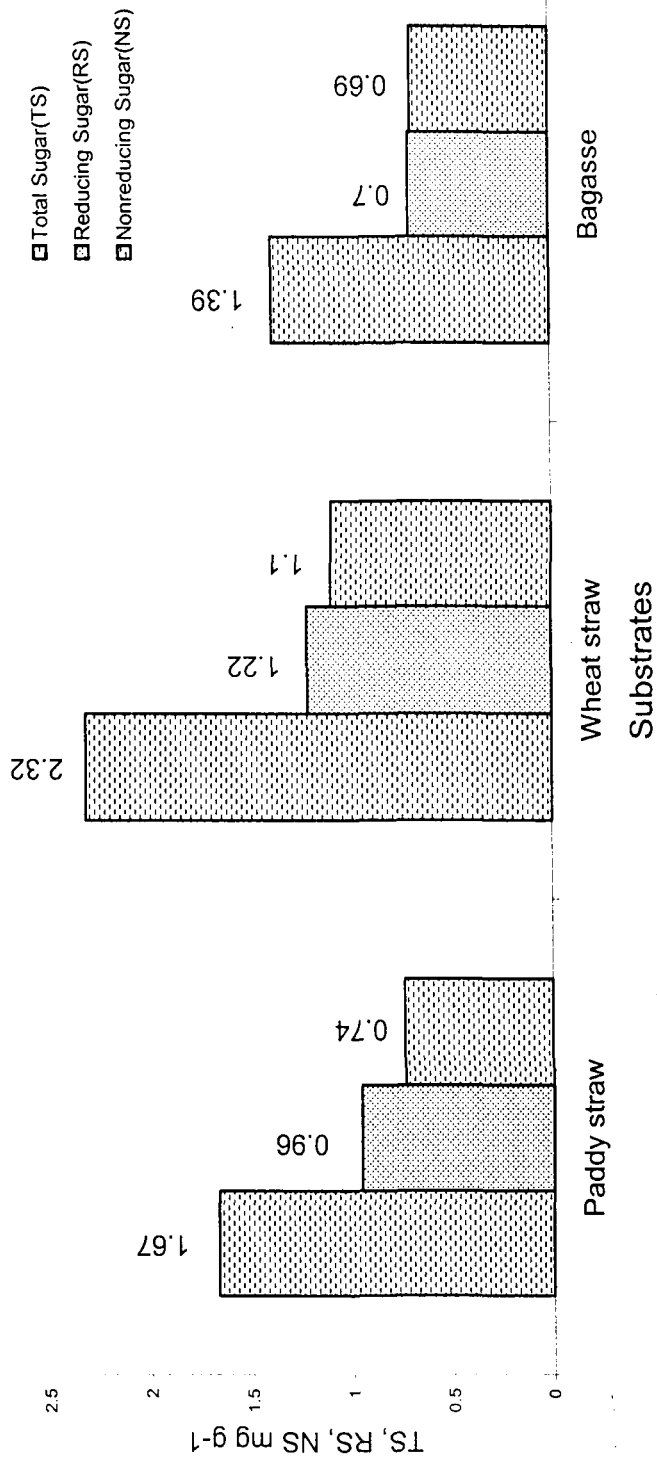


Fig. 4: Effect of crude cellulase enzyme (from *Trichoderma reesei*) on release of sugars in three substrates

4.6.2. Reducing sugar

The reducing sugar released from paddy straw, wheat straw and bagasse when inoculated with *T. reesei* cellulase is recorded in Table-7.

The highest reducing sugar content (1.22 mg g^{-1}) was recorded when wheat straw was pretreated with *T. reesei* crude extract which was found significantly superior over other substrates. The next best substrate was paddy straw (0.96 mg/g) in the release of reducing sugar.

4.6.3. Non reducing sugar

The maximum non reducing sugar released was recorded, in wheat straw (1.10 mg g^{-1}) pretreated with *T. reesei* cellulase, which is found significantly superior over other substrate. The next best substrate was found to be paddy straw (0.70 mg g^{-1}) in the release of non reducing sugar as observed in table-7.

4.7 ETHANOL PRODUCTION IN FUNGAL PRE TREATED PADDY STRAW (WITH SUBSTRATE)

The ethanol yield in paddy straw was found to be increased when fungal pre treated paddy straw was inoculated with different yeast cultures. Accordingly, residual total sugar, reducing sugar and non-reducing sugar content were decreased (Table-8a).

The data regarding ethanol production from fungal pretreated paddy straw when inoculated with different yeast cultures is presented in Table-8a. Ethanol yield was recorded maximum, when fungal pretreated paddy straw

Table 8a: Ethanol production in fungal pre treated, Paddy straw (with substrate)

Sl. No	Treatments	Residual Total sugar (mg g ⁻¹)	Residual Reducing sugar (mg g ⁻¹)	Residual Non Reducing sugar (mg g ⁻¹)	Ethanol mg L ⁻¹
1	Control	2.47	1.27	1.20	025.33
2	<i>Pachysolen tannophilus</i>	0.98	0.50	0.48	129.60
3	<i>Pichia stipitis</i>	1.16	0.56	0.60	206.40
4	<i>Candida shehate</i>	1.09	0.51	0.58	192.00
5	BCY-108	1.00	0.45	0.55	264.00
6	<i>Saccharomyces cerevisiae</i>	1.15	0.49	0.66	384.00
7	<i>Zymomonas mobilis</i>	0.96	0.44	0.52	451.20
	SEM±	0.025	0.025	0.025	3.496
	CD at 1%	0.076	0.076	0.076	10.40

