

धान के पुआल की इथेनाल उत्पादन क्षमता

**ETHANOL PRODUCTION POTENTIAL OF
RICE STRAW**

Ms. SHEETAL K. RADHAKRISHNAN



**DIVISION OF ENVIRONMENTAL SCIENCES
INDIAN AGRICULTURAL RESEARCH INSTITUTE**

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ETHANOL PRODUCTION POTENTIAL OF RICE STRAW

By

Ms. SHEETAL K. RADHAKRISHNAN

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Approved by:

Chairman : Dr. Shiv Prasad _____

Co-chairman : Dr. Navindu Gupta _____

Members : Dr. Lata _____



**Division of Environmental Sciences
Indian Agricultural Research Institute
New Delhi -110012**

Dr. Shiv Prasad
Senior Scientist

CERTIFICATE

This is to certify that the thesis entitled “**Ethanol Production Potential Of Rice Straw**” submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of Master of Science in Environmental Sciences, embodies the results of bona fide research work carried out by Ms. Sheetal K. Radhakrishnan, Roll No. 20056 under my guidance and supervision, and that no part of this thesis has been submitted for any other degree or diploma.

I further certify that any help or information received during the work of this thesis has been duly acknowledged.

(Dr. Shiv Prasad)
Chairperson
Advisory committee

Place: New Delhi
Date:

*Dedicated to my parents, brother
and teachers ...*



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Introduction



1. INTRODUCTION

The energy and environmental crisis which the world is experiencing, forced man to find alternative uses for renewable resources and to use clean technologies. Global energy market has relied heavily on fossil fuels like oil, natural gas, and coal, which provide almost 80 per cent of the world's supply of primary energy needs. Being non-renewable, however, they have brought with them globally destabilizing price shocks. The extensive worldwide use of fossil fuels has not only threatened energy security but also caused serious environmental concerns – particularly climate change. The global uproar about rapidly declining reserves of fossil fuels, increasing demand and spiraling fuel prices have prompted both developed and developing countries alike, to examine other non-petroleum based, commercially viable and environmentally-friendly alternatives. One of the key challenges facing the developing world is to meet its growing energy needs and sustain economic growth without contributing to climate change. Cleaner renewable sources of energy are the ultimate solution to the global energy crisis and biofuels are renewable energy sources, which have been receiving growing attention in recent years. Biofuels especially ethanol, is being increasingly looked at as a substitute for fossil fuels, mainly in the transportation sector. Biofuels can positively impact socio-economic development, e.g., by alleviating poverty, creating jobs, reducing reliance on imported oil and by increasing access to modern energy services (United Nations Foundation, 2006).

India is a country with a positive outlook towards renewable energy technologies (RETs). Several biofuel programs have been initiated over the years. The 11th Five-Year Plan (2007-2012) suggested increasing ethanol blending mandates to 10 per cent once E5 blends were put in place across the country. Ethanol supplies are insufficient even for the 5 per cent ethanol being blended under the EBP currently under implementation in 13 states and three Union territories out of the notified 20 states and four UTs (Economic Times Bureau, 2011). But these programs have suffered setbacks due to supply shortage, sharp fluctuation in oil prices, and global concerns for food security. To remedy this, India adopted a National Policy on Biofuels in December

2009. The program proposes a non-mandatory 20 per cent blending target by both biodiesel and ethanol by 2017. India is the fourth largest producer of ethanol in the world and the second largest producer in Asia after China. Currently ethanol is being produced from molasses, which is a by-product of the manufacture of sugar. But the annual supply of molasses is sufficient only for producing approximately 2.7 billion litres of ethanol. Of this, only a minor share is available for fuel use. Also, Government of India does not allow import of ethanol or other biofuels for fuel purposes, in order to boost domestic production. Therefore, ethanol produced from lignocellulosic biomass is an important option (Amit, 2010). Ethanol is also one of the better tools to fight pollution, as it is an oxygenate fuel that helps in complete combustion of fuel and thus reduces harmful emissions.

The use of first generation biofuels produced from food resources such as high-value sugars and starchy substrates being controversial, the use of non-food substances such as lignocelluloses i.e. second generation biofuels is anticipated. Hence, the conversion of cellulosic biomass to bioethanol is considered as one of the best viable alternative fuel to solve the problems associated with first generation fuels. Biomass appears to be an attractive feedstock for three main reasons: (1) it is a renewable resource that could be sustainably developed in the future, (2) it appears to have formidably positive environmental properties resulting in no net releases of carbon dioxide and very low sulfur content, and (3) it appears to have significant economic potential provided that fossil fuel prices increase in the future. The 73.9 Tg dry wasted crop material worldwide could potentially produce 49.1 GL year⁻¹ of bioethanol (Kim & Dale, 2004), about 16 times higher than the current world ethanol production. The potential bioethanol production could replace 353 GL of gasoline i.e. 32 per cent of the global gasoline consumption.

India being an agrarian nation holds great potential for ethanol production from crop residues due to its widespread availability, abundance and relatively low cost of cellulosic materials (Prasad *et al.*, 2007). India produces ~600 million tons of agricultural residues every year. Theoretically these residues can produce 156 billion liters of ethanol, which can take care of 42 per cent of India's oil demand by 2012. Lignocellulosic biomass is, therefore, considered as the only foreseeable, feasible and

sustainable resource for renewable fuel. However, an accurate estimation of biomass availability in India is non-existent and the only statistics that are available are on agricultural production. According to a survey conducted by NIIST on the surplus availability of biomass residues in India, sugarcane tops is the most surplus residue (79.4 MMT), followed by rice straw (8.5 MMT), wheat straw (9.1 MMT) and other cereal straws ($\sim$ 6 MMT) (Sukumaran *et al.*, 2010).

Bioconversion of lignocellulosic biomass to ethanol is difficult due to: (1) the resistance of biomass to breakdown, (2) the variety of sugars which are released when the hemicellulose and cellulose polymers are broken and the need to find or genetically engineer organisms to efficiently ferment these sugars, and (3) costs for collection and storage of low density lignocellulosic feedstock (Balat and Balat, 2009). Generally, lignocellulosic biomass is a heterogeneous complex containing $\sim$ 40-50% cellulose, a glucose polymer; $\sim$ 25-35% hemicellulose, a sugar heteropolymer; $\sim$ 15-20% lignin, a non-fermentable phenyl-propene unit; plus lesser amounts of minerals, oils, soluble sugars, and other components (Holtzapple, 1993). The relative proportions of these three components in lignocellulosic feedstock vary, depending on the species involved. The typical process for lignocellulosic ethanol production consists of biomass pretreatment and detoxification followed by enzymatic hydrolysis (saccharification) using cellulolytic enzymes (cellulases), subsequent fermentation of the sugar rich hydrolysate using yeasts into ethanol (Margeot *et al.*, 2009). Lignin is not fermented to produce liquid biofuels, but instead can be recovered and used as a fuel for heat and electricity at an ethanol production facility (Larson, 2008). A successful pretreatment must improve formation of sugars or the ability to subsequently form sugars by hydrolysis, avoid the degradation or loss of carbohydrate, avoid the formation of by-products inhibitory to the subsequent hydrolysis and fermentation processes, and be cost-effective (Balat and Balat, 2009). A wide range of physical, chemical and biological pretreatment methods and their combinations have been employed to overcome the recalcitrant nature of lignocellulose, increase enzyme efficiency and improve the yields of monomeric sugars.

Rice is an important crop and rice straw is the most abundant lignocellulosic waste on the earth. As per FAO statistics, world annual rice production in 2011 was

about 480.1 million tons (FAO, 2011). Every kilogram of grain harvested is accompanied by production of 1–1.5 kg of the straw (Maiorella, 1985). It gives an estimate of about 650–975 million tons of rice straw produced per year globally and a large part of this is going as cattle feed and rest as wasted or burned. Asia is the primary region with over 90 per cent of global production and hence the largest potential producer of bioethanol from wasted rice grain and rice straw. India acquires significant position in the global scenario because it is the second largest producer of rice, with a production of 103.4 million tons in 2011 (Hindu business line, 2012). In India, 136.5–150 million tons of paddy straw is estimated to be produced annually. Globally rice straw can produce 205 GL of bioethanol, which is the largest amount from single biomass (Kim and Dale, 2004). Combine harvesting technologies, which have become common in rice–wheat system in India, also leave behind large quantities of straw, which is mainly disposed of by open field burning; especially in the North and North-Western parts, mainly Punjab and Haryana.

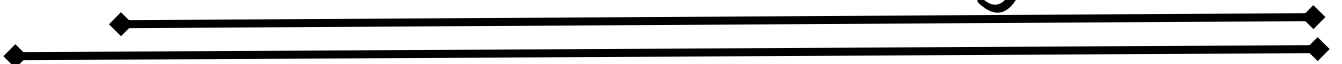
Approximately 85–95 million tons of paddy straw is disposed of by burning, leading to emission of greenhouse gases and particulate matter, loss of plant nutrients, thus adversely affecting pedology, environment and consequently public health (Gupta *et al.*, 2004). It could be a useful and cheap resource for bioethanol production if the technology for cellulose conversion is made available and economical. The ethanol productivity of rice straw varies with the variety. Different cultivars vary in their cellulosic content causing variations in ethanol produced. On an average, 1 kg rice straw has 37% cellulose, 24% hemicellulose and 14% lignin. 1 kg dry straw can theoretically yield 0.42 L ethanol. Projected ethanol production potential from rice straw in India is about 2.6×10^9 litre, therefore it is necessary to screen the suitable variety for ethanol production from rice straw.

The choice of pretreatment method plays an important role in increasing the efficiency of enzymatic saccharification, thereby making the whole process economically viable (Binod *et al.*, 2010). Microwave irradiation has been extensively studied by many researchers because of its high heating efficiency and easy operation. Some studies have shown microwave irradiation to cause changes in the ultra-structure of cellulose, degrade lignin and hemicellulose in rice straw and increase the enzymatic

susceptibility of rice straw. Microwave irradiation could be easily combined with chemical pretreatment which accelerate the productivity of monomers in hydrolysate of rice straw (Zhu *et al*, 2005). Even though globally a lot of research on bioethanol production has been done on rice straw but varietal studies on ethanol production is still to be exploited. Keeping this in view, the present research work has been proposed with the following objectives:

1. To characterize rice straw of different varieties for ethanol production
2. To assess the ethanol production potential of rice straw by efficient saccharification and fermentation process

Background



2. BACKGROUND

At present, the energy crisis and environmental pollution are two major problems the world is facing. Biofuels are going to play an extremely important role in meeting India's energy needs. The country's energy demand is expected to grow at an annual rate of 4.8 per cent over the next couple of decades (Gonsalves, 2006). India's carbon emissions are also growing at an average of 3.2 per cent per annum, making it one of the top 5 contributors to carbon emission. Therefore, developing renewable and clean energy has become a great concern of India and every other country. The transfer of crude oil-based refinery to biomass-based bio-refinery has attracted strong scientific interest which focuses on the development of cellulosic ethanol as an alternative transportation fuel to petroleum fuels (Zheng *et al.*, 2009). The use of bio-ethanol for fuel was widespread in Europe and the United States until the early 1900s. But as it became more expensive to produce than petroleum-based fuel, especially after World War II, bio-ethanol's potential was largely ignored until the oil crisis of the 1970s (Balat and Balat, 2009). This has become even more important today as the most important feedstocks to produce energy in the world i.e. fossil fuels, mainly petroleum, coal, and natural gas, are no longer regarded sustainable, and their availability is much lower. Shafiee and Topal (2009) predicted that oil, coal and gas reserves will last around 35, 107 and 37 years for petroleum, coal and natural gas, respectively.

Due to climate change, dramatic fluctuations of the oil price, decline of oil and increased prices for foodstuffs it becomes more and more important to find alternatives like bioethanol produced from renewable lignocellulosic residues without competition to foodstuff (Kahr and Jager, 2011). Lignocellulosic materials are an attractive feedstock because they are available in large quantities at a relatively low cost (Lynd *et al.*, 2002). Bioethanol from various lignocellulosic feedstocks is now regarded as the most potential biofuel that can alter the petroleum energy dependency in transportation area (Nguyen *et al.*, 2010). The lignocellulosic biomass typically contains 50-80% (dry basis) carbohydrates that are polymers of 5C and 6C sugar units which can be processed either chemically or biologically to yield ethanol (Zhong *et al.*, 2009). Over 150 billion

tonnes of biomass is photosynthesized annually and its major constituents are cellulose, hemicellulose and lignin roughly in the ratio 4:3:3 (Subramanian and Ram, 2011). For efficient hydrolysis of cellulose, lignin component must be separated in order to make cellulose more accessible to the enzymes.

Ethanol is one of the best alternatives to fight pollution, as it is an oxygenate fuel that helps complete combustion of fuel and thus reduces harmful emissions. Bio-ethanol has a higher octane number (108), thus can replace lead, has broader flammability limits, higher flame speeds and higher heats of vaporization. These properties lead to theoretical efficiency advantages over gasoline in an IC engine. An oxygenate fuel such as bio-ethanol provides a reasonable antiknock value and helps in clean-burning gasoline to reduce vehicle exhaust emissions (Balat and Balat, 2009). Ethanol is also more nearly carbon neutral. Besides displacing fossil fuels, the feedstock used to make ethanol requires CO_2 to grow, and they absorb what they need from the atmosphere. Thus, much or all of the CO_2 released when biomass is converted into ethanol and burned in automobile engines is recaptured when new biomass is grown to produce more biofuels.

The ethanol yields and process economics along with the technical maturity and environmental benefits of using ethanol blend fuel are the key parameters that determine the feasibility of bioethanol production (Nguyen and Saddler, 1991). However there are still technical and economical hurdles, which make the bioethanol program unsuccessful at commercial scale. Lignocellulosic biomass as substrate for bioethanol presents a different set of challenges as compared to the molasses or starch based alcohol production (Sukumaran *et al.*, 2010). This chapter reviews the various aspects of ethanol production from lignocellulosic biomass in particular rice straw under various sub-headings as follows.

2.1. Rice straw as a substrate for ethanol

Rice is an important crop in the tropics. Rice straw, one of the cheap lignocellulosic biomasses is produced to the extent of 2 to 3 tonnes per acre. In terms of total production, rice is the third most important grain crop in the world behind wheat and corn. According to the Food and Agriculture Organization (FAO) of the U.N (2009-

10), India acquires an important position in the global scenario because it is the second largest producer of rice accounting for 26%. Globally rice was grown in an area of 161 mha in 2010. Global paddy production is expected to touch 713 million tons in 2011 (476 million tons on milled rice basis).