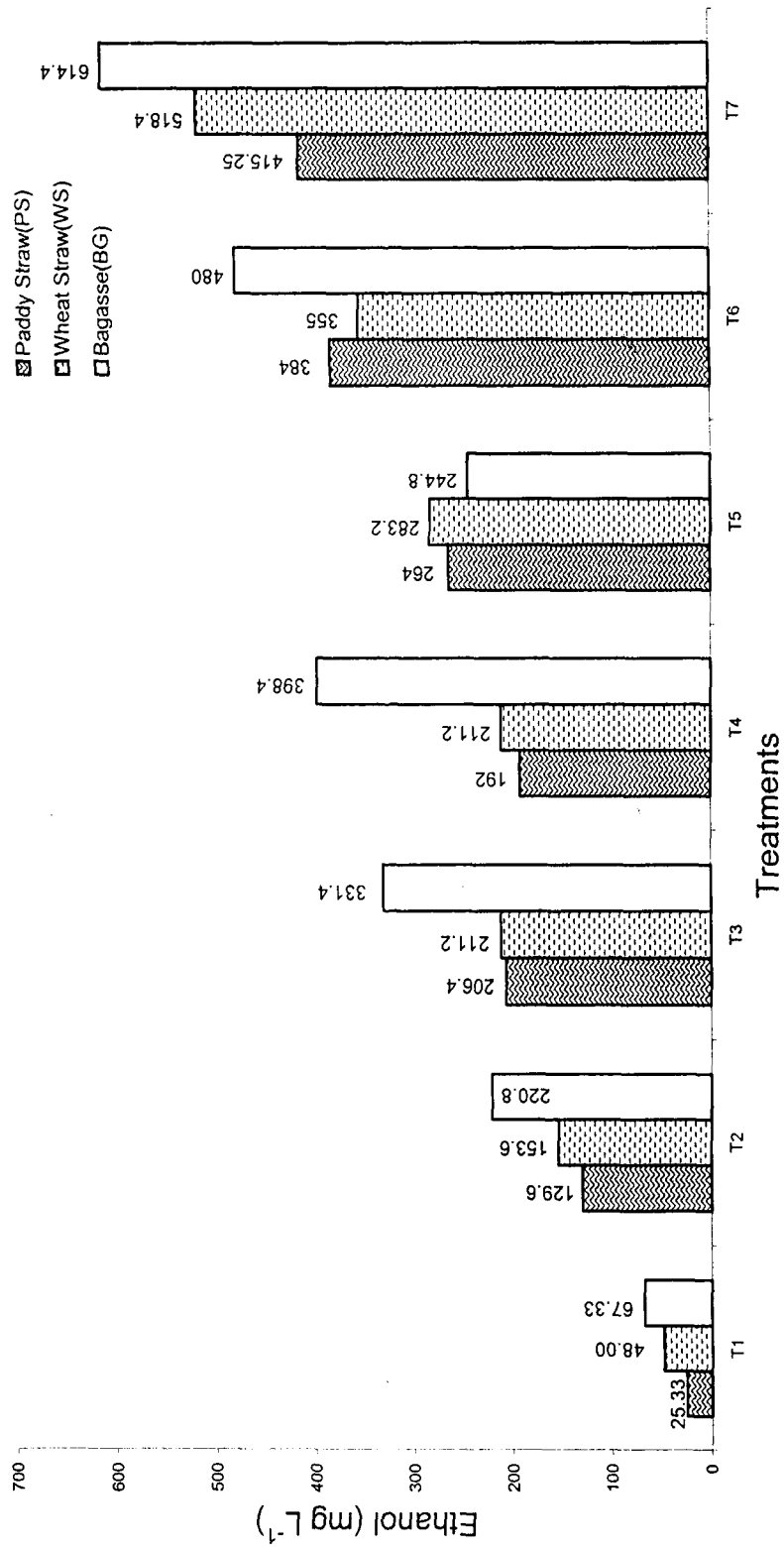


Fig. 5: Ethanol Production in fungal pretreated Paddy, Wheat straw and Bagasse(with substrates) mg L⁻¹

was inoculated with *Z. mobilis* (451.2 mg L⁻¹), which was found significantly superior over rest of the treatment and also over uninoculated control. The next best treatment was fungal pretreated paddy straw inoculated with *S. cerevisiae*, which recorded an yield of 384 mg L⁻¹.

Accordingly, there is decrease in the total sugar content (Table-8a). The maximum decrease in total sugar was observed in *Z. mobilis* treatment (0.96 mg g⁻¹) followed by *P. tannophilus* (0.98 mg g⁻¹) when compared with control, which recorded 2.47 mg g⁻¹. Also reduction in the reducing sugar content was observed (Table-8a). The maximum reduction in the reducing sugar observed in *Z. mobilis* treatment (0.44 mg g⁻¹) followed by BCY-108 (0.45 mg g⁻¹) compared to uninoculated control, which recorded 1.27 mg g⁻¹. The maximum reduction of residual non-reducing sugar observed in paddy straw inoculated with *P. tannophilus* (0.48 mg g⁻¹) followed by *Z. mobilis* treatment (0.52 mg g⁻¹) compared to uninoculated control, which recorded 1.20 mg g⁻¹.

4.8 ETHANOL PRODUCTION IN FUNGAL PRETREATED, WHEAT STRAW (WITH SUBSTRATE)

The ethanol yield in fungal pretreated wheat straw was found to increase due to inoculation of the efficient yeast cultures. Accordingly, the residual total sugar content, residual reducing sugar and residual non-reducing sugar content, were found to be decreased (Table-8b).

The ethanol yield increased significantly in the fungal pretreated wheat straw, when inoculated with *Z. mobilis*, which recorded 518.4 mg L⁻¹, which was found significantly superior over other treatments and uninoculated control. The next best treatment was wheat straw inoculated with *S. cerevisiae*, which recorded a yield of 355.2 mg L⁻¹ (Table-8b).

Table 8b: Ethanol production in fungal pre treated, wheat straw (with substrate)

Sl.No	Treatments	Residual Total sugar (mg g ⁻¹)	Residual Reducing sugar (mg g ⁻¹)	Residual Non Reducing sugar (mg g ⁻¹)	Ethanol mg L ⁻¹
1	Control	2.43	1.46	0.97	048.00
2	<i>Pachysolen tannophilus</i>	1.11	0.61	0.50	153.60
3	<i>Pichia stipitis</i>	0.94	0.50	0.44	211.20
4	<i>Candida shehate</i>	1.22	0.63	0.59	211.20
5	BCY-108	1.19	0.67	0.52	283.20
6	<i>Saccharomyces cerevisiae</i>	1.09	0.51	0.58	355.20
7	<i>Zymomonas mobilis</i>	0.92	0.45	0.47	518.40
	SEM±	0.025	0.025	0.025	3.720
	CD at 1%	0.076	0.076	0.076	11.07

Similarly the residual total sugar content was found to be decreased significantly in yeast inoculated in wheat straw. The minimum content of total sugar observed in wheat straw inoculated with *Z. mobilis* (0.92 mg g^{-1}) followed by *P. stipitis* treatment (0.94 mg g^{-1}) when compared to uninoculated control (2.43 mg g^{-1}).

Accordingly maximum reduction of reducing sugar observed in *Z. mobilis* treatment (0.45 mg g^{-1}) followed by *P. stipitis* which recorded residual reducing sugar content of 0.50 mg g^{-1} compared to control (1.46 mg g^{-1}). The minimum residual non reducing sugar content observed in *P. stipitis* (0.44 mg g^{-1}) and *Z. mobilis* (0.47 mg g^{-1}) strains inoculated to wheat straw, where as uninoculated control recorded 0.97 mg g^{-1} (Table-8b).

4.9 ETHANOL PRODUCTION IN FUNGAL PRETREATED, BAGASSE (WITH SUBSTRATE)

The data pertaining to increase in ethanol yield and residual total sugar, reducing sugar and non-reducing sugar content was presented in Table-8c.

Among the different yeast cultures inoculated to fungal pretreated bagasse *Z. mobilis* has shown significantly higher yield of ethanol (614.4 mg L^{-1}), which was found significantly superior over other treatments and uninoculated control. Next best treatment in production of ethanol was found to be *S. cerevisiae*, which recorded 480 mg L^{-1} of ethanol.

The residual total sugar content was found to be decreased in all treatments compared to uninoculated control table (6c). The minimum content of residual total sugar was found in bagasse inoculated with BCY-108 (0.93 mg g^{-1}) followed by *Z. mobilis* (0.95 mg g^{-1}). Similarly, minimum residual

Table 8c: Ethanol production in fungal pretreated, bagasse (with substrate)

Sl. No	Treatments	Residual Total sugar (mg g ⁻¹)	Residual Reducing sugar (mg g ⁻¹)	Residual Non Reducing sugar (mg g ⁻¹)	Ethanol mg L ⁻¹
1	Control	2.41	1.60	0.81	067.33
2	<i>Pachysolen tannophilus</i>	1.14	0.61	0.53	220.80
3	<i>Pichia stipitis</i>	1.26	0.72	0.54	331.40
4	<i>Candida shehate</i>	1.08	0.52	0.56	398.40
5	BCY-108	0.93	0.46	0.47	244.80
6	<i>Saccharomyces cerevisiae</i>	0.98	0.44	0.54	480.00
7	<i>Zymomonas mobilis</i>	0.95	0.37	0.58	614.40
	SEM±	0.025	0.025	0.025	3.720
	CD at 1%	0.076	0.076	0.076	11.07

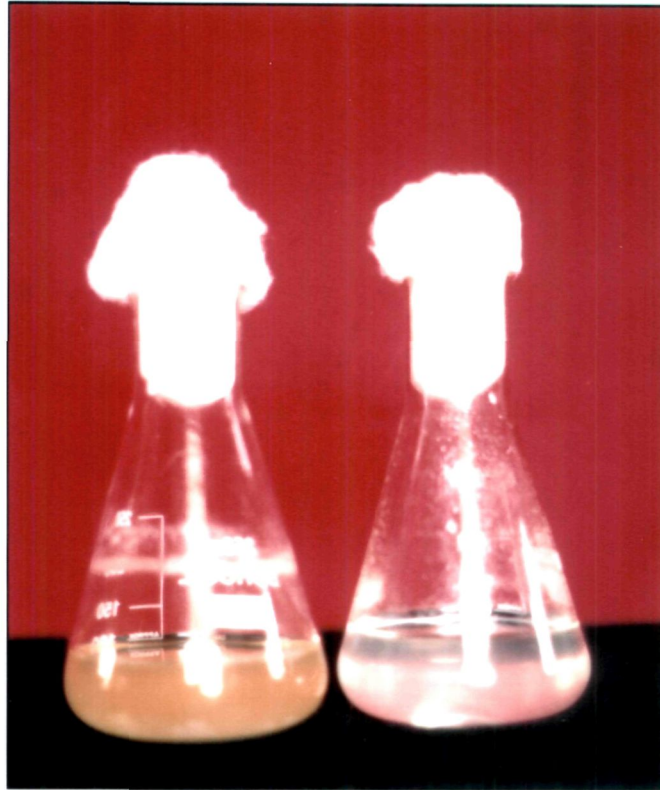


Plate 4: Cellulase enzyme extracted from M and R media inoculated with *Trichoderma reesei*



Plate 5: Experimental setup for fermentation

reducing sugar content was observed in *Z.mobilis* treatments (0.37 mg g⁻¹) followed by *S. cerevisiae*, which recorded 0.44 mg g⁻¹ (Table-8c). The minimum residual non reducing sugar content was observed in BCY-108 inoculated to fungal pretreated bagasse (0.47 mg g⁻¹) followed by *P. tannophilus* which recorded 0.53 mg g⁻¹ compared to uninoculated control (0.81 mg g⁻¹).

4.10 ETHANOL PRODUCTION IN FUNGAL PRETREATED, PADDY STRAW (WITHOUT SUBSTRATE)

The data regarding ethanol yield and residual total sugar, reducing sugar, non reducing sugar content in fungal pretreated filtrate of paddy straw (Table-9a).

Among the different yeast cultures fungal pretreated filtrate of paddy straw inoculated with *Z. mobilis* has recorded maximum ethanol yield (588.73 mg L⁻¹) and found to be significantly superior over other treatments, and uninoculated control. This was followed by *S. cerevisiae* which recorded an yield of 494.4mg L⁻¹.

Accordingly, data regarding decrease in residual total sugar, reducing sugar and non reducing sugar is presented in Table-9a. The minimum content of residual total sugar observed in fungal pretreated filtrate of paddy straw inoculated with *S. cerevisiae* (1.15 mg g⁻¹) followed by *Z. mobilis* (1.17 mg g⁻¹) compared to uninoculated control, which recorded 2.50 mg g⁻¹. The minimum residual reducing sugar content observed in *Z. mobilis* (0.45 mg g⁻¹) followed by *P.tannophilus* (0.57 mg g⁻¹) compared to control (1.25 mg g⁻¹). The maximum decrease in residual non reducing sugar content was observed in *S. cerevisiae* and BCY-108 inoculated to fungal pretreated filtrate of paddy straw,

Table 9a: Ethanol production in fungal pre treated Paddy straw (without substrate)

Sl.No	Treatments	Residual Total sugar (mg g ⁻¹)	Residual Reducing sugar (mg g ⁻¹)	Residual Non Reducing sugar (mg g ⁻¹)	Ethanol mg L ⁻¹
1	Control	2.50	1.25	1.25	038.40
2	<i>Pachysolen tannophilus</i>	1.36	0.57	0.79	134.40
3	<i>Pichia stipitis</i>	1.59	0.58	1.01	096.00
4	<i>Candida shehate</i>	1.71	0.62	1.09	264.00
5	BCY-108	1.29	0.63	0.66	225.60
6	<i>Saccharomyces cerevisiae</i>	1.15	0.60	0.55	494.40
7	<i>Zymomonas mobilis</i>	1.17	0.45	0.72	588.73
	SEM±	0.025	0.025	0.025	2.652
	CD at 1%	0.076	0.076	0.076	7.895

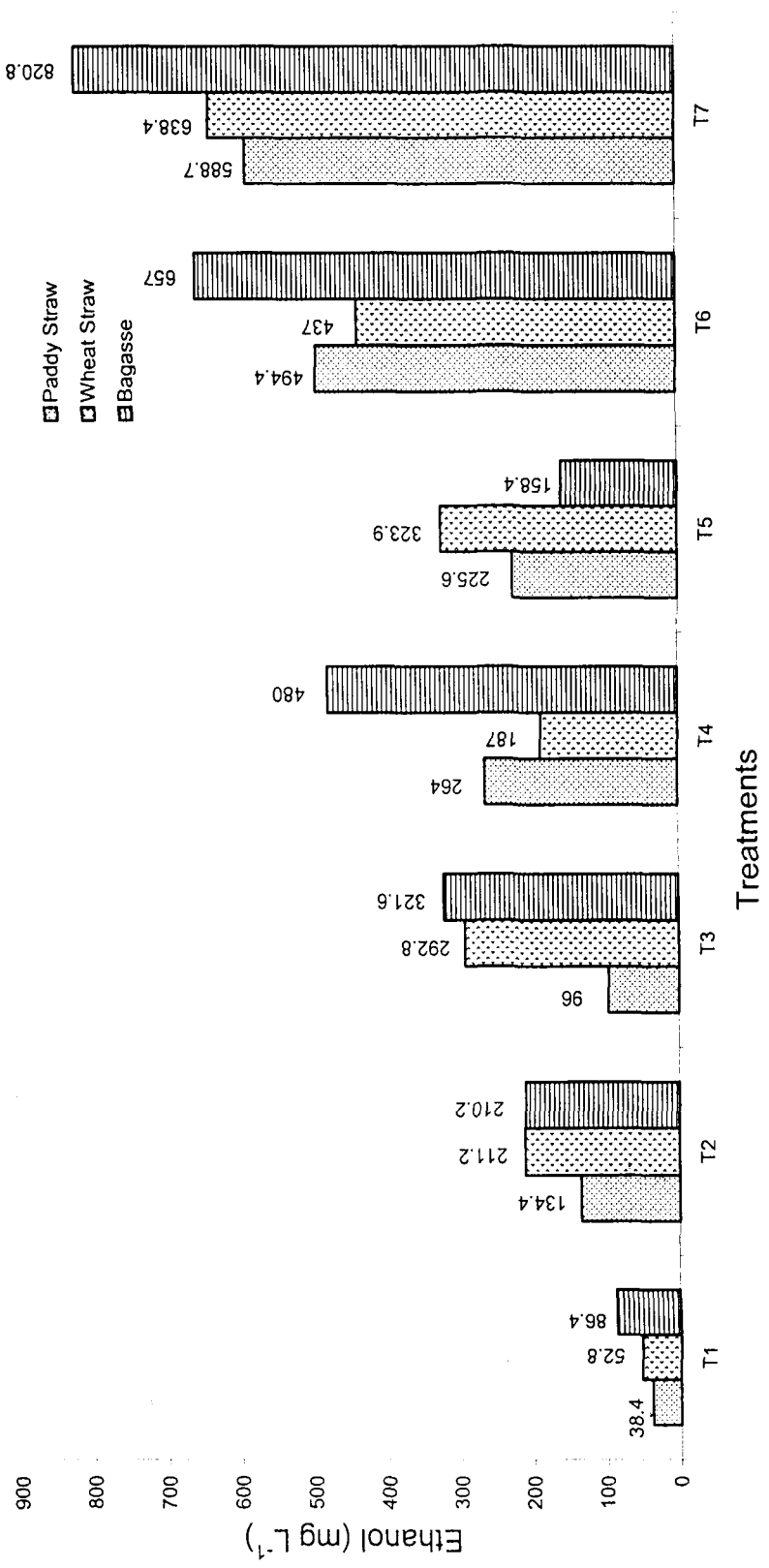


Fig. 6: Ethanol Production in fungal pretreated Paddy, Wheat straw and Bagasse (without substrate) mg L⁻¹

that is 0.55mg g⁻¹ and 0.66 mg g⁻¹ respectively as compared to uninoculated control, which recorded 1.25 mg g⁻¹ of residual non reducing sugar.