According to the third advance estimate of the Agriculture Ministry, rice production in India in the 2010-11 season is pegged at 94.11 million tons (FAO, 2011). Rice is the most important food crop of the developing world and the staple food for more than 60 per cent of the Indian populace. Rice is the most important cereal food crop of India, which occupies about 24 per cent of gross cropped area of the country. It contributes 42 per cent of total food grain production and 45 per cent of total cereal production of the country. The average productivity of rice in India, at present, is 2.2 tons/ha, which is far below the global average of 2.7 tons/ha (CRRRI, 2011).

One kg of rice straw will contain roughly 400 g of cellulose which is theoretically enough for producing 220 g or 283 mL ethanol (Subramanian and Ram, 2011). It gives an estimation of about 650–975 million tons of rice straw produced per year globally and a large part of this is going as cattle feed and rest as wasted or burned. In India, 136.5-150 million tons of paddy straw is estimated to be produced annually. Globally rice straw represents the largest potential of ethanol from single biomass (Kim and Dale, 2004).

The ways of disposition of rice straw are limited by the great bulk of material, slow degradation in the soil, harboring of rice stem diseases, and high mineral content. In India, 23 per cent of rice straw residue produced is surplus and is either left in the field as uncollected or to a large extent open-field burnt, Punjab and Haryana alone constituting 48 per cent of this. About 48 per cent of rice straw produced is subjected to open-field burning in Thailand, and in the Philippines it is 95 per cent (Gadde *et al.*, 2009).

In India, large quantities of paddy straw is disposed of by burning mainly in north-western India causing air pollution and global warming. It could be a useful and cheap resource for bioethanol production if the technology for cellulose conversion is made available and cost-effective. In Punjab alone, which has about 2,647,000 ha

under paddy cultivation and yields roughly 100 million tonnes of rice straw (10 per cent of rice production of India); about three-fourth of crop residue amounting to 70–80 million tonnes of rice is disposed-off by burning (Punia *et al.*, 2008).

The ethanol productivity of rice straw varies with the variety. Different cultivars vary in their cellulosic content causing variations in ethanol produced. 1 kg dry straw can theoretically yield 0.42 L ethanol. The choice of pretreatment method plays an important role in increasing the efficiency of enzymatic saccharification, thereby making the whole process economically viable (Binod *et al.*, 2010). Yeast and bacterial strains i.e. *Pichia stipitis*, *Saccharomyces cerevisiae* can be effectively used for the fermentation of the pretreated straw for ethanol production.

2.2. Components of lignocellulosic biomass

The plant cell wall is built of lignocelluloses. The major components of the complex are cellulose (poly- β -1,4 glucopyranoside), hemicellulose (C5 sugars with degree of polymerization 200 or less) and lignin (polyphenols). Cellulose is the fibre material, therefore giving strength and flexibility to the cell wall. Cellulose fibers are assembled into bundles and covered by lignin, which has an impregnating, and protecting role. This coat makes the fibre more resistant to biological and chemical attack. The matrix is then fixed by different types of hemicelluloses, such as xyloglucan, rhamnogalactan or glucurono-arabinoxylan, or pectins like homogalacturonan; forming a chemical bond between cellulose and lignin.

2.2.1. Cellulose

Cellulose, the most abundant polymer on earth, is a homopolymer of β -linked D-glucopyranose units linked by β -1, 4 glycosidic bonds, usually present in a highly ordered crystalline structure. The basic repeating unit is the disaccharide cellobiose. Cellulose is a high molecular weight polysaccharide; the degree of polymerisation (DP) is usually between 500 and 15,000. The secondary and tertiary conformation of cellulose, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, makes cellulose a hydrolysis resistant molecule.

Intra molecular hydrogen bonds are present between the hydroxyl group of the C3 and the oxygen atom of the C5 atom in the adjacent glucose units. Cellulose fibres

form bundles, which are stabilized by intermolecular hydrogen bonds between the cellulose chains. Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils. These microfibrils build up macrofibrils and these are in turn assembled into cellulose fibres (Lynd *et al.*, 2002). There are crystalline and amorphous regions in the structure of cellulose. Amorphous regions are less organized than the crystalline, thus these regions are less resistant to enzymatic attack. Cellulose is hydrophilic, but not water-soluble. Cellulose is hydrolysed by acids, but not with alkalis, in contrast to hemicellulose, which can be hydrolyzed and solubilised both with acids and with concentrated bases. It can also be hydrolyzed enzymatically.

2.2.2. Hemicellulose

Hemicellulose is a highly branched heteropolymer containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose), pentoses (D-xylose, L-arabinose), and uronic acids (D-glucuronic acid). Hemicelluloses can also be partially acetylated. It is more easily hydrolyzed than cellulose (Brigham *et al.*, 1996). The composition of hemicellulose depends on the source of the raw material (Wiseloge *et al.*, 1996).

The linkage of the monomers can be different glycosidic bonds. The pentoses are dominant in hemicelluloses among which xylose is the most important sugar followed by arabinose and hexoses (Yoswanthana *et al.*, 2010). The classification of hemicelluloses can be upon their structure. The classification is based on the composition of the main chain: (a) xylans, (b) mannans, (c) β -glucans, (d) xyloglucans and (e) arabinogalactans (Brigham *et al.*, 1996). The main chain is built up from β -1,4-xylopyranose subunit, which is usually substituted with D-arabinose, L- galactose or methyl-glucuronic acid side-chains. Mannans have basically two main groups: glucomannans and galactomannans. The main chain of the glucomannans are built up from glucose and mannose subunits linked by β -1,4-glycosidic bonds, whereas galactomannans have β -1,4-homomannan main chain substituted with D-galactose by α -1,6 bonds. Mannans are mostly presented in softwoods. β -glucans are randomly linked by β -1,3 and β -1,4 linkages and have role during the growth of the cell. The xyloglucans are the hemicelluloses of the primary cell wall, therefore are present in all

plants. The degree of substitution depends on the xyloglucan source (Hayashi, 1989). Arabinogalactans have β -1,4 or α -1,4 galactose main chains substituted with L-arabinofuranose or short oligomers built up from arabinose and galactose monomers. The presence of hemicellulose reduces the mean pore size of the substrate and therefore reduces the accessibility of cellulose to hydrolytic enzymes.

2.2.3 Lignin

Lignin, the most abundant aromatic polymer in nature, is a macromolecule of phenolic character, being the dehydration product of three monomeric alcohols (lignols), *trans-p*-coumaryl alcohol, *trans-p*-coniferyl alcohol, and *trans-p*-sinapyl alcohol, derived from *p*-cinnamic acid (Kirk *et al.*, 1977). Among the three major biopolymers that constitute wood, lignin is distinctly different from the other two macro-molecular polymers. It is formed by 4-hydroxy-phenylpropanoids (monolignols) that differ in their degree of methoxylation (Nagy, 2009). The three most common building blocks are the 4-hydroxyphenylpropane, the guaiacylpropane, and the syringylpropane subunits. The precursors of these units during the biosynthesis of the lignin are *p*-coumaryl alcohol, *trans*-coniferyl alcohol and *trans*-sinapyl alcohol, respectively 4-hydroxyphenylpropane unit is not methoxylated, whereas guaiacyl and syringyl units have one or two methoxyl groups adjacent to the phenolic hydroxyl group, respectively.

Softwood lignin contains mainly guaiacyl, while hardwood lignin contains both guaiacylpropane and syringylpropane units. Grass lignin contains all three possible building monomers. The building blocks of lignin are connected with different linkages ether and carbon-carbon, and dibenzodioxocin. The dominant linkages in softwood lignins are β -o-4; β -5; biphenyl or dibenzodioxocin linkages. It has a very complex three-dimensional cross-linked structure and high molecular weight. Lignin is insoluble in acids, while it can be solubilised using concentrated alkali bases or organic solvents.

Lignin acts as a physical barrier, preventing the digestible parts of the substrate from being hydrolysed, and binds non-productively to the cellulolytic enzymes (Esteghlalian *et al.*, 2001). Cellulose, hemicellulose and lignin give 75-95% of the total dry mass of lignocelluloses, of which around 70% is the polysaccharide fraction. The

ratio between these three components is different in various species. Trace amount of minerals (ash) are also present in lignocelluloses.

2.2.4. Composition of Rice straw

Rice straw is composed of cellulose (30-56%), hemicellulose (10–27%), lignin (3–30%), protein (3.6-7.2%) and ash (16-17% which contains 83 % silica) (Subramanian and Ram, 2011; Sherief *et al.*, 2010). The content of lignin in rice straw is less than in other common feedstocks, such as corn stover and wheat straw (Hsu *et al.*, 2010). The low feedstock quality of rice straw is primarily determined by a high ash content (10– 17%) and also high silica content in ash (SiO₂ is 75% in rice). On the other hand, rice straw as feedstock has the advantage of having a relatively low total alkali content (Na₂O and K₂O typically comprise <15% of total ash) (Baxter *et al.*, 1996).

2.3. Technological challenges in lignocellulosic ethanol production

Lignocellulosic biomass from agricultural wastes, such as rice straw, wheat straw, and corn stover contains two structural polysaccharides, cellulose and xylan, that are the main sources of fermentable sugars i.e. glucose and xylose, respectively (Park *et al.*, 2010). In contrast to sugar-containing crops, the utilization of lignocellulose as a substrate for ethanol production has a barrier in its complex structure, which resists degradation. The difficulties for rice straw to be effectively converted into fermentable sugars are from two facts: the strong crystalline structure of cellulose in rice straw and the presence of the complex structure of lignin and hemicellulose with cellulose, which together limit the accessibility of rice straw to hydrolytic enzymes. Therefore, various pretreatments of rice straw have been developed to disrupt the crystalline structure of cellulose and increase its exposure to hydrolytic enzymes and thus increase its enzymatic hydrolysis (Zhu *et al.*, 2005). The pore size of the substrate in relation to the size of the enzymes is another limiting factor in the enzymatic hydrolysis of lignocellulosic biomass. Cellulases can get trapped in the pores if the internal area is much larger than the external area which is the case for many lignocellulosic materials (Chandra *et al.*, 2007). Thus, it is necessary to remove the lignin and hemicellulose,

decrease the crystallinity of cellulose, and increase the accessible surface area and porosity by pretreatment technology for achieving acceptable enzymatic digestibility.

High amounts of different types of enzymes are required to achieve high sugar yields from both cellulose and hemicellulose fractions, and the enzymatic saccharification of cellulose incurs high cost. The vulnerability of cellulases to inhibition by end products and inhibitors produced during pretreatment processes also decreases the efficiency (Panagiotou and Olsson, 2006). Thus, development of cellulases and other accessory enzymes needed for complete degradation of lignocellulose components is an important issue. There is also need to standardize the pretreatment technologies for effective and economic utilisation of lignocellulosic biomass, minimizing formation of inhibitory by-products and maximising fermentable sugar recovery for ethanol production.

2.4. Pretreatment technologies for lignocellulosic biomass

Pretreatment refers to a process that converts lignocellulosic biomass from its native form, in which it is recalcitrant to cellulase enzyme systems, into a form for which cellulose hydrolysis is much more effective. Cellulose and hemicellulose are densely packed by layers of lignin, which protect them against enzymatic hydrolysis. For most types of biomass, the enzymatic digestibility of cellulose without some type of pretreatment is very low (<20%). So, it is necessary to have a pretreatment step to break the lignin seal to expose cellulose and hemicellulose for faster and more efficient enzymatic action. According to Taherzadeh and Karimi (2008), an effective and economical pretreatment should meet several requirements like avoiding destruction of hemicelluloses and cellulose, avoiding formation of possible inhibitors for hydrolytic enzymes and fermenting microorganisms and production of less residues, minimizing the energy demand, reducing the cost of size reduction for feedstocks and cost of material for construction of pretreatment reactors, and consumption of little or no chemical and using a cheap chemical. Pretreatment has been viewed as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion (Mosier *et al.*, 2005). In general, pretreatment methods can be classified into three

categories, including physical, chemical, and biological pretreatment, according to the different forces of energy applied in the process.

2.4.1. Physical pretreatment

Mechanical comminution, extrusion, irradiation, freeze pretreatment etc are the methods employed for physical pretreatment of lignocellulosic substrates. The use of physical pretreatment increases the accessible surface area and size of pores, and decreases the crystallinity and degrees of polymerization of cellulose (Binod *et al.*, 2010).

Mechanical comminution

Straw can be comminuted by a combination of grinding and milling, which is the initial step of most pretreatments. The objective is a reduction in particle size and crystallinity of lignocellulose in order to increase the surface area and reduce the degree of polymerization. Also its effect, in combination with other treatments has been tried to reduce cellulose crystallinity. The enzymatic hydrolysis of the superfine ground straw gained the highest hydrolytic rate and yielded a very high reducing sugar (Jin and Chen, 2006). The fermentation of rice straw pretreated with ball milling led to a small improvement in the ethanol yield (Okamoto *et al.*, 2011).

Freeze pretreatment

Recently the freeze pretreatment was tried to enhance enzymatic saccharification of lignocellulosic biomass and it was found highly effective and safe for environment because no chemicals are used in this method. The freeze pretreatment at -20°C in acetate buffer was found to significantly increase the enzyme digestibility of rice straw from 48% to 84 % (Chang *et al.*, 2011).

Hot water pretreatment

Hot-water pretreatment is often called auto-hydrolysis, and the major advantages are less expense, lower corrosion of equipment, less xylose degradation. Water under high pressure can penetrate into the biomass, hydrate cellulose, and remove hemicellulose and part of lignin. Hot water pretreatment has been reported to have the potential to enhance cellulose digestibility, sugar extraction, and pentose recovery, with

the advantage of producing prehydrolyzates containing little or no inhibitor of sugar fermentation. Perez *et al.* (2008) continued to optimize process variables (temperature and residence time) in LHW pretreatment of wheat straw and achieved 80% and 91% xylose recovery and enzymatic hydrolysis, respectively. Hydrothermal treatment using steam or hot-compressed water (HCW) is an effective process for the enzymatic hydrolysis of hardwoods and agricultural residues. A temperature of 180°C for 30 min can be the most efficient process for hot compressed water pretreatment of rice straw (Yu *et al.*, 2009).

Irradiation

The cellulosic component of the lignocellulosic materials is degraded by irradiation to fragile fibers, low molecular weight oligosaccharides and cellobiose (Kumakura and Kaetsu, 1983). Effect of irradiation with gamma rays (Begum *et al.*, 1988), X-rays, electron beams (Jin *et al.*, 2009; Bak *et al.*, 2009b), on rice straw have shown positive effects on reducing sugar production. Microwave irradiation degrades lignin and hemicellulose and also changes the ultra-structure of cellulose. Enzymatic hydrolysis of rice straw could be enhanced by microwave pretreatment in presence of water (Azuma *et al.*, 1984; Ooshima *et al.*, 1984) and also in glycerin medium with lesser amount of water (Kitchaiya *et al.*, 2003). Rice straw treated by microwave irradiation alone had almost the same hydrolysis rate and reducing sugar yield compared to the raw straw (Zhu *et al.*, 2005). Microwave pretreatment removed 7.5% of acid insoluble ash (silica content) in rice straw (Ma *et al.*, 2009). Because these methods do not involve the use of extreme temperatures, the generation of inhibitory substances produced during acid or alkali pretreatment can be either avoided or minimized.

2.4.2. Chemical pretreatment

Chemical pretreatments were originally developed and have been extensively used in the paper industry for delignification of cellulosic materials to produce high quality paper products. And now it is the most studied technique among pretreatment methods for biofuels production. Chemical pretreatments can be simple; such as

soaking the biomass in NaOH at room temperature, or as complicated as treating the material with high-temperature steam (acidic) or ammonia explosion.

2.4.2.1. Alkali pretreatment

Delignification is the major effect of alkali treatment. The alkali pretreatment results in a sharp increase in saccharification. The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross-linking xylan, hemicelluloses and other components. Sodium, calcium and ammonium hydroxides are suitable for the process. Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Fan *et al.*, 1987). Pretreatment of rice straw with 4% NH₄OH under 15 bar at 120°C and further with 1 % sulfuric acid (1:2 v/v) at 15 bars and 110°C for 2 minutes was reported to be highly effective in the study by Subramanian and Ram (2011). This method has been reported to be more effective on agricultural residues than on wood materials (Kumar and Wyman, 2009). Approximately 44.7–84.9% of lignin was removed and marked decrease was found in hemicellulose levels from 18.9 to 9.7% of dry rice straw by ammonia percolation at temperatures range between 100-190 °C and gave a maximum digestibility of 96.9% at 24 hr (Kim *et al.*, 2011). Alkaline peroxide pretreatment is also useful in removing waxes, silica and the waterproof cutins that coat plant tissue. Treatment of rice straw with mild alkaline peroxide at 40 °C for 24 h gave an enzymatic saccharification ratio of 83.23 % (Li *et al.*, 2011).

2.4.2.2. Acid pretreatment

The main objective is to solubilise the hemicellulose fraction of the biomass and to make the cellulose more accessible to enzymes. Dilute acid pretreatment is probably the most commonly applied method among the chemical pretreatment methods. Acid treatment is highly effective in cleaving the β -glycosidic linkages of hemicelluloses and liberating monomeric five-carbon sugars (Lee *et al.*, 1994). The main disadvantage is the generation of high concentration of by-products including acetic acid, furfural, and hydroxymethylfurfural (HMF), which inhibit enzyme activity and microbial growth

(Hsu *et al.*, 2010). Acid treatment at 121°C, 15 min was reported to be effective pretreatment method for converting lignocellulose in rice straw to sugar (Yoswathana *et al.*, 2010).

Organosolv process

Organosolv pretreatment enhances the enzymatic digestibility mainly by delignification and hemicellulose removal leaving a cellulose-rich residue, which can be hydrolyzed with enzymes at high rates and to almost theoretical glucose yield. Organosolv provides treated cellulose suitable for enzymatic hydrolysis, using an organic or aqueous organic solvent (Curreli *et al.*, 1997). Jahan (2006) reported acetic acid and formic acid pretreatment of rice straw with the variation of reaction variables. Acetic acid dissolved pentosans more slowly than formic acid.

Oxidative delignification

Oxidative pretreatment involves the addition of an oxidizing compound, like hydrogen peroxide or peracetic acid, to the biomass, which is suspended in water. This pretreatment remove the hemicellulose and lignin to increase the accessibility of the cellulose. The main reactions in wet oxidation pretreatment are the formation of acids from hydrolytic processes, as well as oxidative reactions.

Ozonolysis

Pretreatment of lignocellulosic materials can be performed by treatment with ozone, referred to as “ozonolysis” pretreatment. Ozone is a powerful oxidant, is soluble in water and is readily available. Ozonolysis method can effectively degrade lignin and part of hemicellulose. The pretreatment is usually carried out at room temperature, and does not lead to inhibitory compounds (Sun and Cheng, 2002). However, ozonolysis might be expensive since a large amount of ozone is required.

Ionic liquids (ILs) pretreatment

Ionic liquids can dissolve large amounts of cellulose at considerably mild conditions. According to a study by Poornejad *et al.* (2011), 3 hr 85% N-methylmorpholine-N-oxide (NMMO) (an industrial solvent) treated substrate showed significant improvement in hydrolysis. The glucose concentration in hydrolysate after

24 hr increased significantly by pretreatment. The rate of hydrolysis and the glucose concentration increased, showing significant improvement in enzymatic digestibility of rice straw after treatment with NMMO, due to destruction of intermolecular interactions.

2.4.3. Physico-chemical pretreatment

Physico-chemical pretreatment of lignocellulosic biomass are combinations of physical and chemical treatments and may be accomplished by the following methods.

Steam explosion

Steam explosion is the most commonly used method for the pretreatment of lignocellulosic materials (McMillan, 1994). The high temperature causes degradation of hemicellulose and transformation of lignin, thus increasing the potential of cellulose hydrolysis and enzymatic digestion. Jin and Chen (2006) studied the effect of the combination of low severity steam explosion and superfine grinding of rice straw on enzymatic hydrolysis, and found the method to shorten the grinding time, save the energy cost, avoid the inhibitors, and obtain high enzymatic hydrolysis. The results by Ibrahim *et al.* (2011) indicated that higher glucose concentration (58.4 g/l) can be obtained from the alkali-extracted steam-exploded rice straw.

Ammonia fiber explosion (AFEX)

Biomass in this process is treated with liquid anhydrous ammonia at 60-100°C and high pressure for varying periods of time (Dale, 1986; Holtzapple *et al.*, 1991). The pressure is then released causing a rapid expansion of the gas that result in swelling and physical disruption of biomass fibres and partial decrystallization of cellulose. However, the process does not work for raw materials with high lignin content (Wyman *et al.*, 2005). It improves saccharification rates. AFEX was reported as an effective pretreatment process for rice straw as it resulted in only 3% sugar loss during pretreatment (Zhong *et al.*, 2009).

Ultrasound pretreatment

The effect of ultrasound on lignocellulosic biomass has been employed for extracting hemicelluloses, cellulose and lignin (Sun and Tomkinson, 2002). The

positive effect of ultrasonic treatment during acid pretreatment of rice straw on polysaccharide conversion into sugar, yielding up to 44% sugar was shown by Yoswathana *et al.* (2010).

CO₂ explosion

CO₂ explosion is also used for pretreatment of lignocellulosic materials. It was hypothesized that CO₂ would form carbonic acid and increase the hydrolysis rate. In attempts to develop improved lignocellulosic pretreatment techniques, the idea of using supercritical CO₂ explosion, which would have a lower temperature than steam explosion and possibly a reduced expense compared to ammonia explosion, was developed. Carbon dioxide molecules are comparable in size to water and ammonia and should be able to penetrate small pores accessible to water and ammonia molecules. Carbon dioxide was suggested to be helpful in hydrolyzing hemicelluloses as well as cellulose. Moreover, the low temperature prevents any appreciable decomposition of monosaccharides by the acid. The delignification with CO₂ at high pressures can be improved by co-solvents such as ethanol–water or acetic acid–water, and can efficiently increase the lignin removal (Pasquini *et al.*, 2005).

Microwave assisted chemical pretreatment

Microwave is emerging as an important and efficient pretreatment method when applied in combination with other methods. The microwave/chemical pretreatment is reported more effective than the conventional heating/chemical pretreatment by accelerating reactions during the pretreatment process to enhance the enzymatic hydrolysis of rice straw (Zhu *et al.*, 2005). Zhu *et al.* (2006b) examined three microwave/chemical processes for pretreatment of rice straw – microwave/alkali, microwave/acid/alkali and microwave/acid/alkali/H₂O₂ – for enzymatic hydrolysis and xylose recovery. The results showed that the straw pretreated by microwave/acid/alkali/H₂O₂ had the highest hydrolysis rate and glucose content in the hydrolysate. Ma *et al.* (2009) demonstrated the positive role of microwave irradiation on biomass digestion. Rice straw treated by microwave/alkali showed more weight loss, higher cellulose content and lower moisture, lignin and hemicellulose content than that by alkali alone. The hydrolysate from microwave/NaOH pretreated substrate after 72-hr

enzymatic hydrolysis had a higher glucose content and lower xylose and reducing sugar of 34.9 g l⁻¹ (Zhu *et al.*, 2005). The rice straw treated by microwave irradiation after alkali treatment had almost the same hydrolysis rate and yield as its control and when intermittent microwave irradiation was used, initial hydrolysis rate was greatly accelerated but the yield was decreased slightly (Zhu *et al.*, 2006).

2.4.4. Biological Pretreatment

Biological pretreatments are non-energy intensive processes, normally performed using lignocellulolytic species from the ascomycetes (e.g. *Trichoderma reesei*), basidiomycetes including white-rot fungi (e.g. *Phanerochaete chrysosporium*), brown-rot fungi (e.g. *Fomitopsis palustris*) which degrade lignin and hemicellulose but very little part of cellulose, since cellulose is more resistance than the other parts of lignocelluloses to biological attack. The most promising microorganisms for biological pretreatment are white-rot fungi that belong to class Basidiomycetes (Taniguchi *et al.*, 2005). Biological pretreatments are safe, environment friendly and less energy intensive, compared to other pretreatment methods (Talebna *et al.*, 2010), but they are slow and they consume cellulose and hemicellulose in addition to lignin.

Taniguchi *et al.* (2005) evaluated biological pretreatment of rice straw using four white-rot fungi (*P. chrysosporium*, *Trametes versicolor*, *Ceriporiopsis subvermispora*, and *Pleurotus ostreatus*) on the basis of quantitative and structural changes in the components of the pretreated rice straw as well as susceptibility to enzymatic hydrolysis. Pretreatment with *P. ostreatus* resulted in selective degradation of the lignin rather than the holocellulose component, and increased the susceptibility of rice straw to enzymatic hydrolysis. A number of cellulolytic microorganisms are used for biological pretreatment, but fungi and bacteria are the main natural agents for cellulose degradation. The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa. However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular. Cellulolytic fungi belonging to *Aspergillus* spp, *Trichoderma* spp, *Fusarium* spp. are extensively used in biological pretreatment of lignocellulosic biomass.

2.5. Enzymatic hydrolysis or Saccharification

Enzymatic hydrolysis refers to the breakdown of cellulose to monomer sugars by cellulase enzymes. Hydrolysis of both cellulose and hemicellulose can be accomplished during the pretreatment stage with the application of a sufficiently high pretreatment severity. However, the overall sugar recovery and fermentation is compromised when the pretreatment severity is high enough to even degrade hemicellulosic sugars. Therefore, while the hydrolysis of hemicellulose is partially accomplished depending on the type and condition of pretreatment, hydrolysis of cellulose is performed with an additional enzymatic hydrolysis step.

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Beguin and Aubert, 1994). Many fungal species such as *Trichoderma*, *Penicillium*, *Aspergillus* and *T. emersonii* are able to produce large amounts of extracellular cellulases and hemicellulases. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions: pH 4.8 and temperature 45–50°C and does not have corrosion problem (Duff and Murray, 1996). Cellulases are produced as a multicomponent enzyme system comprised usually of three enzymes that act synergistically in the hydrolysis of cellulose:

- Endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-end.
- Exoglucanase or cellobiohydrolase (CBH, 1,4- β -D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends;
- β -glucosidase (EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988).

Endoglucanases or carboxymethylcellulases, initiate attack randomly at multiple internal sites in the low crystalline regions of the cellulose fibre opening-up sites for subsequent attack by the cellobiohydrolases creating free chain ends (Wood,

1991). Cellobiohydrolase, the major component of the fungal cellulase system can hydrolyse highly crystalline cellulose (Bhat and Bhat, 1997). They remove monomers and dimers from the end of the glucose chain. β -glucosidase hydrolyse glucose dimers and in some cases cello-oligosaccharides to glucose. In addition to the three major groups of cellulase enzymes, there are also a number of ancillary enzymes that attack hemicellulose, such as glucuronidase, acetylerase, xylanase, β -xylosidase, galactomannanase and glucomannanase (Duff and Murray, 1996). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts to ethanol. The decrease in hydrolysis rate over time has been attributed to inhibition by the accumulated end products. It is also probable that the solubilization of the hemicellulose fraction in the pretreatment step produces potential inhibitors like furans and organic acids in low concentrations.

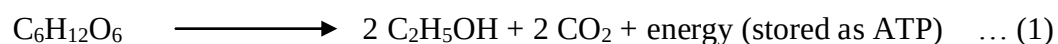
Genetic engineering is used to produce super strains, which are capable of hydrolyzing cellulose and xylan along with fermentation of glucose and xylose to ethanol. The utilization of cellulose by microorganisms involves a substantial set of fundamental phenomena beyond those associated with enzymatic hydrolysis of cellulose (Lynd *et al.*, 2002). The enzymatic hydrolysis of cellulose (saccharification) is affected by several factors, *viz.*, substrate concentration, degree of polymerization, degree of crystallinity, structural composition and availability of surface area, amount of cellulase, reaction conditions such as pH, temperature etc. Therefore, pretreatment is one of the key strategies for enhanced enzymatic saccharification of lignocellulosics. Monomeric sugars released after enzymatic saccharification of the free cellulose, can be converted into bioethanol (Kuila *et al.*, 2011).

2.6. Fermentation

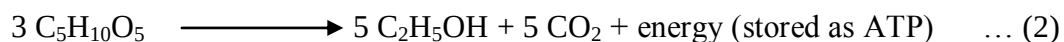
The conversion of lignocellulosic biomass into ethanol is more difficult than for simple sugar and starch because from lignocellulosic materials, first fermentable sugars need to be produced via hydrolysis. The general steps for producing bio-ethanol, include pretreatment to break open the bonds to release the fermentable sugars from polysaccharides through hydrolysis, fermentation of released sugars and finally distillation step to separate bio-ethanol. Lignocelluloses on hydrolysis, give pentose and

hexose sugars that are fermented to ethanol with the help of specific microorganisms. The pentoses are dominant in hemicellulose, in which xylose is the most important sugar accounting for 14.8–20.2% of the straw. Consequently, fermentation of xylose is considered an important feature of microorganisms to use in industrial scale fermentation of rice straw hydrolyzates (Karimi *et al.*, 2006b). The biochemical reactions involved in fermentation of pentose and hexose sugars to ethanol is given in Eqs. (1) and (2).

- 2 moles of ethanol and CO₂ for every mole of hexose consumed as per the equation:



- 5 moles of ethanol and CO₂ each for every 3 moles of pentose consumed as per the equation:



Fungi, bacteria, and yeast can be used for fermentation. *Saccharomyces cerevisiae* also known as Bakers' yeast, is most frequently used to ferment glucose to ethanol. Theoretically, 100 g of glucose will produce 51.2 gram of ethanol and 48.8 g of carbon dioxide. However, in practice, the microorganisms use some of the glucose for growth and the actual yield is less than 100% (Badger, 2002). But it is capable of converting only hexoses into ethanol.