4.11 ETHANOL PRODUCTION IN FUNGAL PRE TREATED WHEAT STRAW (WITHOUT SUBSTRATE)

The ethanol yield in fungal pretreated filtrate of wheat straw was found to increase due to inoculation of different yeast cultures. Accordingly the residual total sugar, reducing sugar and non reducing sugar content decreased (Table-9b).

The ethanol yield increased significantly in the fungal pretreated filtrate of wheat straw when inoculated with *Z.mobilis* (638.4 mg L⁻¹), followed by *S. cerevisiae* (437.46 mg L⁻¹). The strain *Z. mobilis* was found to be significantly superior over other treatments and over uninoculated control, which recorded 52.8 mg L⁻¹ of the substrate (Table- 9b)

Accordingly, there was reduction in residual total sugar content. The minimum content of residual total sugar observed in *P. tannophilus* inoculated to filtrate of wheat straw was 1.02 mg g⁻¹ followed by *P. stipitis* which recorded 1.14 mg g⁻¹ respectively compared to control (2.43 mg g⁻¹). The maximum reduction in residual sugar content was recorded in *Z. mobilis* (0.32 mg g⁻¹) and BCY-108 (0.36 mg g⁻¹) compared to uninoculated control which recorded residual reducing sugar content of 1.46 mg g⁻¹. The maximum reduction in non-reducing sugar was observed in filtrate inoculated with *P.tannophilus* (0.64 mg g⁻¹) followed by *C.shehate* (0.70 mg g⁻¹) compared to uninoculated control, which recorded 0.97 mg g⁻¹ indicating their potential in conversion to ethanol.

Table 9b: Ethanol production in fungal pre treated wheat straw (without substrate)

Sl.No	Treatments	Residual total sugar (mg g ⁻¹)	Residual reducing sugar (mg g ⁻¹)	Residual Non reducing sugar (mg g ⁻¹)	Ethanol mg L ⁻¹
1	Control	2.43	1.46	0.97	052.80
2	<i>Pachysolen tannophilus</i>	1.02	0.38	0.64	211.20
3	<i>Pichia stipitis</i>	1.14	0.38	0.76	292.80
4	<i>Candida shehate</i>	1.15	0.45	0.70	187.20
5	BCY-108	1.27	0.36	0.91	323.93
6	<i>Saccharomyces cerevisiae</i>	1.42	0.52	0.90	437.46
7	<i>Zymomonas mobilis</i>	1.18	0.32	0.86	638.40
	SEM±	0.025	0.025	0.025	3.462
	CD at 1%	0.076	0.076	0.076	10.30

4.12 ETHANOL PRODUCTION IN FUNGAL PRETREATED BAGASSE (WITHOUT SUBSTRATE)

The data regarding ethanol production and residual total sugar, reducing sugar, and non reducing sugar content after inoculation of different yeast cultures to fungal pretreated filtrate of bagasse (Table-9c).

The maximum ethanol yield was recorded, when fungal treated filtrate of bagasse was inoculated with *Z. mobilis* (820.8 mg L⁻¹), which was found significantly superior over other treatments and over uninoculated control. The next best treatment was found to be *S. cerevisiae* treatment, which recorded ethanol yield of 657.6 mg L⁻¹.

Similarly maximum reduction in residual total sugar was observed in *S. cerevisiae* and in *Z. mobilis* which recorded 1.16 mg g⁻¹ and 1.19 mg g⁻¹ respectively compared to uninoculated control 2.41 mg g⁻¹ (Table-9c). The decreased in residual reducing sugar content is presented in table (7c). Minimum sugar content was observed in *P. tannophilus* treatment (0.41 mg g⁻¹) and also in *P. stipitis* and *Z. mobilis* treatment, which recorded sugar content of 0.42 mg g⁻¹ compared to control (1.3 mg g⁻¹).

The maximum reduction in non reducing sugar content observed in *S. cerevisiae* inoculated filtrate of bagasse which recorded minimum content of 0.63 mg g⁻¹ followed by *Z. mobilis* (0.77 mg g⁻¹), when compared to uninoculated control, which recorded a non reducing sugar content of 1.02 mg g⁻¹ (Table – 9c).

Table 9c: Ethanol production in fungal pretreated bagasse (without substrate)

Sl.No	Treatments	Residual total sugar (mg g ⁻¹)	Residual reducing sugar (mg g ⁻¹)	Residual Non reducing sugar (mg g ⁻¹)	Ethanol mg L ⁻¹
1	Control	2.41	1.39	1.02	086.40
2	<i>Pachysolen tanophillus</i>	1.23	0.41	0.82	210.20
3	<i>Pichia stipitis</i>	1.29	0.42	0.87	321.60
4	<i>Candida shehate</i>	1.58	0.44	1.14	480.00
5	BCY-108	1.42	0.49	0.93	158.40
6	<i>Saccharomyces cerevisiae</i>	1.16	0.53	0.63	657.60
7	<i>Zymomonas mobilis</i>	1.19	0.42	0.77	820.80
	SEM±	0.001	0.001	0.001	50.536
	CD at 1%	0.076	0.076	0.076	17.27

Discussion

V. DISCUSSION

The continuous exploitation of natural fossil fuels has resulted in its faster depletion. The increase in price of non renewable energy source and enhanced environmental pollution necessitates to search for alternate energy sources, which were renewable, cost effective and non toxic.

In next few years the renewable energy source could eventually occupy the position of natural fossil fuels. Ethanol is one of the biofuel, which can substitute diesel upto 20 percent. Presently, five percent of ethanol is being blended along with diesel for transportation in few states in India. This indicates the demand for ethanol. Ethanol can be produced from various biomass such as sudan grass, sweet sorghum, sugarcane, sugar beets, corn, wheat, barley, potato etc. which are available abundantly. In India, ethanol is produced by fermentation of molasses using strain of *S. cerevisiae*. In recent years, due to the increase in the cost of molasses, it has been emphasized to search alternate substrates for alcohol production. Some of the alternate biomass sources tried successfully are the pineapple, cannery waste (Nigam, 1999); Starch (Verma et al., 2000) and carob pods (Marakis and Marakis, 1996).

Paddy, wheat and sugarcane are the major crops in India and in Karnataka and is reported that 606 mt of paddy straw, 299 mt of wheat straw and 887 thousand tones of bagasse are being produced every year. These crop residues do have the potential for production of ethanol as they contain considerable amount of sugars bound in the form of cellulose and hemicellulose. The present investigation was undertaken to know the possible extent of release of maximum reducing sugars by following microbiological

pretreatment methods and to identify suitable yeast and bacterial strains for fermentation for ethanol production.