Among the wild-type xylose-fermenting yeast strains for ethanol production, *Pichia stipitis* reportedly provides one of the best overall performances in terms of complete sugar utilization, minimal by-product formation, low sensitivity to temperature, and substrate concentration. *Pichia stipitis* is a promising strain able to utilize both C₅ and C₆ sugars. An adapted strain of *P. stipitis* with enhanced inhibitor tolerance was able to give a good ethanol yield of 0.44 g g⁻¹, which is equivalent to 87% of the maximum possible ethanol conversion, when used with NaOH-neutralized rice straw hydrolysate under weakly-acidic conditions of pH 5.0 (Huang *et al.*, 2009). The ethanol production by *Pichia stipitis* was evaluated in a stirred tank bioreactor using semi-defined medium containing xylose (90.0 g/L) as the main carbon source. Fed batch cultures of *P. stipitis* have been reported to produce up to 47 g/L of ethanol

from xylose at 30°C under low aeration conditions (Jeffries *et al.*, 2007). Ethanol yield efficiencies can be as high as 84.7 to 90.7% per unit substrate consumption (Suh *et al.*, 2003).

2.6.1. Fermentation condition

The success of fermentation depends upon the existence of defined environmental conditions for biomass and product formation. The environmental parameters like pH, temperature, oxygen availability, among others, and the culture medium composition are important factors affecting the bioconversion processes. Among these factors, aeration is considered to be the most important factor affecting xylose fermentation by yeasts, since it determines the partitioning of the substrate carbon flow between growth and product formation (du-Preez, 1994). The optimum pH range for growth of *Pichia stipitis* and fermentation on xylose was found to be 4-7 at 25°C (Slininger *et al.*, 1990).

2.6.2. Types of fermentation

Ethanol can be produced by four main types of industrial operations: batch, continuous, fed-batch and semi-continuous. In the continuous process, feed, which contains substrate, culture medium and other required nutrients, is pumped continuously into an agitated vessel where the microorganisms are active. The product, which is also taken out continuously from the bioreactor, contains ethanol, cells, and residual sugar.

The fed-batch operation may be regarded as a combination of the batch and continuous operations. In this operation, the feed solution, which contains substrate, yeast culture and the required minerals and vitamins, are fed at constant intervals while effluent is removed discontinuously. The main advantage of the fed-batch system is that intermittent feeding of the substrate prevents inhibition and catabolite repression. In semi-continuous processes, a portion of the culture is withdrawn at intervals and fresh medium is added to the system.

Batch fermentation process

During batch fermentation, the rate of ethanol production per milligram of cell protein is maximal for a brief period early in this process and declines progressively as

ethanol accumulates in the surrounding broth. In this partially closed system, feed is loaded into sterile fermentor and then removed at the end. Several potential causes for the decline in fermentative activity have been investigated. Viability remained at or above 90%, internal pH remained near neutrality, and the specific activities of the glycolytic and alcohologenic enzymes (measured in vitro) remained high throughout batch fermentation. None of these factors appears to be causally related to the fall in fermentative activity during batch fermentation (Dombek and Ingram, 1987).

Materials and Methods



3. MATERIALS AND METHODS

The study was undertaken on ethanol production from straw of different rice varieties by microwave assisted acid and alkali pretreatment. This chapter includes the details of the materials and various methods used in determination of physico-chemical characteristics, pretreatment of rice straw and ethanol production from saccharified hydrolysate. The samples were analyzed as per standard analytical methodology during the course of entire study. The study was undertaken in the laboratories of Division of Environmental Sciences, Indian Agricultural Research Institute, New Delhi. The materials and methods used are presented under the following heads.

3.1. Compositional Analysis of rice straw

3.1.1. Feedstock

The rice straw of different varieties PRH-10, Pusa Basmati 1121, Pusa 44, Taraori basmati and IR-36 (Plate 1) were collected from rice crop grown at the fields of Indian Agricultural Research Institute, New Delhi by random selection in triplicate. The rice straw (the residue remaining in the field after harvest) collected was air dried, cleaned, dried, cut into smaller pieces and then powdered using a laboratory grinder, so that it passes through 20-40 mesh sieve. The ground straw was stored at room temperature till further use. This was used directly in chemical pretreatment studies. The compositional analysis of straw was carried out following standard protocols.

3.1.2. Estimation of total solids

Total solids in the samples were determined by evaporating a known amount of slurry to dryness on a steam bath followed by drying at 105⁰C in an oven for 8 hours, to constant weight (APHA, 1995; Sluiter *et al.*, 2008). The total solids in samples weight was calculated in percent as per following formulae.

$$\% \text{ Total solids} = \frac{B-C}{B-A} \times 100$$

Where, A is weight of empty crucible, B is weight of crucible + wet slurry/ biomass and C is weight of crucible + oven dried material



Plate 1. Rice varieties collected from IARI field

3.1.3. Estimation of total nitrogen

Total nitrogen in the sample was estimated by Kjeldahl method and expressed as percentage on dry weight basis (Piper, 1950). The method involved the digestion of the sample so as to convert organic N to NH_4^+ -N using KJELPLUS Supra LX and then determined NH_4^+ -N in the digested material by a titrimetric procedure. The amount of N in samples was calculated in per cent as per following formulae.

$$\% \text{ Nitrogen} = \frac{V \times 0.0014}{W} \times 100$$

Where, W is weight of sample in gm; V is volume of 0.1 N H_2SO_4 used for neutralization of NH_3 in boric acid in mL.

3.1.4. Estimation of phosphorus and potassium

The estimation of phosphorus involved the preparation of extract in which phosphorus was estimated colorimetrically. A known weight (0.5g) of oven dried sample was taken in a 100 mL conical flask and digested with tri-acid mixture (HNO_3 - H_2SO_4 - HClO_4 in 10:1:4 ratio) on an electric hot plate as per procedure described by Jackson (1973). Digested material was cooled, diluted with water, filtered through Whatman No.1 paper to get clear aliquot. Aliquot thus, obtained was diluted to a known volume and then phosphorus content was estimated. Phosphorus concentration in aliquot was determined by vanadomolybdophosphoric acid colorimetric method described by Jackson (1973) using Spectrophotometer GS 5071. Potassium was estimated directly from the aliquot by Systronics Flame Photometer 128 following the method described by Piper (1950).

3.1.5. Estimation of ash and silica content

The estimation was done by controlled ashing as described by Thimmiah (1999). 5g of powdered oven dry sample was transferred into a previously weighed silica crucible. It was kept in a muffle furnace for ashing. The temperature was increased gradually until a final temperature of about 500-550°C and kept for required time. The crucible was cooled in a dessicator and weighed to get the ash content. The ash was moistened with little water, 40 mL of dilute HCl (1:1) was added, covered and digested on a water bath for 20-30 min. Then 1 mL of conc. HNO_3 was added and the contents

evaporated to dryness. The dehydrated contents were moistened with 10mL dilute HCl, 50 mL water and warmed. This was then filtered through a Whatman no.44 filter paper. The filter paper along with residue was transferred to a pre-weighed crucible and ignited to remove filter paper and any carbon present in the crude ash. The weight of the ash corresponds to the silica content.

3.1.6. Estimation of organic fibers

The organic fibres were analyzed as cellulose, hemicellulose and lignin (Goering and Van Soest, 1975) using 2022 Foss Tecator Fibertec Analyzer (following Foss Tecator application note, AN 380).

3.1.6.1. Neutral Detergent Fiber (NDF)

Oven dried rice straw was ground to pass through 1 mm sieve. 1 g of sample was weighed to an accuracy of ± 2 mg into a previously weighed fiber cap capsule (W_1). Capsule was placed in the Fibertec cold extraction unit containing 120 mL acetone, agitated for 30 seconds and left for 10 minutes and filtered. The process was repeated three times and washed with water. Capsule was placed in 2022 hot plate Foss Tecator hot extraction unit and 100 mL of cold Neutral Detergent Solution (NDS), 0.5 g Na_2SO_3 and 2-4 drops of n-Octanol (to prevent foaming) added and heated to boiling for 60 minutes. Samples were filtered, and, washed two times with distilled water and sucked as dry as possible. Capsule was again placed in the Fibertec cold extraction unit containing 120 mL acetone and washed twice. The solvent was evaporated and the capsules dried at $130\pm 2^\circ\text{C}$ for 2 hr or at $105\pm 2^\circ\text{C}$ for at least 5hr. Cooled to room temperature in a desiccator and weighed (W_2). The sample in the capsule was ashed at $525\pm 25^\circ\text{C}$ for at least 3 hr. Cooled to room temperature in a desiccator and weighed (W_3). The amount of NDF was calculated in per cent as per the following formulae.

$$\text{Neutral Detergent Fiber} = \frac{(W_2 - W_3)}{W_1} \times 100$$

Where, W_1 is weight of sample, W_2 is weight of capsule and sample after oven drying and W_3 is weight of capsule and sample after ashing.

3.1.6.2. Acid Detergent Fiber (ADF)

Oven dried samples were ground to pass through 1mm sieve. 1 g of sample was weighed to an accuracy of ± 2 mg into a previously weighed fiber cap capsule (W_1). Capsule was placed in the Fibertec cold extraction unit containing 120 mL acetone. It was agitated for 30 seconds, left for 10 minutes and filtered. The process was repeated three times. It was followed by washing with distilled water. Capsule was again placed in Fibertec hot extraction unit and 350 mL of cold Acid detergent solution (ADS) and 2-4 drops of n-Octanol was added and heated to boiling for 60 minutes. Filtered and washed two times with distilled water and sucked as dry as possible. Then the capsule was again placed in the Fibertec cold extraction unit containing 120 mL acetone. Filtered and repeated. The solvent was evaporated and the capsules dried at $130 \pm 2^\circ\text{C}$ for 2 hr or at $105 \pm 2^\circ\text{C}$ for at least 5hr. Cooled to room temperature in desiccator and weighed (W_2). The amount of ADF was calculated in per cent as per following formulae.

$$\text{Acid Detergent Fiber} = \frac{(W_2 - W_3)}{W_1} \times 100$$

Where, W_1 is weight of sample, W_2 is weight of capsule and sample after oven drying and W_3 is weight of capsule.

3.1.6.3. Acid Detergent Lignin (ADL)

Oven dried sample was ground to pass through 1mm sieve. 1 g of sample was weighed to an accuracy of ± 2 mg into a previously weighed capsule (W_1). Capsule was placed in the fibertec cold extraction unit containing 120 mL acetone, left for 10 minutes and filtered. The process was repeated three times and washed with distilled water. Capsule was again placed in Fibertec hot extraction unit and 350 mL of Acid Detergent Solution (ADS) and 2-4 drops of n-Octanol was added and heated to boiling for 60 minutes. Then it was filtered. It was washed two times with distilled water and sucked as dry as possible. Capsule was placed in the Fibertec cold extraction unit again and filled with 25 mL acetone, filtered and the process repeated. The capsules were placed in the unit containing 120 mL 72 % H_2SO_4 , stirred with glass rod and filtered

off after 3 hr. It was washed with water until the sample was free from acid. Solvent was evaporated and capsules dried at $130 \pm 2^\circ\text{C}$ for 2 hr or at $105 \pm 2^\circ\text{C}$ for at least 5hr. Cooled to room temperature in desiccators and weighed (W_2). The samples were ashed in the crucible at $525 \pm 25^\circ\text{C}$ for at least 3 hr, cooled to room temperature in a desiccator and weighed (W_3).

$$\text{Acid Detergent Lignin} = \frac{(W_2 - W_3)}{W_1} \times 100$$

Where, W_1 is weight of sample; W_2 is weight of capsule and sample after 72% H_2SO_4 treatment and oven drying and W_3 is weight of capsule and sample after 72 % H_2SO_4 treatment and ash drying.

The calculation and estimation of cellulose, hemicelluloses and lignin was done using following formulae:

Hemicelluloses = Neutral Detergent Fiber - Acid Detergent Fiber

Lignin = Acid Detergent Lignin

Cellulose = Acid Detergent Fiber - Acid Detergent Lignin

3.1.7. Estimation of total soluble sugar

Total soluble sugar was estimated by anthrone method (Thimmiah, 1999). Anthrone reagent (100 mL) was prepared by adding 200 mg anthrone into 100 mL of ice cold 95 % (v/v) sulphuric acid. In the anthrone reaction carbohydrates are dehydrated by conc. H_2SO_4 to form furfural. Furfural condenses with anthrone to form a blue-green colored complex which is measured in ECIL Spectrophotometer at 630nm.

Total soluble sugar in rice straw

A 100 mg of the sample was weighed into a boiling tube. It was hydrolyzed by keeping it in a boiling water bath for 3 hr with 5 mL of 2.5 N HCl and cooled to room temperature followed by neutralization with solid sodium carbonate until the effervescence ceases. The volume was made up to 100mL and centrifuged. The supernatant was collected and 1 mL of aliquot was taken. Then 4mL of anthrone reagent was added and heated for 8 minutes in a boiling water bath. Cooled rapidly and the green to dark green color read using ECIL spectrophotometer at 630nm. Calibration curve was prepared with standard solutions of glucose and plotting sugar concentration

on X axis and absorbance on Y axis. The amount of total soluble sugar in the solution was estimated by referring to standard curve of glucose in mg/mL from the following equation.

$$\text{Amount of carbohydrate present} = \frac{\text{Sugar value from graph (mg)} \times \text{Total vol. of extract (mL)} \times 100}{\text{Aliquot sample used (mL)} \times \text{Weight of sample (mg)}}$$

3.1.8. Determination of reducing sugar

Total reducing sugar was estimated by DNS method (Thimmiah, 1999).

Solution A: Sodium hydroxide (10g) was dissolved in 1000 mL distilled water.

Solution B:

Dinitrosalicylic acid	10g
Crystalline phenol	2g
Rochelle's salt	200 mg

All these ingredients were dissolved in 1000 mL of solution A.

Solution C: Sodium sulphite (5g) was dissolved in 100mL distilled water. This solution was prepared fresh every time.

DNS reagent: Working DNS reagent was freshly prepared before use by mixing

Solution B	99 mL
Solution C	1 mL

Estimation of reducing sugars

100 mg of the sample is weighed and the sugars extracted with hot 80% alcohol twice (5mL each time). Supernatant was collected and evaporated on water bath. 10 mL of distilled water was added and the sugars dissolved. The aliquot of alcohol free extract (0.5 mL) was pipetted out into test tubes and the volume made up to 3mL with distilled water, followed by addition of 3mL DNS reagent, mixed and heated for 16 minutes in a boiling bath, and cooled to room temperature. The color developed was measured at 575 nm. The standard curve was prepared by plotting amount of reducing sugar in µg on X axis and absorbance on Y axis.

$$\text{Amount of reducing sugar present} = \frac{\text{Sugar value from graph (}\mu\text{g)} \times \text{Total vol. of extract (mL)}}{\text{Aliquot sample used (mL)} \times \text{Weight of sample (100 mg)} \times 1000}$$

3.2. Pretreatment of rice straw

3.2.1. Autoclave pretreatment

The autoclave assisted acid and alkali pretreatment was carried out as follows: 1g of oven dry, powdered rice straw was taken in glass tubes and 10 mL 1% sulfuric acid (H_2SO_4) (v/v) and 1% sodium hydroxide (NaOH) (w/v) was added in separate tubes keeping a solid loading ratio of 1:10 (solid: liquid). The tubes were placed in an autoclave and treated at 121°C at 15 bar pressure for 15 minutes. The pretreatment experiment was conducted using completely randomized design (CRD) with three replications. Rice straw submerged in distilled water and treated with autoclave was taken as control.

The treated slurry was filtered through a Whatman No. 1 filter paper to separate residues and liquid. The residues were collected, and washed 3-4 times with distilled water, till it was almost neutralized. After washing, the filtered cake were dried at 35°C and stored for enzymatic hydrolysis. Their weight loss was also determined. While the liquid fraction was collected and centrifuged at 0°C at 5000 rpm for 10min and the supernatant were used to determine the fermentable sugar (Keshwani *et al.*, 2007 and Ma *et al.*, 2009). Total soluble sugars or fermentable sugars of the filtrate were determined by the anthrone method described in section 3.1.7.

3.2.2. Microwave assisted acid and alkali pretreatment

A WD700 (MG-5062T) type domestic microwave convection oven produced by LG was used in this study for microwave assisted pretreatment of rice straw. Sample preparation procedure was followed as was done for autoclave pretreatment. The tubes were positioned at the centre of the rotating circular glass plate in the oven for microwave treatment. Different sets of treatments were given at all combinations of 3 sets of temperatures (120°C, 140°C and 160°C) and residence time (5, 10 and 15 minutes). Rice straw submerged in distilled water and treated with microwave was taken as control. The treated material was processed similarly to that done after autoclave treatment.

3.3. Enzymatic saccharification of pretreated rice straw

Enzymatic saccharification of pretreated rice straw of different cultivars was carried out according to protocol described by NREL LAP-009 (Brown and Torget, 1996). The enzymes i.e. cellulase and β -glucosidase used in the study were procured from MERCK, India.

3.3.1. Preparation of Citrate buffer (0.05 M, pH 4.8)

Stock solution A: 0.05 M solution of citric acid (10.51 g in 1000 mL distilled water)

Stock solution B: 0.05 M solution of sodium citrate (14.71 g in 1000 mL distilled water)

To prepare 0.05 M citrate buffer of pH 4.8, 440 mL of stock solution of A was mixed with 560 mL of stock solution B.

3.3.2. Saccharification

Pretreated samples were used in wet form for the enzymatic digestibility test. The samples obtained from the pretreatment, after washing and neutralization, were used in the studies of enzymatic saccharification. Untreated paddy straw was used as reference substrate in the process. The samples were mixed with the required reagents. Sample equal to the equivalent of 0.1g of cellulose was taken in 250 mL conical flasks, and to each flask 5 mL of 0.05 M sodium citrate buffer (pH 4.8), cellulase enzyme to equal approximately 60 FPU/g cellulose, β -glucosidase to equal 64pNPGU/g cellulose, 20 mg/mL sodium azide as antibiotic to prevent growth of organisms during the process and then made up to 10 mL with distilled water.

A substrate blank and enzyme blank was also prepared. The flasks were plugged with cotton and incubation was done at 50°C and 68 rpm in a BOD incubator-cum-shaker for 96 hrs. All the experiments were done in triplicates. Samples were taken every 24 h of the hydrolysis and subjected to total soluble sugar analysis by anthrone method (Thimmiah, 1999). The saccharified slurry was filtered through a Whatman No. 1 filter paper to separate residues and filtrate. The filtrate was stored for further fermentation. The hydrolyzed residue was washed, dried and weighed to get the weight loss.

3.4. Ethanol fermentation of rice straw

3.4.1. Fermentative microorganism and growth media

The yeast strain, *Pichia stipitis* NCIM 3498 procured from NCIM (National Collection of Industrial Microorganisms), National Chemical Laboratory, Pune was used for ethanol fermentation. The strain was maintained on malt extract glucose yeast extract peptone (MYGP) agar slants with the composition

Malt extract	3 g/L
Glucose	10 g/L
Yeast extract	5 g/L
Peptone	5 g/L
Agar	24 g/L

pH was maintained at 6.4-6.8 with H₂SO₄. The media was sterilized at 121°C temperature and 15 lbs sq⁻¹ inch pressure for 20 minutes. Slants were incubated at 30°C for 2 days.

3.4.2. Inoculum preparation

A loopful culture from 24 hr old yeast culture maintained on slants was inoculated in 100 mL MGYB broth (malt extract, 3 g/L; glucose, 10 g/L; yeast extract, 5 g/L and peptone 5 g/L) contained in 250 mL flask and incubated at 30°C on gyratory shaker for 12 hr. This culture was used to inoculate 50 mL medium in a 250 mL flask which was incubated in the same conditions till O.D. reached about 0.6. The inoculum rate used was 10% (v/v).

3.4.3. Fermentation study

Fermentation was carried out in sterile 250 mL Erlenmeyer flasks containing 125 mL of neutralized, autoclaved growth media, containing equal proportions of the pretreated filtrate and the hydrolysed filtrate of rice straw. 24 hr old culture of *Pichia stipitis* NCIM 3498 was inoculated into the media @ 10% (v/v). The flasks were incubated at 100 rpm at a temperature of 35°C and at a controlled pH of 4.8. Samples were collected at an interval of 6 hr. The samples collected were analyzed for residual sugar and ethanol. The fermentation parameters i.e. pH, temperature and inoculum rate

for the fermentation studies was followed as per described by Prasad *et al.* (2009) which were found optimum for those studies in the previous studies.

3.4.4. Batch fermentation

The strain of *P. stipitis* was separately used in batch fermentation study. A 24 hr old slant washed with MGYB broth was used to inoculate 100 mL of broth in a 1 litre flask and was incubated on a gyratory shaker at 30°C. 30 mL of the 24 hr old culture was used to prepare 300 mL of inoculum. Fermentation was carried out in a sterilized 7 litre capacity Appilkon fermentor. 300 mL of 24 hr old inoculum was used to inoculate 3 liter hydrolysate from rice straw. The temperature of bioreactor was maintained at 30°C and the culture was allowed to grow for 72 hr. The residual sugars, ethanol content and pH was measured in the samples taken at intervals of 24 hrs.

3.4.5. Estimation of Ethanol

The fermented liquor was analyzed for sugars (residual sugar). Ethanol concentration was analyzed by gas chromatography (Shimadzu GC-14B, Japan, Solid phase: polyethylene glycol PEG-20M, carrier gas: nitrogen, oven temperature 180°C, injection temperature 200°C, flame ionization detector temperature 230°C; and isopropanol as an internal standard). Theoretical ethanol yield is calculated as:

$$\text{Theoretical ethanol yield} = \text{Total sugar used} * 92/180$$

where 92 is the mass of 2 moles of ethanol formed from 1 mole of hexose and 180 is the molar mass of hexose.

Percentage of conversion efficiency or yield efficiency (Ey) was calculated as:

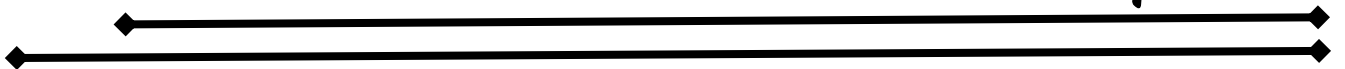
$$E_y = Y_{ps} * 100 / 0.51$$

where Y_{ps} is ethanol yield expressed as g ethanol per g sugar utilized (g g^{-1}) and 0.51 is the maximum theoretical ethanol yield of glucose consumption (Prasad *et al.*, 2009).

3.5. Statistical analysis

The results of the three replicates were pooled and expressed as mean ($n = 3$), and $\pm$ standard deviations. Statistical analysis of the data was carried using Statistical Analysis System (SAS) for windows version 6.11.

Research Paper I



Characterization and pretreatment of rice straw of different varieties for ethanol production

Abstract

In the last decade, most research has tended to focus on developing an economical and eco-friendly ethanol production process. Much emphasis is being given to the production of ethanol from agricultural and forestry residues and other forms of lignocellulosic biomass. With over 90% of the worldwide production, most rice straw is produced in Asia. Therefore, rice straw is considered to be an attractive alternative for fuel ethanol production in Asia. An objective of the study was screening selected popular rice varieties grown in northern India which has not been comparatively studied before with respect to ethanol production potential. Characterization of the rice varieties on the basis of fibre, sugar content and other chemical constituents, showed rice straw to be a potential feedstock for ethanol production. Pretreatment is considered the most important step in determining the cost and success of the process of enzymatic saccharification of lignocellulosic biomass. The effectiveness of pretreatment using autoclave and microwave assisted dilute acid and alkali to produce maximum fermentable sugars in terms of total soluble sugar from the straw was investigated. The results showed that microwave assisted alkali pretreatment is a promising approach to enhance fermentable sugar yields. Fermentable sugars were found to increase with the increase in temperature and irradiation time in case of microwave treatment. Sugar recovery was found maximum by microwave assisted alkali (NaOH) treatment, followed by acid (H_2SO_4) and minimum in hot water. In the treatments with microwave, Taraori Basmati was found to give highest yield, followed by Pusa Basmati 1121 with respect to the release of soluble sugars.

Keywords: rice varieties, fibres, sugars, pretreatment, microwave, autoclave

4.1. Introduction

Research in utilization of lignocellulosic plant materials for bioethanol production has grown significantly over the last few decades as the depletion of non-renewable fuels and increasing greenhouse gas emissions continue to create an increasing need for an alternative non-fossil transportation fuel (Ibrahim *et al.*, 2011). Rice straw is one of the abundant lignocellulosic waste materials in the world. Approximately 731 Mt of rice straw worldwide can potentially be converted to 205 Gt of bioethanol annually (Hideno *et al.*, 2011). It is also one of the most abundant agricultural by-products in India. Rice straw has high cellulose and hemicelluloses content that can be readily hydrolyzed, making it a potential feedstock for fuel ethanol production (Chen *et al.*, 2011). But the composition of cellulose, hemicellulose as well as those of inhibitory compounds such as lignin and silica varies with the variety of rice. Hence selection of high fermentable sugar yielding varieties from among the multitude of rice varieties grown throughout India becomes of significant importance. Also, for cellulosic ethanol more challenging technological interventions are required than for first generation bioethanol from starch and sugary substrates. The process of cellulosic ethanol from biomass involves firstly two cost intensive steps namely pretreatment of biomass and saccharification of carbohydrates for subsequent fermentation with microorganisms to produce ethanol.

The strong crystalline structure of cellulose and the presence of the complex structure of lignin and hemicellulose with cellulose in rice straw make the pre-treatment of rice straw especially important before its saccharification. It greatly affects the saccharification efficiency and cost of ethanol production from rice straw (Zhu *et al.*, 2005b). Many physical, chemical, biological processes and their combinations have been worked out but a universal pretreatment process is difficult to envision owing to the diverse nature of different biomass feedstocks (Alvira *et al.*, 2010). The primary goal of pretreatment is to break down the lignin carbohydrate complexes and make carbohydrates more vulnerable to enzyme attack (Wan and Li, 2010). Among many pretreatment methods of rice straw, the alkali pretreatment is one of the most widely employed (Chadha *et al.*, 1995). Dilute-acid hydrolysis has been successfully developed

for pretreatment of lignocellulosic materials. Depending on the substrate and the conditions used, up to 95% of the hemicellulosic sugars can be recovered by dilute-acid hydrolysis from the lignocellulosic feedstock (Jefferies and Jin, 2000). Studies have shown microwave irradiation could change the ultra structure of cellulose, degrade lignin and hemicellulose in rice straw and increase the enzymatic susceptibility of rice straw. Microwave irradiation could also be easily combined with chemical reactions and accelerate the chemical reaction rate (Zhu *et al.*, 2005).

The present study was done with the aim of screening a few selected popular rice varieties grown in northern India which has not been studied before with respect to ethanol production potential. The effectiveness of pretreatment using microwaves combined with dilute acid and alkali to produce fermentable sugars in terms of total soluble sugars from the straw was also investigated.

4.2. Materials and Methods

4.2.1. Feedstock

The feedstock used was rice straw of varieties PRH-10, Pusa Basmati 1121, Pusa 44, Taraori basmati and IR-36 collected from the fields of Indian Agricultural Research Institute, New Delhi by random selection in triplicate. The rice straw (the residue remaining in the field after harvest) collected was air dried, cleaned, cut into smaller pieces and then powdered using a laboratory grinder, so that it passes through 20-40 mesh sieve. The ground straw was stored at room temperature till further use. This was used directly in chemical pretreatment studies. The composition analysis of straw was carried out following standard protocols.

4.2.2. Characterization of feedstock/rice varieties

Total solids were estimated by method described by Sluiter *et al.*, 2008. The fibres i.e. cellulose, hemicellulose and lignin in rice straw was analyzed using 2022 Foss Tecator Fibretec Analyzer (following Foss Tecator application note, AN 380, Goering and Van Soest, 1975) (Plate 2). The estimation of silica was done by controlled ashing as described by Thimmiah (1999). Total nitrogen, phosphorus and potassium was estimated by Kjeldahl method described by Piper (1950); colorimetrically as



Plate 2. Fibre analysis using Foss Tecator Fibretec Analyzer

described by Jackson (1973) and flame photometrically as described by Piper (1950) respectively. Total soluble sugar was estimated by anthrone method and reducing sugar by DNS method given by Thimmiah (1999).

4.2.3. Microwave assisted acid and alkali pretreatment of rice straw

The microwave assisted acid and alkali pretreatment was carried out with 1g of oven dry, powdered rice straw. The sample was taken in 50 mL glass tubes and immersed in 1% sulfuric acid, 1% sodium hydroxide and hot water, maintaining a solid loading ratio of 1:10 (solid: liquid). The tubes were positioned at the centre of the rotating circular glass plate in the oven for microwave treatment (Plate 3). Different sets of treatments were given at all combinations of temperatures (120°C, 140°C and 160°C) and residence time (5, 10 and 15 minutes). Rice straw submerged in distilled water (hot water) and treated with microwave was taken as control. All the treated sample slurry was filtered (Plate 4). The filtrate was collected, neutralized and stored at -20°C. The pretreated straw residues were thoroughly washed with distilled water, dried at 35°C and stored at -20°C for enzymatic hydrolysis.