All lignocellulosic plant materials provide abundant source of renewable energy (Eva and Barbel, 2000). Since they contain polysaccharides like cellulose 50%, hemicellulose 25% and lignin 25% (Ashok *et al.* 2000). These polysaccharides after pretreatment break down into simple sugars, which is subsequently fermented to ethanol by fermenting microorganisms. In the present study initial chemical constituents of the raw substrates were estimated. Paddy straw and wheat straw contain reducing sugar (0.03 mg g^{-1} and 0.02 mg g^{-1}), total sugar (0.56 mg g^{-1} and 0.60 mg g^{-1}) non reducing sugar (0.53 mg g^{-1} and 0.58 mg g^{-1}), carbon (52.2% and 54.6%), nitrogen (0.41% and 0.35%), total solids (90% and 89%), moisture (10% and 11%). Bagasses contain reducing sugar (0.16 mg g^{-1}), total sugar (0.69 mg g^{-1}), non reducing sugar (0.53 mg g^{-1}), carbon (54.4%), nitrogen (0.43%), total solids (92%) and moisture (8%). The composition indicates that there is fair amount of available sugars for conversion. Further, microbiological and enzymatic pretreatment methods were carried out to assess the release of maximum reducing sugars. It is well established that certain fungi are able to saccharify the substrate through their enzymes, which cleave the complex polymer into simple sugars. Microbiological pretreatment is an attractive method as it could be cheap, efficient and ecofriendly (Vidyavathi 2003).

In the present study, six different fungal cultures were inoculated to paddy straw, wheat straw and bagasse individually and in combinations to know their effect on release of sugars. After the incubation period, reducing sugar, total sugar, non reducing sugar, cellulase, carbon and nitrogen content were estimated. It was found that reducing sugar content was increased in

all the three substrates with all the twenty-one treatments when compared with the control. This indicated that all the fungal cultures efficiently degrade complex lignocelluloses. The break down of cellulose and hemicellulose resulted in the release of fermentable hexose sugar (glucose), which are in reducing form. In some case organisms utilize non reducing sugar as carbon source and convert non reducing sugar to reducing sugar, thus resulting in over all increase in the final reducing sugar content. Among the different treatments, tested combined inoculation of *P. chrysosporium* and *Pleurotus spp* was found to be efficient in releasing the reducing sugar both in paddy straw and bagasse (1.27 mg g⁻¹ and 1.97 mg g⁻¹ respectively). The next best treatment in case of paddy straw was *Pleurotus spp* (1.21 mg g⁻¹) and in case of bagasse, the combined inoculation of *T. reesei* and *Pleurotus spp* (1.60 mg g⁻¹). The maximum release of reducing sugar in wheat straw was found in combined inoculation of *T. viride* and *P. chrysosporium* (1.47 mg g⁻¹), which was followed by, combined inoculation of *P. chrysosporium* and *Pleurotus spp* (1.44 mg g⁻¹). When different treatments were compared, combined inoculation of fungal cultures were showed maximum release of reducing sugar than single inoculation in all the three substrates. This may be because of mutual synergism between two organisms in release of reducing sugar. Similar work was done by Yadav *et al.* (1992), showed that maximum degradation of sugarcane trash was observed with the combined application of *Penicillium sp* and *Bacillus megatherium*. Gupte and Madamwar (1997) reported that co-culturing of two fungal strains showed improved hydrolytic activity when compared to their single inoculation. Zayed and Meyer (1996) examined *T. viride* and *A. niger* for their ability to produce fermentable sugars from cellulosic waste and achieved reducing sugar extraction of 27 g from 50 g delignified wheat straw at 25 to 30°C within 3 days. Similarly Geeta *et al.*

(2002) observed maximum saccharification of paddy straw due to inoculation of fungal cultures viz. *P. chrysosporium*, *Aspergillus foetidus*, *A. niger* and *T. viride* for bioethanol production. Among the fungal cultures *P. chrysosporium* was found to be superior in releasing fermentable sugars, which yielded 534 mg L⁻¹ accounting upto 53.4 percent. Similar studies on biological pretreatment for maximum release of reducing sugar in yam was observed by Ramanathan (2000) and Roche and Durand (1996) observed in sugar beet pulp.

The total sugar includes both reducing sugar and non reducing sugar (Chen and Chou, 1993). Increase or decrease in the reducing and non reducing sugar results in the change of total sugar content. In the present study total sugar content was found to be increased in all the substrates with all the treatments compared to the control. Increase in the total sugar content after microbiological pretreatment may be because of maximum release of reducing sugar and non reducing sugar. Among the different treatments in all the substrates (Paddy straw, wheat straw and bagasse) combined inoculation of *P. chrysosporium* and *Pleurotus spp* showed maximum total sugar content (2.48 mg g⁻¹, 2.54 mg g⁻¹ and 2.41 mg g⁻¹ respectively). The next best treatment to release the total sugar in case of paddy straw was single inoculation of *Phanerochaete chrysosporium* (2.10 mg g⁻¹) and combined inoculation of *T. viride* and *P. chrysosporium* (2.43 mg g⁻¹) in case of wheat straw. In case of bagasse second best treatment was combined inoculation of *T. reesei* and *Pleurotus spp* (2.27 mg g⁻¹).

Non reducing sugar content in paddy straw showed variations depending upon the treatment. Maximum increase of non reducing sugar in paddy straw was observed in the combined inoculation of *P. chrysosporium*

and *Pleurotus spp* (1.21 mg g⁻¹) followed by combined inoculation of *T. viride* and *Pleurotus spp* (0.96 mg g⁻¹). In wheat straw maximum non reducing sugar was observed in combination of *P. chrysosporium* and *Pleurotus spp* (1.10 mg g⁻¹) followed by combined inoculation of *T. reesei* and *P. chrysosporium* (1.04 mg g⁻¹). In case of bagasse maximum release of non reducing sugar was observed in single inoculation of *P. chrysosporium* (0.89 mg⁻¹), which was followed, by combined inoculation of *T. viride* and *T. reesei* (0.16 mg⁻¹).

Cellulase is an extra cellular, inducible enzyme having three components such as endoglucanases, exoglucanases and β -glucosidases (Umar and Hanif, 1996). In the present investigation, cellulase released by different fungal cultures both individually and in combination were tested with all the substrates paddy, wheat straw and bagasse. The maximum release of cellulase in paddy straw was observed in individual treatment of *P. chrysosporium* (4.29 IU), which is followed by combined inoculation of *P. chrysosporium* and *Pleurotus spp* (3.33 IU). In wheat straw the maximum release of cellulase was found in single inoculation of *Pleurotus spp* (4.73 IU) followed by the combined inoculation of *T. viride* and *A. awamori* (4.47 IU). Maximum release of cellulase in bagasse was found in the combined inoculation of *T. viride* and *T. reesei* (4.79 IU), which was followed by the combined inoculation of *T. viride* and *P. chrysosporium*, which yielded 3.80 IU of cellulase. Similar work was done by Gupte and Madamwar (1997). They reported that production cellulolytic enzyme under SSF by co-culturing of two fungal strains showed improved hydrolysis and production of β -glucosidase enzyme as compared to single inoculation. Modi *et al.* (1994) reported higher yield of cellulase from strain of *Streptomyces spp* HM29 when grown on bagasse instead of rice straw. Umar and Hanif (1996) extracted maximum cellulase enzyme when *Aspergillus fumigatus* was grown on wheat straw.

Microorganisms require carbon source for their energy and development and to carry out different metabolic activity. In the present investigation organisms utilized paddy straw, wheat straw and bagasse as their source of carbon for efficient saccharification. When all the substrates with different treatments analysed for carbon content, it was found that carbon content was decreased after incubation period, as compared to control. This indicated the activity of inoculated fungal strains in efficient degradation of substrates. The maximum utilization of carbon in case of paddy straw was observed in combined inoculation of *A. niger* and *Pleurotus spp* (47.3%). In case of wheat straw maximum utilization of carbon was observed in combined inoculation and *T.viride* and *Pleurotus spp* and also in combined inoculation of *A. niger* and *A.awamori* (52.6%). *A.awamori* showed maximum utilization of carbon (50.1%) in case of bagasse. Similar result was observed by Sharath (2003). They reported that carbon content in wood was decreased after fungal inoculation. Umar and Hanif (1996) used wheat straw as carbon source for the production of cellulase by *Aspergillus fumigatus*. Breccia *et al.* (1997) reported that fungal cultures utilized hemicellulose in bagasse as carbon source for the production of cellulase.