4.2.4. Autoclave pretreatment

Autoclave assisted acid and alkali pretreatment was also carried out. The feedstock was prepared as done for microwave pretreatment in section 4.2.3. The tubes were placed in an autoclave and treated at 121°C at 15 psi pressure for 15 minutes. The pretreatment experiment was conducted using completely randomized design (CRD) with three replications. Rice straw submerged in distilled water and treated with autoclave was taken as control. The residue was also treated as done in above section.

4.3. Results & Discussion

4.3.1. Characterization of rice varieties

General information of rice varieties i.e. maturity, yield, nature is given in Table 1. The rice straw was characterized prior to pretreatment and saccharification. The results are presented in Table 2. Total solids were found to be maximum in IR 36, and the values were observed to range from 88.97 to 94.47%. Nitrogen content ranged from 0.45 to 0.55% and maximum was found in PRH 10 followed by Pusa Basmati 1121. Phosphorus content ranged from 0.11 to 0.17% with Taraori basmati having highest

Table 1. Characteristics of rice varieties

Varieties	Pusa 44	IR 36	Pusa Basmati 1121	PRH-10	Taraori Basmati
Characters	Non-basmati, Semi-dwarf	Non-basmati, Semi-dwarf	Basmati , Semi-dwarf	Basmati, Hybrid Semi-dwarf	Basmati, Tall
Maturity	140-145 days	105-115 days	140-145 days	115 days	145 days
Yield	7 t/ha.	4-6 t/ha	4 t/ ha	6.5 t/ha	3.38t/ha

Table 2. Chemical characterization of rice varieties

Characters (% oven dry weight basis)	Varieties				
	Pusa 44	IR 36	Pusa Basmati 1121	PRH 10	Taraori Basmati
Total solids	92.54±1.81	94.47±1.98	90.68±3.25	93.89±1.95	88.97±2.61
Nitrogen	0.45±1	0.46±0.76	0.49±0.25	0.55±0.18	0.46±0.52
Phosphorus	0.12±0.005	0.16±0.004	0.11±0.002	0.14±0.006	0.17±0.012
Potassium	1.16±0.05	1.58±0.1	0.97±0.05	1.15±0.08	1.19±0.17
Silica	9.7±0.14	12.35±1.5	13.57±0.125	10.55±0.35	6.5±0.115
Cellulose	41.76±0.34	35.51±0.35	40.7± 0.7	39.7 ±0.36	36.21±1.35
Hemicellulose	15.25±0.15	22.74±0.8	15.8±0.4	20.15±0.25	22.95±1.19
Lignin	5.54±0.64	6.15±0.45	4.95±0.45	4.5±0.17	3.75±0.65
Total soluble sugars	17.35±2.34	16.55±3.12	16.94±2.01	16.6±0.95	23.86±3.07
Reducing sugars	0.16±0.011	0.19±0.002	0.31±0.028	0.22±0.018	0.4±0.003

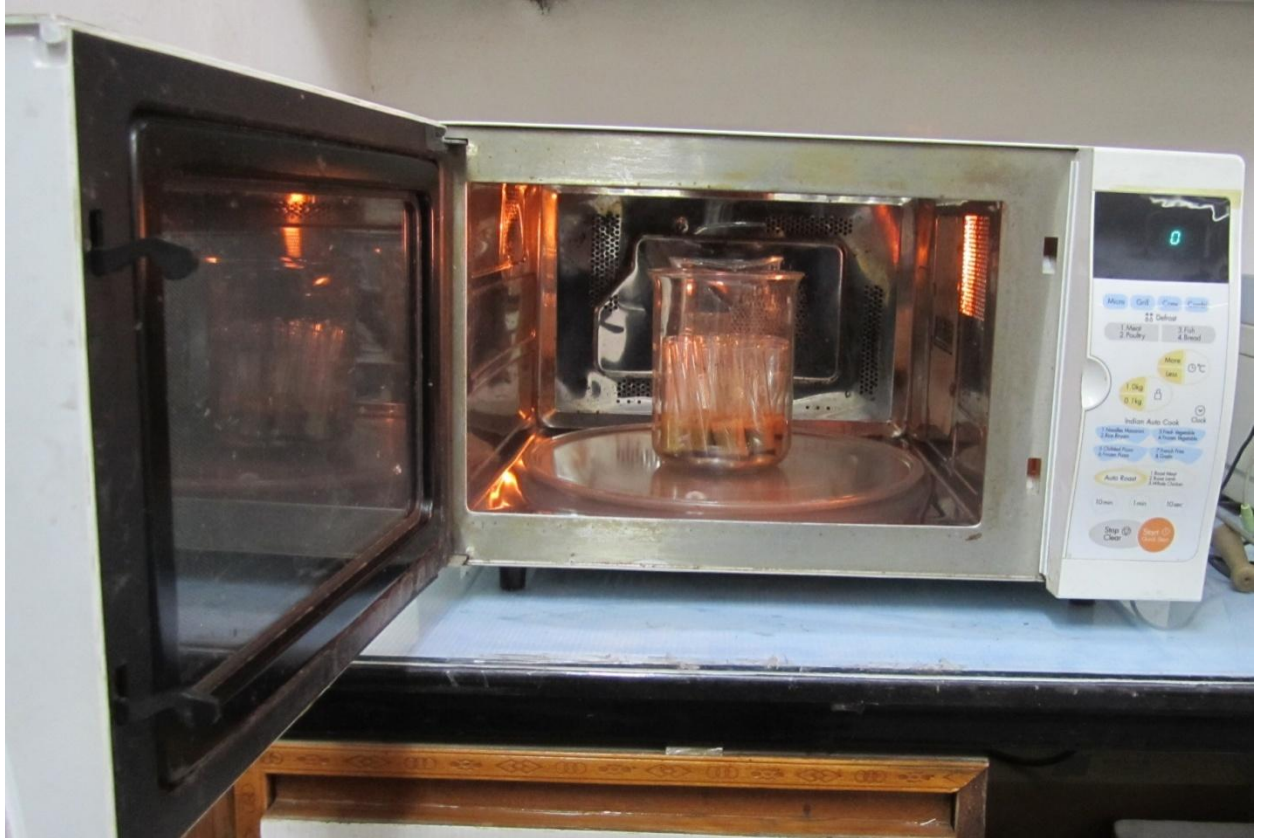


Plate 3. Microwave pretreatment of rice straw



Plate 4. Filtration of pretreated straw

value followed by IR 36. The values for potassium ranged from 0.97 to 1.58% led by IR 36 followed by Taraori basmati. This result is consistent with other several studies by Dobermann and Fairhurst, 2002; Nader and Robinson, 2010. Silica was found in highest concentration in Pusa basmati 1121 followed by IR 36 with values ranging from 6.5 to 13.57%. The value for silica was in agreement with that obtained by Subramanian and Ram (2011) and Jin and Chen (2006).

Fibre content was analyzed as cellulose, hemicellulose and lignin. Cellulose content ranged from 35.51 to 41.76%. Maximum content was found in Pusa 44 followed by Pusa basmati 1121. Taraori basmati had highest hemicellulose content followed by IR 36 with values ranging from 15.25 to 22.95%. IR 36 had maximum lignin content of 6.15% followed by Pusa 44 with 5.54%. Lowest amount of lignin was found in Taraori basmati (3.75%). Similar results on fiber content (cellulose, hemicellulose and lignin) in the rice straw have also been reported by Sharma *et al.*, 2001; Sun and Cheng, 2002 and Singh *et al.*, 2010. The lignin complex and silica incrustation in rice straw is reported to shield the microbial action and hence restrict paddy straw digestibility, making removal or degradation of lignin and silica necessary for economical utilization of the straw (Phutela *et al.*, 2011). Total soluble sugar was found in greatest content in Taraori basmati followed by Pusa 44 and the content ranged from 16.55 to 23.86 %. Reducing sugar content ranged from 0.16 to 0.40. Taraori basmati had the highest content followed by Pusa basmati 1121(0.31%). Poornejad *et al.* (2011) in his work also reported similar value for total sugar.

4.3.2. Pretreatment of rice straw

4.3.2.1. Effect of autoclave pretreatment of rice straw on total sugar recovery

The effect of autoclave pretreatment was studied at 121°C, 15 psi pressure for 15 min and results are shown in Fig 1(Table 1, Annexure). The sugar recovery by treatment with sulphuric acid ranged from 17.91 to 28.68 %, sodium hydroxide 6.42 to 15.82%, and hot water pretreatment 4.97 to 11.31%. In all the treatments of autoclave with acid, alkali as well as hot water, the variety Pusa Basmati 1121 was found to produce higher content of soluble sugars. Yoswathana *et al.* (2010) obtained up to 21.45% sugar after 1% sulfuric acid treatment at 121°C, similar to the present results.

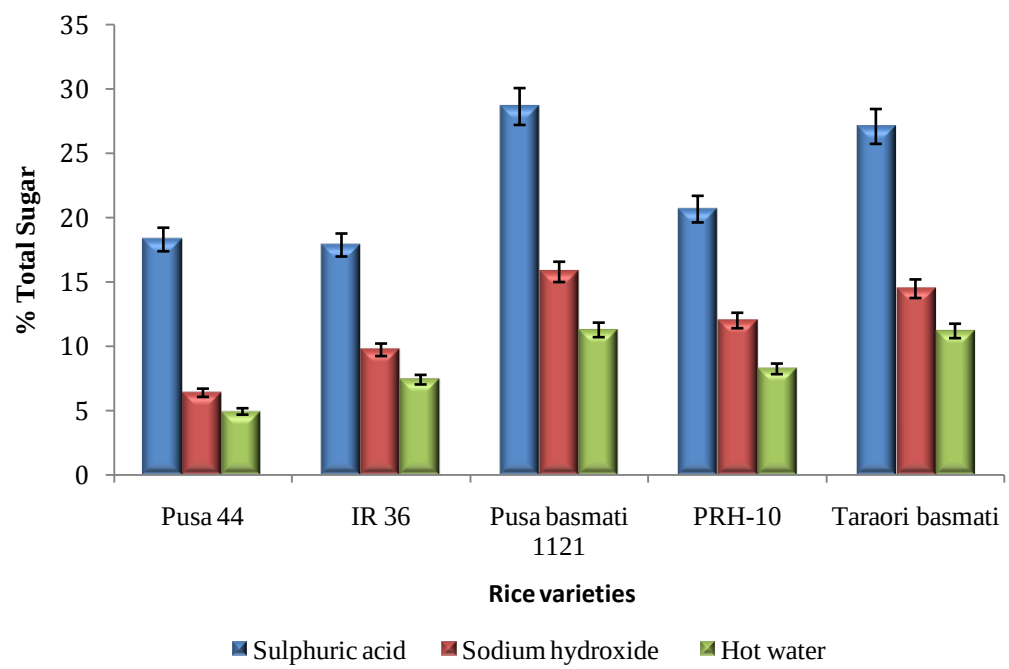


Fig. 1. Total sugars recovery by autoclave pretreatment from rice straw

There was no significant difference between Pusa Basmati 1121 and Taraori basmati, but had significantly higher recovery than other varieties. The autoclave treatment resulted in highest sugar recovery by sulphuric acid followed by sodium hydroxide and minimum with distilled water. Combination of chemical pretreatment and subsequent enzyme treatment increased the sugar yield drastically. Up to 37 and 28% sugar w/w could be achieved for acid and alkali pretreated samples respectively (Yoswathana *et al.*, 2009).

4.3.2.2. Effect of microwave assisted pretreatment of rice straw on total sugar recovery

Microwave irradiations are reported to cause acceleration of ions, collision with other molecules which generates sufficient heat for the solubilization of hindering components such as lignin (soluble only at high temperatures) and disruption of silicified waxy surface and breakdown of lignin-hemicellulose complex which make these irradiations highly suitable for enhancing paddy straw digestibility (Phutela *et al.*, 2011).

Upon treatment with sulphuric acid (Fig. 2) at 120 °C for 5 min the sugar content recovered ranged from 3.96 to 8.37%, at 10 min values lay between 7.04 to 20.79% and at 15 min, the range was from 8.5% to 21.2%. At 140 °C, the sugar recovery ranged from 4.2 to 10.5% for 5 min, 7.9 to 22.3% for 10 min and from 10 to 23.1% for 15 min. And at 160 °C, the sugar recovery was found to vary from 4.87 to 13.13% for 5 min, 8.79 to 23.4% for 10 min and 12.81 to 25.6% for 15 min (Table 2 Annexure).

Treatment with sodium hydroxide (Fig. 3) at 120° C for 5 min the gave a sugar recovery ranging from 4.2 to 10.2%, at 10 min from 10.4 to 22.2% and at 15 min, the range was from 11.97 to 25.6%. At 140 °C, the sugar recovery ranged from 4.4 to 12.8% for 5 min, 11 to 23.4% for 10 min and from 14 to 27.2% for 15 min. And when temperature was increased to 160 °C, the sugar recovery was found to vary from 5.6 to 13.8% for 5 min, 11.9 to 25.7% for 10 min and 15.8 to 30.3% for 15 min of treatment (Table 3 Annexure).