After the pretreatment the nitrogen content in all the substrates with different treatments was found to be increased when compared to the uninoculated control. Increase in the nitrogen content may be because of decrease in the mass of the substrates after 7 days of incubation period (Sharath, 2003). Maximum nitrogen content in paddy straw was observed in the combined inoculation of *T.viride* and *P. chrysosporium* (0.57%). In wheat straw the maximum increase was observed in combined inoculation of *A. niger* and *A.awamori* (0.49%). Where as in bagasse the highest nitrogen content was recorded in *T.viride* treatment (0.58%). Similar results were obtained by

Sharath (2003). They reported that the nitrogen content of wood increased after degradation.

After microbiological pretreatment the substrates were also analysed for release of reducing sugar and cellulase. It was found that bagasse was the best substrate. It released maximum sugar (1.97 mg g^{-1}) when inoculated with combined pretreatment of *P. chrysosporium* and *Pleurotus spp* and maximum cellulase (4.74 IU) when inoculated with both *T. viride* and *T. reesei*. This may be because of low ash content in bagasse (2.4%). Where as paddy straw and wheat straw contain 17.5 percent and 11 percent of ash respectively. So the low ash content offers numerous advantages like bioconversion process using microbial cultures as hypothesized by (Ashok *et al.* 2000).

The enzymatic degradation of cellulose is catalyzed by three major enzymes of the cellulase complex (endoglucanases, exoglucanases and β -glucosidases). The efficiency of enzymatic degradation of different biomass depends upon different parameters like structural properties of the substrate, mode of action of the cellulase, adsorption and desorption phenomenon etc. (Sattler *et al.* 1998).

The crude enzyme extracted on 5th and 10th day were pretreated to the substrates and analysed for the parameters like total sugar, reducing sugar and non reducing sugar.

Among the 5th and 10th day extracted crude enzyme pretreated to the paddy straw, the 10th day extracted enzyme showed maximum release of reducing sugar (0.98 mg g^{-1}), total sugar (1.54 mg g^{-1}) and non reducing sugar (0.98 mg g^{-1}). Where as 5th day extracted enzyme released comparatively low amount of reducing sugar (0.88 mg g^{-1}), total sugar (1.45 mg g^{-1}) and non

reducing sugar (0.57 mg g^{-1}). Similarly Parminder *et al.* (1997) obtained maximum reducing sugar of 268.5 mg g^{-1} of cellulase after 48 hours of saccharification.

Similarly wheat straw treated with the 5th and 10th day extracted enzyme was analysed for the above mentioned parameters. The 10th day extracted enzyme treatment showed highest release of total sugar, reducing sugar and non reducing sugar (1.83 mg g^{-1} , 1.42 mg g^{-1} and 0.41 mg g^{-1} respectively), compared to 5th day released enzymes treatment, which released reducing sugar (0.84 mg g^{-1}) total sugar (1.16 mg g^{-1}) and non reducing sugar (0.32 mg g^{-1}). Similarly Lars and Karl (1990) used wheat straw for enzymatic hydrolysis. They reported 2-fold increase in glucose and 4-fold increase in xylose content after enzymatic pretreatment.

In case of bagasse also the 10th day extracted enzyme showed maximum reducing sugar (1.16 mg g^{-1}), total sugar (1.60 mg g^{-1}) and non reducing sugar (0.44 mg g^{-1}) as compared to 5th days released reducing sugar (0.81 mg g^{-1}), total sugar (1.21 mg g^{-1}) and non reducing sugar (0.40 mg g^{-1}). Similarly Sanjeev *et al.* (2002) reported that release of reducing sugar was 375.7 mg g^{-1} when sunflower stalk was pretreated with crude enzyme extract from *Trichoderma*.

The release of sugars by 10th days enzymatic extract was found to be more compared to 5th days enzymatic extract this may be because of concentration of enzyme released due to more days of incubation period and may be lesser concentration of enzyme on 5 days of incubation period. This higher concentration of enzyme on 10th days extracted treatment resulted in the release of maximum sugars. Similar results were observed by Sanjeev *et*

al. (2002). They reported the yield of reducing sugar increased from 112.6 to 373.7 mg g⁻¹ when concentration of enzyme was increased from 5 FPU g⁻¹ to 25 FPU g⁻¹. When substrates were compared for release of sugar, wheat straw showed higher results. The minimum release of sugar in paddy straw and bagasse may be because of crystalline lignocellulosic complex, which may restrict or resist the action of enzymes or these two substrates may require large quantity of enzymes for saccharification as hypothesized by Ashok *et al.* (2000).

In next pretreatment method, cellulase enzyme was extracted from 1% cellulose based M and R media using *T. reesei* (Schaffner and Toledo, 1991). When cellulase extracted from M & R media, inoculated to all the substrates, it was found that wheat straw released maximum reducing sugar (1.22 mg g⁻¹), total sugar (2.3 mg g⁻¹) and non reducing sugar (1.1. mg g⁻¹). Whereas paddy straw and bagasse released comparatively lower sugar (0.9 mg g⁻¹ and 0.7 mg g⁻¹), total sugar (1.6 mg g⁻¹ and 1.3 mg g⁻¹) and non reducing sugar (0.7 mg g⁻¹ and 0.6 mg g⁻¹) respectively.

From the results obtained due to different pretreatments, it could be concluded that all the methods of pretreatment had the potential to release fermentable sugars. But when microbiological pretreatment and enzymatic pretreatment were compared to release maximum reducing sugar, microbiological pretreatment was effective. This may be because of efficiency of fungal cultures to degrade the complex crystalline structure of biomass compared to enzymatic degradation or saccharification. The minimum release of reducing sugar by enzymatic pretreatment compared to microbiological pretreatment may be because of some inhibitory compounds, which inhibit the action of cellulase enzyme. Marsden and Gray (1986) reported that during

hydrolysis, cellulases are very susceptible to end product inhibition such as cellobiose and to a lesser degree by glucose. Sutcliffe and Saddler (1986) reported that large amount of cellulase remain attached to undegraded lignocellulosic residues and become unavailable for more susceptible substrate. Ramo *et al.* (1993) reported that both endoglucanases and cellobiohydrolases are inhibited by increased concentration of cellobiose, whereas β -glucosidases are more sensitive to glucose concentration.

All the substrates in combination with respective efficient fungal cultures were used for the further fermentation. In first experiment, fungal pretreated filtrate of paddy straw, wheat straw and bagasse was inoculated with five different yeasts and one bacterial strain for fermentation.

All the treatments with filtrate of paddy straw showed increased production of ethanol, compared to control. The maximum ethanol yield in paddy straw filtrate was observed in *Z.mobilis* treatment (588.7 mg L⁻¹) followed by *S. cerevisiae* treatment (494.4 mg L⁻¹). The uninoculated control also showed 38.4 mg L⁻¹ of ethanol indicating natural fermentation due to the competent air microflora. The reducing sugar, total sugar and non reducing sugar were decreased significantly in all the treatments indicating the ability of yeasts and bacterial strain to utilize available sugars. The maximum utilization of reducing sugar was observed in *Z.mobilis* treatment (0.45 mg g⁻¹). The maximum utilization of total sugar and non reducing sugar in *S. cerevisiae* treatment, recorded 1.15 mg g⁻¹ and 0.55 mg g⁻¹ respectively.