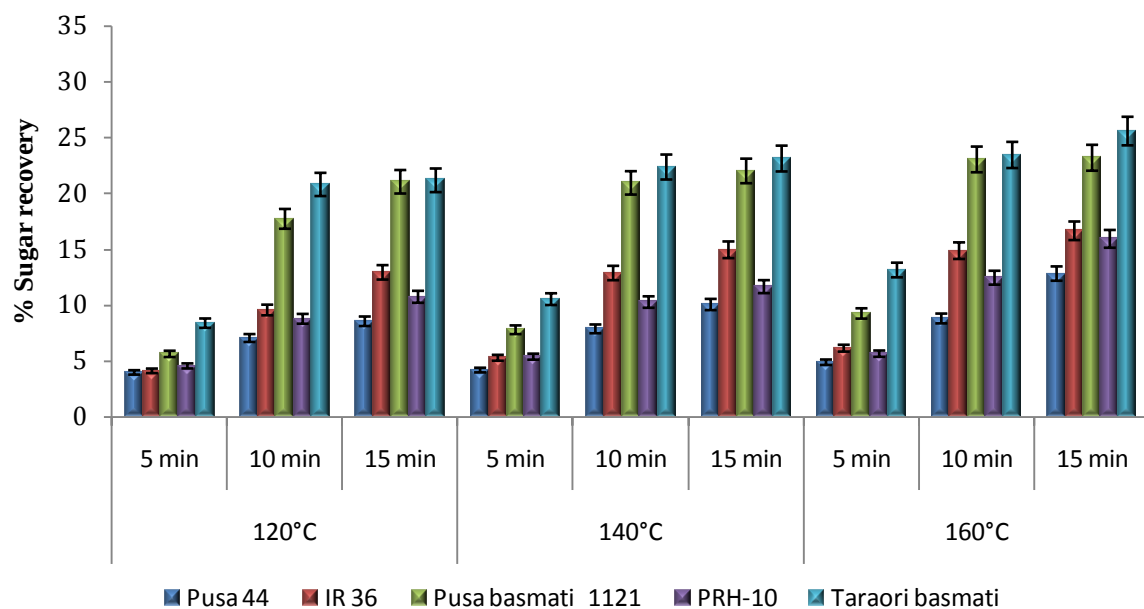


Fig 2. Total sugars recovery by microwave assisted H_2SO_4 pretreatment of rice straw

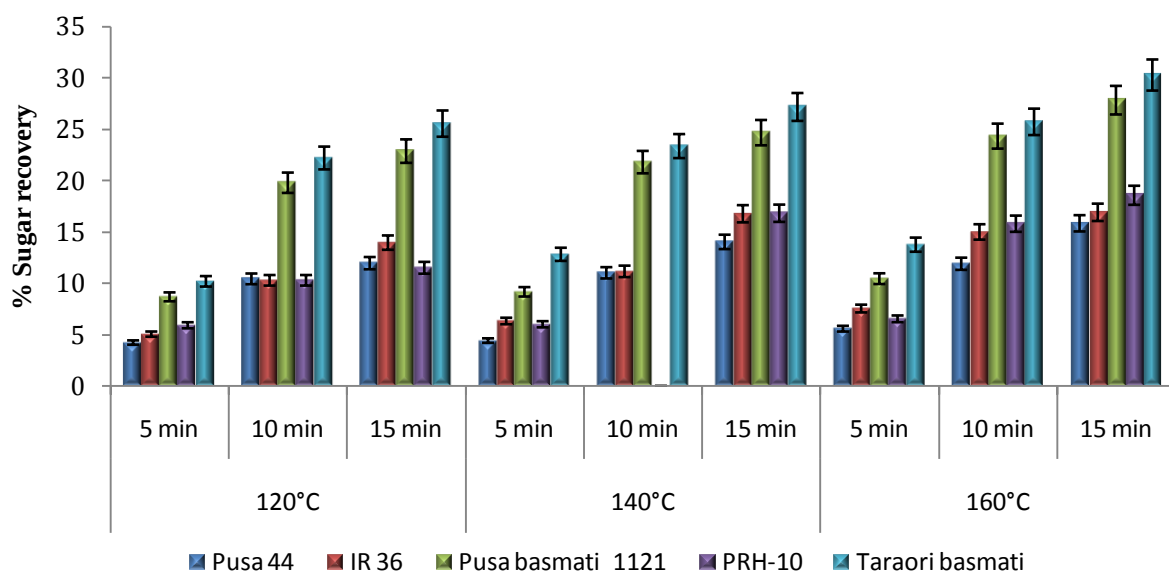


Fig 3. Total sugars recovery by microwave assisted $NaOH$ pretreatment of rice straw

Hot water treatment (Fig. 4) at 120° C resulted in a sugar content ranging from 3.3 to 7.2% for 5 min, values between 6.9 to 19.7% at 10 min and at 15 min it ranged from 8.1 to 21.97%. At 140°C, the sugar recovery ranged from 4.6 to 9.2% for 5 min, 7.8 to 20.6% for 10 min and from 10.8 to 22.4% for 15 min. And at 160 °C, the sugar recovery was found to vary from 4.8 to 12.6% for 5 min, 8.5 to 22.97% for 10 min and 10.97 to 23.3% for 15 min (Table 4 Annexure). Among the rice straw treated with acid for 5, 10 and 15 min at 120° C, sugar recovery was found maximum with Taraori basmati with 8.37, 20.79 and 21.16%, respectively. When it was treated at 140 ° C for 5, 10 and 15 min, sugar recovery was 10.52, 22.35 and 23.11%, respectively. On treatment with 160 ° C for 5, 10 and 15 min, the recovery was 13.13, 23.43 and 25.57%, respectively (Fig. 4).

Rice straw treated with alkali for 5, 10 and 15 min at 120 ° C, gave maximum sugar recovery with Taraori basmati with 10.2, 22.21 and 25.6% yield respectively. When treated at 140 ° C for 5, 10 and 15 min, it showed sugar recovery of 12.83, 23.4 and 27.2%, respectively. On treatment with 160 ° C for 5, 10 and 15 min, recovery was 13.8, 25.7 and 30.3%, respectively (Fig. 3). Alkaline pretreatment is basically a delignification process, in which a significant amount of hemicellulose is solubilized as well. Alkaline pretreatment of lignocellulosic materials causes swelling, leading to decreased degree of polymerization and crystallinity, increased internal surface area, disruption of the lignin structure, and separation of structural linkages between lignin and carbohydrates (Fan et al, 1987). These may be the causes for the higher sugar recovery obtained after microwave assisted alkali treatment over others.

In rice straw treated with hot water for 5, 10 and 15 min at 120 ° C also, sugar recovery was found maximum with Taraori basmati with 7.24, 19.7 and 21.97%, respectively. When it was treated at 140 °C for 5, 10 and 15 min, sugar recovery of 9.2, 20.6 and 22.4%, respectively was obtained. On treatment with 160 ° C for 5, 10 and 15 min, recovery was 12.6, 22.97 and 23.28% respectively.

The soluble sugar in hydrolysate was found to increase with the increase in temperature and irradiation time. Sugar recovery was found maximum by alkali treatment, followed by acid and minimum in hot water. This result is consistent with

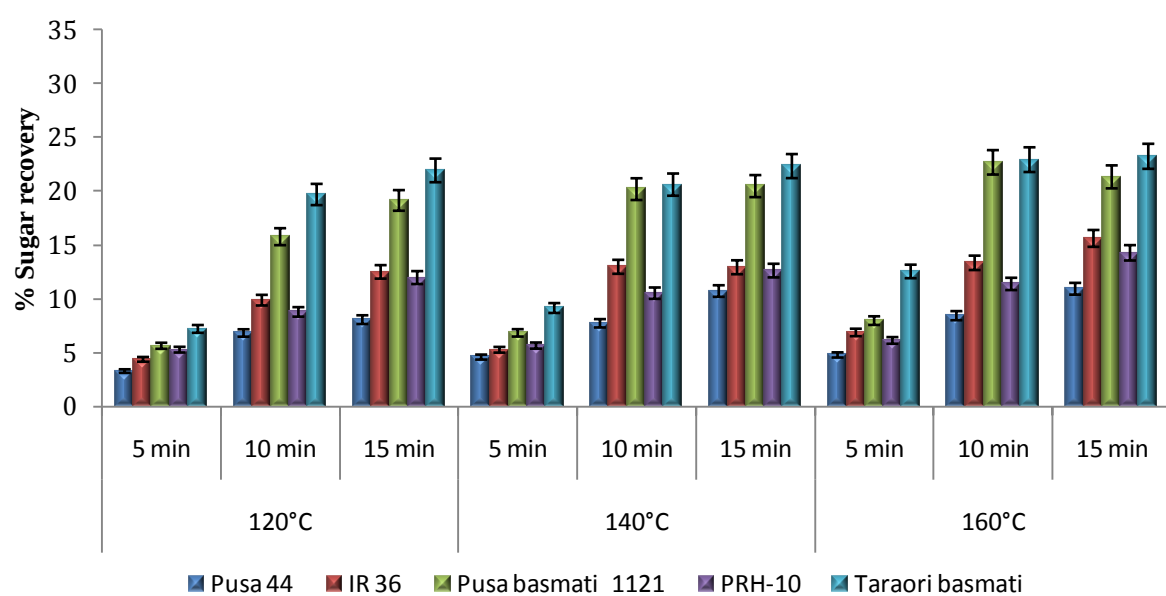


Fig 4. Total sugars recovery by microwave assisted distilled water pretreatment of rice straw

studies done by Gasper *et al.* (2007), who have reported that alkali pretreatment is more effective in breaking bonds between lignin, hemicellulose and cellulose, compared to acid or oxidative agents. Many researchers have also reported that sodium hydroxide treatment produced higher sugars than sulphuric acid in case of biomass (Antonopoulou and Lyberatos, 2011).

In the treatments with microwave, Taraori Basmati was found to be the best variety followed by Pusa Basmati 1121 with respect to the release of soluble sugars. Taraori Basmati had significantly higher value in terms of sugar than other varieties. This may be because of lower content of silica in Taraori basmati, compared to others. Jin and Chen (2006) reported that cellulose and hemicellulose contents affect hydrolysis performance positively while silica and lignin in the rice straw had a negative effect on sugar recovery.

4.3.2.3. Effect of pretreatment of rice straw on weight loss by autoclave pretreatment

Weight loss of the rice straw is an important index for the effectiveness of the pretreatment. The weight loss by autoclave treatment was highest with sulphuric acid followed by sodium hydroxide and minimum when pretreated with distilled water. The maximum weight loss ranged from 29.48 to 39.52 % with autoclave assisted sulphuric acid pretreatment. It was observed to be maximum in Pusa basmati 1121, followed by IR 36 and PRH 10. Weight loss was found minimum with distilled water, the values ranging from 7.35 to 12.85%. Taraori basmati showed least weight loss in all treatments. The effect of pretreatment by autoclave is given in Table 3.

4.3.2.4. Effect of pretreatment of rice straw on weight loss by microwave

With treatment by H_2SO_4 at 120 °C for 5 min, the loss ranged from 6.6 to 14.62%. At 10 min the loss increased with range between 15.5 to 17.42%. and from 15.3 to 19.6% in 15 min treatment. When the treatments were given at 140 °C, the loss showed increase. At 140 °C, for 5 min the loss ranged from 14.6 to 19.3%, in 10 min treatment, between 17 and 23.35% and for 15 min treatment, the values ranged from 18 to 28.5%. At 160 °C, the losses ranged from 14.88 to 21.52% for 5 min, from 18.85 to 21.52% for 10 min and from 19.5% to 26.75% for 15 min (Table 4).

Table 3. Weight loss (%) by autoclave pretreatment

Varieties	Sulphuric acid (H ₂ SO ₄)	Sodium hydroxide (NaOH)	Hot water (DW)
Pusa 44	30.58±3.06	29.47±1.2	11.45±0.9
IR 36	35.48±5.02	26.38±6.41	12.85±2.3
PB 1121	39.52±3.84	25.58±4.71	10.45±0.5
PRH 10	34.72±0.61	29.43±1.2	11.10±3.5
Taraori basmati	29.48±1.7	26.63±1.77	7.35±1.7

Table 4. Weight loss (%) by microwave assisted H₂SO₄ pretreatment

Variety	120°C			140°C			160°C		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Pusa 44	6.6±4.8	13.68±2.55	15.95±2.55	15.3±3.04	17.05±0.7	18±2.7	15.38±0.6	18.85±1.48	24.25±1.15
IR 36	14.62±1.5	15.5±4.2	19.6±3.3	14.6±1.84	23.35±0.4	28.5±1.2	14.88±1.3	22.7±1.01	22.7±0.5
PB 1121	6.7±0.1	13.35±1.75	17.32±0.75	15.73±1.5	21.1±1.55	24.1±0.7	17.85±1.01	19.05±1.18	19.8±0.8
PRH-10	8.65±2.25	13.92±1.69	15.3±1.3	15.65±2.6	17.77±0.51	23±1.3	16.68±1.33	18.85±0.19	19.55±0.93
Taraori	13.35±5.45	17.42±2.15	18.2±3	19.33±2.2	19.9±2.05	22.25±3.25	21.52±0.06	21.95±0.09	26.75±0.6

Treatments with NaOH showed higher weight loss than other treatments. At 120 °C, for 5 min the loss ranged from 13.45 to 18.5%, after 10 min from 15.5 to 20.8% and from 18.45 to 25.9% for 15 min. At 140 °C, range of loss in weight was seen to vary from 20.7 to 25.2% in 5 min treatment, from 21.9 to 26.25% in 10 min and from 23.9 to 34.05% in 15 min. After treatment at 160 °C, the sugar recovery ranged from 18.9 to 25.65% in 5 min, from 22.8 to 31.1% in 10 min and from 26.1 to 34.2% in 15 min (Table 5). Weight loss up to 44.6% has been reported after 30 min of microwave-alkali pretreatment by Verma *et al.*, 2011.

Treatment with hot water, at 120° C for 5 min the gave sugar recovery ranging from 6.1 to 15.9%, at 10 min from 13.5 to 19.4% and at 15 min, the range was from 16.5 to 22.35%. At 140 °C, the sugar recovery ranged from 11.5 to 20.9% for 5 min, 16.45 to 22.15% for 10 min and from 18.5 to 25.3% for 15 min. And when temperature was increased to 160 °C, the sugar recovery was found to vary from 12.5 to 21.6% for 5 min, 15.4 to 22.3% for 10 min and from 18.05 to 27.15% after 15 min of treatment (Table 6).

It is obvious that the rice straw had more final weight loss treated by microwave/alkali and microwave/acid than by microwave/hot water without any chemicals. Highest weight loss was observed with NaOH after treatment at 160 °C for 15 min. The maximum loss was 34.2% by Taraori basmati. It may be that microwave/alkali pretreatment could remove more lignin and hemicellulose from rice straw.

On comparing the total sugar recovery and weight loss, it can be seen that the sugar recovery showed increase with the increase in the weight loss. It might indicate a direct relation between sugar recovery and weight loss. This may be because of degradation of lignin and efficient conversion of sugars from fibres in terms of weight loss.

4.4. Conclusion

Characterization of the rice varieties on the basis of fibre, sugar content and other chemical constituents, showed it as a potential feedstock for ethanol production.