In wheat straw filtrate, maximum yield of ethanol was recorded in *Z.mobilis* treatment (638.4 mg L⁻¹) followed by *S. cerevisiae* (437.4 mg L⁻¹). The uninoculated control also showed 52.8 mg L⁻¹ of ethanol yield indicating

natural contamination. The reducing sugar, total sugar, non reducing sugar content were found to decrease significantly as compared to un inoculated control. Maximum utilization of reducing sugar was observed in *Z. mobilis* treatment (0.32 mg g⁻¹), total sugar and non reducing sugar in *P.tannophilus* treatment (1.02 mg L⁻¹ and 0.64 mg g⁻¹ respectively).

In case of bagasse filtrate, the highest ethanol yield was recorded in *Z.mobilis* treatment (820.8 mg L⁻¹) followed by *S. cerevisiae*, which recorded 657.6 mg L⁻¹. The uninoculated control showed ethanol yield of 86.4 mg L⁻¹. The residual reducing sugar content decreased significantly in *P.tannophilus* treatment (0.41 mg g⁻¹). Maximum reduction in residual total sugar and non reducing sugar were recorded in *S. cerevisiae* treatment (1.16 mg g⁻¹ and 0.63 mg g⁻¹ respectively). Similar study conducted by Geeta *et al.* (2001) indicated maximum ethanol yield of 40–43 g L⁻¹ by local isolate BCY-107 followed by BCY-108 (36.06 g L⁻¹) Sohn and Seu (1994) achieved maximum ethanol concentration of 86.6 g L⁻¹ by strain *Kluyveromyces marxianus* from 20 percent (w/v) glucose medium at 40°C in 96 hours of fermentation period. Nimbkar *et al.* (1989) studied the sugar utilization by the yeast during fermentation of juice of sweet sorghum with 20 percent fermentable sugar at 48, 72, and 120 hours and observed decrease in the sugar content to 2, 4 and 1 percent at 48, 72 and 120 hours respectively.

The difference in the performance of different fermenting organism may be due to the preferential utilization of pentose or hexose sugars present in the hydrolysate of different substrates. The decrease in the residual sugar content may be due to their high potential in utilization of reducing sugar and non reducing sugar.

In simultaneous saccharification and fermentation (SSF) of paddy straw, among the different treatments maximum ethanol was observed in *Z.mobilis* treatment (451.2 mg L⁻¹) followed by *S. cerevisiae* (384 mg L⁻¹). The uninoculated control did show 25.33 mg L⁻¹ indicating natural contamination. The residual reducing sugar, total sugar and non reducing sugar decreased significantly indicating efficient utilization of sugars for the ethanol production. Maximum decrease or utilization of reducing sugar and total sugar recorded in *Z.mobilis* treatment 0.44 mg g⁻¹ and 0.96 mg g⁻¹ respectively. Maximum utilization of non reducing sugar observed in *P.tannophilus* treatment (0.48 mg g⁻¹).

Maximum ethanol yield in wheat straw after simultaneous saccharification and fermentation (SSF) was recorded in *Z.mobilis* treatment (518.4 mg L⁻¹) followed by *S. cerevisiae* (353.2 mg L⁻¹). The uninoculated control also showed ethanol content of 48 mg l⁻¹. The residual reducing sugar, total sugar and non reducing sugar content decreased significantly compared to control. This indicates the efficient utilization of sugars while fermentation. Maximum reduction in reducing sugar, total sugar, were observed in *Z.mobilis* pretreatment that is 0.45 mg g⁻¹, 0.92 mg g⁻¹ respectively and non reducing sugar in *P. stipits* treatment (0.44 mg g⁻¹).

In case of bagasse ethanol yield was increased with all the cultures. Maximum ethanol was recorded in *Z.mobilis* treatment (614.4 mg L⁻¹) followed by *S. cerevisiae* treatment (480 mg L⁻¹). The uninoculated control also showed ethanol content of 67.3 mg L⁻¹ indicating natural contamination by air microflora. The residual reducing sugar content decreased significantly in *Z.mobilis* (0.37 mg g⁻¹). Maximum decrease in total sugar and non reducing sugar content recorded in BCY-108 treatment, which recorded 0.93 mg g⁻¹ and

0.47 mg g⁻¹ respectively. Similar work was done by Krishna *et al.* (1998) who carried out ethanol production by simultaneous saccharification and fermentation (SSF) of sugarcane leaves using *T. reesei* (QM 9414) and *S. cerevisiae* (NRRL-y-132). They observed 92% conversion of and increased ethanol production.

When both fermentation methods were compared, maximum ethanol was produced in filtrate fermentation method (without solids). This may be due to the available sugar for the yeast strains as carbon source. Where as in simultaneous saccharification and fermentation (SSF) there may be chance of utilization of sugar also by fungal cultures as carbon source. This results in decrease in availability of sugars to yeast strain for fermentation. Among the organisms, *Z. mobilis* recorded maximum production of ethanol in all the substrates and also in both the fermentation methods. This may be because of its higher ability of sugar up take and ethanol production, lower biomass production, higher ethanol tolerance (Gunasekaran and Chandra, 1999).

Thus it can be concluded that the fungal pretreatment of the selected substrates can release the reducing sugars though not substantially significant. Further conversion to ethanol can be achieved by *S. cerevisiae* and *Z. mobilis*. But the yield of ethanol was not be economical. Further extensive research is essential to exploit completely in releasing the bound sugars.

Summary

VI. SUMMARY

A constant increase in the price of petrol and diesel has recently developed the concept of blending ethanol with petrol or diesel @ 20 percent. Majority of the distilleries are using molasses. Due to the decrease in availability and high cost of molasses, alternate sources need to be identified. Therefore, present investigation was carried out using agricultural crop residues like paddy straw, wheat straw and bagasse with the aim of optimizing the pretreatment conditions for obtaining maximum reducing sugars for conversion to ethanol.

1. The naturally available sugar constituents of these crop residues were estimated and it was observed that paddy straw, wheat straw and bagasse contain reducing sugar (0.03 mg g^{-1} , 0.02 mg g^{-1} and 0.16 mg g^{-1}), total sugar (0.56 mg g^{-1} , 0.60 mg g^{-1} and 0.69 mg g^{-1}) non reducing sugar (0.53 mg g^{-1} , 0.58 mg g^{-1} and 0.53 mg g^{-1}) carbon (52.2%, 54.6% and 54.4%) nitrogen (0.41%, 0.35% and 0.43%), total solids (90%, 89% and 92%) and moisture content (10%, 11% and 8%).
2. To maximize the release of reducing sugar microbiological and enzymatic pretreatment were carried out. In microbiological pretreatment to paddy straw, combined inoculation of *P. chrysosporium* and *Pleurotus spp* showed maximum release of reducing sugar (1.27 mg g^{-1}), total sugar (2.48 mg g^{-1}) and non reducing sugar (1.21 mg g^{-1}). Single inoculation of *P. chrysosporium* showed significant release of cellulase (4.29 IU). Maximum utilization of carbon was observed in combined inoculation of *A. niger* and *Pleurotus spp* (47.3%) and increased nitrogen

content was observed in combined inoculation of *T. viride* and *A. awamori* (0.6%).

3. In wheat straw significant increase in the reducing sugar, total sugar and non reducing sugars were observed in combined inoculation of *P. chrysosporium* and *Pleurotus spp* (1.44 mg g⁻¹, 2.54 mg g⁻¹ and 1.10 mg g⁻¹ respectively). Significant increase in the release of cellulase was observed in single inoculation of *Pleurotus spp* (4.73 IU). The maximum utilization of carbon observed in combined inoculation of *Trichoderma reesei* and *P. chrysosporium* (49.8%) and significant increase in nitrogen content was observed in combined inoculation of *A. niger* and *A. awamori* treatment (0.49%).
4. In case of bagasse combined inoculation of *P. chrysosporium* and *Pleurotus spp* showed maximum release of reducing sugar (1.97 mg g⁻¹), total sugar (2.41 mg g⁻¹). Maximum release in non reducing sugar was observed in single inoculation of *P. chrysosporium* (0.89 mg g⁻¹). In releasing of cellulase, combined inoculation of *T. viride* and *T. reesei* showed significant result (4.79 IU). Maximum utilization of carbon was observed in combined inoculation of both *T. viride* and *Pleurotus spp* treatment and *A. niger* and *A. awamori* treatment (52.6%). Significant increase in the nitrogen content was observed in *T. viride* treatment (0.58%).
5. In microbiological pretreatment among three substrates, bagasse released maximum reducing sugar (1.97 mg g⁻¹) and cellulase (4.79 IU) as compared to wheat straw and paddy straw.

6. In crude enzyme pretreatment enzyme extracted on 10th day from bagasse showed maximum release of reducing sugar (0.98 mg g⁻¹) total sugar (1.59 mg g⁻¹) and non reducing sugar (0.61 mg g⁻¹) in paddy straw as compared to 5th day extracted enzyme pretreatment. In wheat straw and bagasse also 10th day extracted enzyme showed maximum release of reducing sugar (1.42 mg g⁻¹ and 1.16 mg g⁻¹ respectively), total sugar (1.83 mg g⁻¹ and 1.60 mg g⁻¹ respectively) and non reducing sugar (0.41 mg g⁻¹ and 0.44 mg g⁻¹ respectively). Among the different substrates wheat straw showed maximum release of reducing sugar (1.42 mg g⁻¹) from 10th day extracted enzyme pretreatment.
7. In crude enzyme pretreatment, the enzyme extracted from 1% cellulose based medium inoculated with *T. reesei*, wheat straw showed maximum release of reducing sugar (1.22 mg g⁻¹) total sugar (2.32 mg g⁻¹) and non reducing sugar (1.10 mg g⁻¹) compared to paddy straw and bagasse.
8. In the filtrate fermentation method of paddy straw *Z. mobilis* (588.73 mg L⁻¹) showed maximum release of ethanol. Significant decrease in the reducing sugar was observed in *Z. mobilis* treatment (0.45 mg g⁻¹). Maximum utilization of total sugar and non reducing sugar was observed in *S. cerevisiae* treatment (1.15 mg g⁻¹ and 0.55 mg g⁻¹ respectively).
9. In wheat straw filtrate, significant release of ethanol was recorded in *Z. mobilis* treatment (638.40 mg L⁻¹). Significant decrease in reducing sugar was recorded in *Z. mobilis* treatment (0.32 mg g⁻¹). Maximum utilization of total sugar and non reducing sugar was observed in *P. tannophilus* treatment (1.02 mg g⁻¹ and 0.64 mg g⁻¹ respectively).

10. In case of bagasse, significant increase in ethanol production observed in *Z. mobilis* treatment (820.3 mg L⁻¹). Significant decrease in reducing sugar observed in *P.tannophilus* (0.41 mg g⁻¹). Maximum decrease in total sugar and non reducing sugar 1.16 mg g⁻¹ and 0.63 mg g⁻¹ respectively in *S. cerevisiae* treatment.

11. In simultaneous saccharification and fermentation (SSF) of paddy straw significant increase in ethanol production (451.2 mg L⁻¹) and significant decrease in total sugar (0.96 mg g⁻¹) and reducing sugar (0.44 mg g⁻¹) was observed in *Z. mobilis* treatment. Maximum decrease in the non reducing sugar observed in *P.tannophilus* treatment (0.48 mg g⁻¹). In wheat straw significant increase in ethanol (518.4 mg L⁻¹) and utilization of reducing sugar (0.45 mg g⁻¹) and total sugar (0.92 mg g⁻¹) was observed in *Z. mobilis* treatment. Significant utilization of non reducing sugar was observed in *P.stipitis* treatment (0.44 mg g⁻¹). In case of bagasse, maximum production of ethanol (614.4 mg L⁻¹) and maximum utilization of reducing sugar (0.37 mg g⁻¹) was found in *Z.mobilis* treatment. Significant decrease in total sugar (0.93 mg g⁻¹) and non-reducing sugar (0.47 mg g⁻¹) was observed in *P.stipitis* treatment.

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Appendices

Appendix I: Potato dextrose agar medium (PDA)

Potato	:	200 g
Dextrose	:	20 g
Yeast extract	:	0.10 g
Agar	:	20 g
Distilled water	:	1000 ml
pH	:	6.0

Preparation

200 g of reeled potatoes were cut into small pieces and suspended in 1000 ml of distilled water and steamed for 30 minutes. The extract was obtained by filtering through the muslin cloth and final volume made upto 1000 ml.

Appendix II: Malt extract glucose yeast extract - peptone (MGYP) agar medium

Yeast extract	:	3 g
Malt extract	:	3 g
Peptone	:	5 g
Glucose	:	10 g
Agar	:	16 g
Distilled water	:	1000 ml
pH	:	6.4 - 6.8

Appendix III: Mandels and Reese Medium (M and R medium)

Stock solutions	Volume in ml to be added to prepare 1 litre medium
(NH ₄) ₂ SO ₄ (10%)	14.00
KH ₂ PO ₄ (1M)	15.00
Urea (10%)	3.00
CaCl ₂ (10%)	3.00
MgSO ₄ .7H ₂ O (10%)	3.00
*Trace metal stock	1.00
Tween 80 (optional)	2.00
Distilled water	1000
*Trace metal stock solution	
FeSO ₄	2.5 g
MnSO ₄ H ₂ O	0.98 g
ZnCl	0.83 g
CaCl ₂	1.00 g
HCl (concentrated)	5 ml
Water	495 ml
pH	5.5

Appendix IV: *Zymomonas mobilis* selective media

Beef extract	:	1%
Sodium chloride	:	0.5%
Peptone	:	1%
Glucose	:	2%
Distilled water	:	100 ml
pH	:	7.0 - 7.2

STUDIES ON ETHANOL PRODUCTION FROM AGRO-RESIDUES

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ABSTRACT

Environmental pollution and demand for energy are the major challenges that the world is facing today, this is due to increase in population and the advances in technologies. Therefore, ethanol is being blended with petrol or diesel @ 20% for conserve the natural resource and decrease the pollution. 90 per cent of the ethanol is being currently produced from molasses. However due of its higher cost, it is necessary to search an alternate source that is cost effective. According to the statistical analysis, paddy, wheat and sugarcane are the major crops in India. Hence, the present study was conducted to know the potential of these three crop residues for ethanol production. The crop residues were subjected to microbiological and crude enzyme pretreatment to obtain maximum reducing sugars. Among these pretreatment methods combined inoculation of *Phanerochaete chrysosporium* and *Pleurotus spp* in paddy straw (1.27 mg g^{-1}) and bagasse (1.97 mg g^{-1}) and combined inoculation of *Trichoderma viride* and *Phanerochaete chrysosporium* (1.47 mg g^{-1}) in wheat straw was found to be effective in releasing the reducing sugar compared to enzymatic pretreatment. Hence, microbiological pretreatment along with respective substrates were subjected for further studies on ethanol production.

The substrates were further subjected to fermentation of filtrates and fermentation along with the solid substrates. Among these, filtrate fermentation showed maximum release of ethanol (820.80 mg L^{-1}) and *Zymomonas mobilis* was efficient in releasing ethanol from all the substrates. Among the substrates, bagasse recorded maximum release of ethanol compared to paddy straw and wheat straw. Thus, it can be concluded that fermentation of microbiologically pretreated filtrate of bagasse using *Zymomonas mobilis* is the best method for ethanol production.