Table 5. Weight loss (%) by microwave assisted NaOH pretreatment

Varieties	120°C			140°C			160°C		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Pusa 44	17.2±4.8	17.28±3.98	18.45±0.15	20.7±0.65	21.9±5.36	23.9±6	18.92±1.51	24.3±0.55	26.1±1.14
IR 36	18.5±0.8	20.82±1.45	25.95±0.85	25±1.59	26.25±3.2	34.05±1.55	23.12±0.64	29.8±0.52	33.8±1.91
PB 1121	18.2±1.7	19.15±0.26	24.45±1.15	21.97±1.62	26.05±3.95	26±2.45	21.48±1.02	22.8±0.74	28±1.42
PRH-10	13.45±0.75	15.55±5.41	22.4±2.1	23.55±0.2	23.8±0.6	37±2.95	20.28±1.01	26.4±1.79	28.1±1.42
Taraori	15.65±0.25	19.48±2.97	24.85±4.35	25.2±2.45	25.97±0.57	28.3±3.9	25.65±0.44	31.1±0.68	34.2±3.22

Table 6. Weight loss (%) by microwave assisted hot water pretreatment

Varieties	120°C			140°C			160°C		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Pusa 44	6.1±3.2	15.45±0.15	16.55±3.05	14.65±0.1	16.55±3.05	18.5±2.2	12.45±0.3	17.7±1.65	18.05±0.5
IR 36	15.8±4	16.9±0.15	19±0.6	18.85±0.45	21.55±1.4	23.1±0.9	15.10±0.35	20.3±0.06	23.1±0.15
PB 1121	12±4.7	13.55±0.6	18.9±4.7	11.5±0.2	18.95±1.9	25.3±2.7	14.90±0.25	15.4±1.75	15.75±0.09
PRH-10	14±0.5	14.25±0.4	18.3±0.5	12.65±1.75	16.45±0.6	22.55±1.75	16.55±1.9	16.85±2.09	19.05±1.7
Taraori	15.9±1.5	19.4±2.35	22.35±1.45	20.9±0.15	22.15±1.15	24.9±4.9	21.65±3.27	22.3±0.05	27.15±2.3

The results show that a combined microwave assisted alkali pretreatment is a promising approach to enhance fermentable sugar yields. Fermentable sugars were found to increase with the increase in temperature and irradiation time in case of microwave treatment. Sugar recovery was found maximum by alkali (NaOH) treatment, followed by acid (H₂SO₄) and minimum in hot water. The highest total sugar yields from rice straw were obtained for microwave assisted alkali pretreatment at 160°C for 15 minutes with 1% alkali solution. The study also concluded that microwave radiation could be utilized for pretreatment process for efficient conversion of rice straw into fermentable sugars at higher temperature in dilute alkali solution. The results showed Taraori basmati and Pusa basmati 1121 had higher productivity in terms of sugar recovery for ethanol production.

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Research Paper II



Ethanol production potential of rice straw by enzymatic saccharification and fermentation

Abstract

Rice straw is composed of heterogeneous complex of carbohydrate polymers, especially cellulose and hemicelluloses, which can be readily hydrolyzed into fermentable sugars by pretreatment and enzymatic saccharification. Ethanol fermenting microorganisms can convert these hydrolyzed sugars into ethanol. In the present investigation, pretreated straw of the rice varieties Pusa 44, PRH 10, IR 36, Pusa basmati 1121 and Taraori basmati were taken for enzymatic saccharification and ethanol fermentation. The samples of microwave assisted chemical pretreated straw of the rice varieties were processed for enzymatic saccharification with cellulase and β -glycosidase enzymes. The total fermentable sugar in terms of total sugar ranged from 9 to 39% after saccharification. The saccharified hydrolysate were fermented into ethanol by using *Pichia stipitis* NCIM 3498. Ethanol yields up to 5.16 per cent (v/v) was obtained by fermentation. The kinetics of ethanol fermentation from Taraori basmati was carried out in Applikon fermentor. Maximum ethanol obtained was 6.63 per cent (v/v), equivalent to 92.9 per cent of the theoretical yield. The ethanol yield coefficient (Y_{ps}) was found to be 0.47 g ethanol g^{-1} sugar utilized. The potential of ethanol production from the rice varieties, according to the results obtained in the study, ranged from 141 to 360 litres per ton of straw. Maximum ethanol production potential was observed to be from the rice varieties Taraori basmati, followed by Pusa basmati 1121. Thus rice straw can be a potential feedstock for fuel ethanol production.

Keywords: Rice straw, sugar, enzymatic saccharification, *Pichia stipitis*, ethanol fermentation

5.1. Introduction

As the demand for non-petroleum based fuels continues to grow, more emphasis is being placed on producing a cost-competitive liquid transportation biofuel such as ethanol. Rice straw is one of the most abundant agricultural residues in India. Heterogeneous complex of carbohydrate polymers, especially cellulose and hemicelluloses from rice straw, can be readily hydrolyzed into fermentable sugars by pretreatment and enzymatic saccharification and subsequently fermented to ethanol. Efficient pretreatment and sequential enzymatic hydrolysis is necessary to maximize sugar productivity and at the same time minimize loss of sugar (Jørgensen *et al.*, 2007). Enzymatic saccharification is accomplished by synergistic action of at least three major classes of enzymes: endo-glucanases, exo-glucanases and β -glucosidases; together called as cellulase. Consequently, the main hydrolysis product of cellulose is glucose, whereas the hemicellulose gives rise to several pentoses and hexoses (Binod *et al.*, 2010).

The utilization of both cellulose and hemicellulose, present in typical lignocellulosic biomass hydrolyzate is essential to obtain a high yield for the economical production of ethanol by fermentation. *S. cerevisiae*, the most commonly used microorganism in fermentation, is capable of converting only hexose sugars to ethanol. The promising yeasts that have the ability to use both pentose and hexose sugars are *Pichia stipitis*, *Candida shehatae* and *Pachysolan tannophilus*. Low efficiency due to the natural recalcitrance of lignocellulose to deconstruction and high cost of enzymatic conversion form the major bottlenecks in this technology. India acquires significant position in the global scenario because it is the second largest producer of rice, with a production of 103.4 million tons in 2011 (Hindu business line, 2012). In India, 136.5-150 million tons of paddy straw is estimated to be produced annually. Hence there is need to develop efficient conversion technology for the production of ethanol from rice straw, in order to economically utilize this huge potential. In this study, the aim was to assess the ethanol production potential of rice straw by efficient saccharification and fermentation process.

5.2. Materials and Methods

5.2.1. Substrate for saccharification

The pretreated rice straw obtained from the previous study was taken as substrate for enzymatic saccharification experiment. The rice straw of the varieties Pusa 44, PRH 10, IR 36, Pusa basmati 1121 and Taraori basmati pretreated with microwave assisted alkali, acid and hot water was washed and neutralized, before the saccharification. The wet material after pretreatment was directly used, as drying is reported to cause irreversible pore collapse in the microstructure of biomass and thus decreased enzymatic release of sugars (Brown and Torget, 1996).

5.2.2. Enzymatic saccharification

Enzymatic saccharification of pretreated rice straw of different cultivars was carried out according to protocol described by NREL LAP-009 (Brown and Torget, 1996). The enzymes i.e. cellulase and β -glucosidase used in the study were procured from MERCK India. The samples obtained from the pretreatment, after washing and neutralization, were used in the studies of enzymatic saccharification. Untreated paddy straw was used as control. Sample equal to the equivalent of 0.1g of cellulose was taken in 250 mL conical flasks, and to each flask 5mL of 0.05 M sodium citrate buffer (pH 4.8), cellulase enzyme to equal approximately 60 FPU/g cellulose, β -glucosidase to equal 64 pNPGU/g cellulose, 20mg/mL sodium azide as antibiotic to prevent growth of organisms during the process and then made up to 10 mL with distilled water.

The flasks were plugged with cotton and incubation was at 50⁰C and 68 rpm in a BOD incubator-cum-shaker for 96 hrs (Plate 5). All the experiments were done in triplicates. Samples were taken every 24 hr of the hydrolysis and subjected to total sugar analysis by anthrone method (Thimmiah, 1999). The saccharified slurry was filtered through a Whatman No. 1 filter paper to separate residues and filtrate. The filtrate was stored at -20°C till fermentation. The hydrolysed residue was washed, dried and weighed to get the weight loss.

5.2.3. Microorganism

Pichia stipitis NCIM 3498 procured from NCIM (National Collection of Industrial Microorganisms), National Chemical Laboratory, Pune was used for ethanol



Plate 5. Saccharification in BOD-cum-shaker

fermentation. The strain was maintained on malt extract glucose yeast extract peptone (MYGP) agar slants (Plate 6) and sub-cultured periodically.

5.2.4. Fermentation

The growth media was inoculated with 24 hr old culture of *Pichia stipitis* NCIM 3498 was inoculated into the media @ 10% (v/v) (Plate 7) and incubated at 100 rpm at a temperature of 30°C and at a controlled pH of 4.8. The samples collected were analyzed for residual sugar and ethanol. The residual sugar and ethanol produced were periodically estimated by anthrone method and by gas chromatography, respectively.

5.2.5. Batch fermentation in bioreactor

Rice straw of the best variety was subjected to kinetic study in fermentor. 150 g of dried, powdered straw suspended in 1.5 L of 1% NaOH (solid loading ratio of 1:10 w/v) was pretreated by microwave for 15 min at 160°C. The residue after filtration and neutralization was saccharified using commercial cellulase and β -glucosidase enzyme. The filtrate from microwave hydrolysis and from saccharification was used as substrate for fermentation, with *P. stipitis* NCIM 3498. Fermentation was carried out in a sterilized 7 litre capacity Aplikon fermentor. The temperature of bioreactor was maintained at 35°C and the culture was allowed to grow for 72 hr. The residual sugars, ethanol content and pH was measured in the sample taken at intervals.

5.2.6. Estimation of ethanol

Ethanol concentration was analyzed by gas chromatography (Shimadzu GC-14B, Japan, Solid phase: polyethylene glycol PEG-20M, carrier gas: nitrogen, oven temperature: 180°C, injection temperature: 200°C, flame ionization detector temperature: 230°C; and isopropanol as an internal standard). Percentage of conversion efficiency or yield efficiency (Ey) was calculated as

$$Ey = Y_{ps} \times 100 / 0.51$$

Where Y_{ps} is ethanol yield expressed as g ethanol per g sugar utilized (g g^{-1}) and 0.51 is the maximum theoretical ethanol yield of glucose consumption (Prasad *et al.*, 2009).



Plate 6a. *Pichia stipitis* 3498 strain



Plate 6b. *Pichia* culture for inoculation



Plate 7. Hydrolysate inoculated with *P. stipitis* culture for fermentation

5.3. Results and Discussion

5.3.1. Saccharification

5.3.1.1. Saccharification of pretreated rice straw for sugar recovery

Pretreated rice straw was subjected to saccharification for the recovery of sugar (fermentable sugar). Total sugar obtained from saccharification of microwave assisted acid (H_2SO_4), alkali (NaOH) and hot water (HW) pretreated straw of rice varieties is given in Fig. 5 (Table 5 Annexure).

Total sugar was found maximum in Taraori basmati and ranged from 14.17 to 39.65%, followed by Pusa basmati 1121 (11.67 to 31.15%), PRH 10 (11.5 to 23.93%) and IR 36 (9.53 to 18.98%). It was observed to be minimum and varied from 9.19 to 17.89% in Pusa 44. Saccharification yields of rice straw pretreated with microwave assisted alkali showed highest sugar recovery and it ranged from 17.89 to 39.65%, followed by acid (10.09 to 19.05%) and hot water (9.19 to 14.17%). The sugar yields from alkali pretreated straw was almost twice the yields obtained from acid or hot water pretreated straw. The results indicate that microwave assisted pretreatment was highly effective in increasing the saccharification yield, compared to the control. Zhu *et al.* (2005) also reported that microwave irradiation could enhance the enzymatic hydrolysis of rice straw by removing more hemicellulose and lignin in its microwave assisted alkali pretreatment and increasing its accessibility to hydrolytic enzymes.

Sun and Cheng (2002) have also reported alkali pretreatments better compared with acid pretreatments and liquid-hot-water pretreatments, as alkalis readily remove lignin and xylan side chains, resulting in a dramatic increase in efficiency of enzymatic saccharification. The current result is also consistent with the results of Zhao *et al.* (2007), who reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose compared with H_2SO_4 pretreatment. However, enzymatic hydrolysis of lignocelluloses with no pretreatment is usually not so effective because of high stability of the materials to enzymatic or bacterial attacks (Taherzadeh and Karimi, 2008). This may be the reason for the low sugar recovery obtained in control.

5.3.1.2. Total sugars from pretreatment and enzymatic saccharification

The result observed for total sugar recovery after both microwave assisted acid, alkali and hot water (HW) pretreatment and saccharification of the different rice

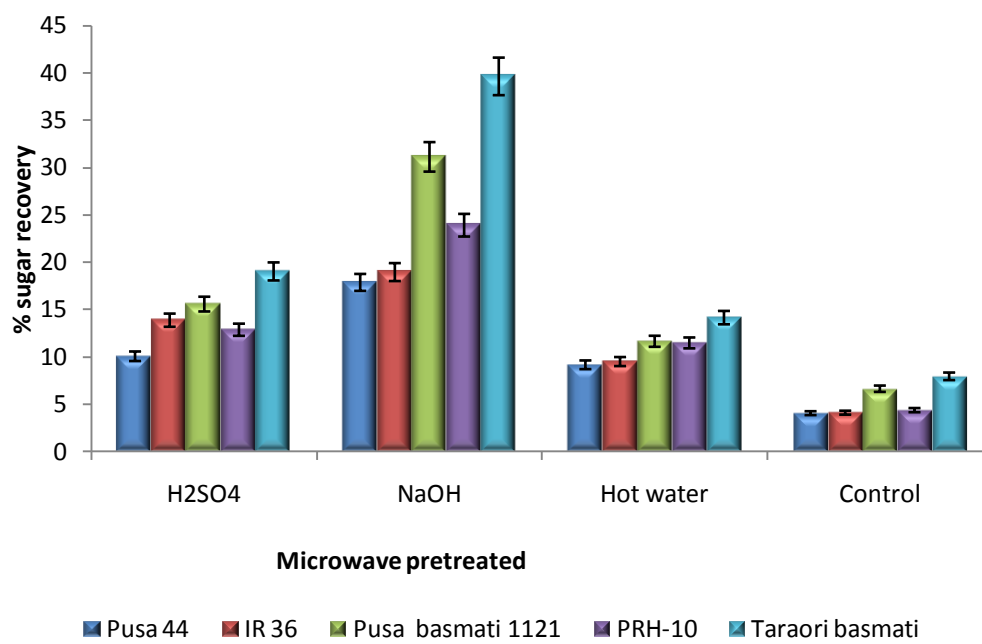


Fig. 5. Soluble sugar recovery by enzymatic saccharification of pretreated rice straw

varieties, is given in Fig. 6 (Table 6 Annexure). The total sugar obtained by microwave assisted acid pretreatment (at 160°C and 15 min) and saccharification of rice varieties, ranged from 22.9 to 44.62%. The sugar recovery varied from 33.74 to 69.94% in alkali pretreated and saccharified straw, while after hot water pretreatment and saccharification the sugar recovery ranged from 20.16 to 37.45%. The total sugar from pretreatment and saccharification was recorded highest in Taraori basmati (44.4%) followed by Pusa basmati 1121 (38.7%), IR 36 (30.54%), PRH 10 (28.8%) and minimum in Pusa 44 (22.9%), when pretreated with microwave assisted sulphuric acid. Treatment with microwave assisted sodium hydroxide gave maximum sugar in Taraori basmati (69.9%) followed by Pusa basmati 1121 (58.9%), PRH 10 (42.51%), IR 36 (35.9%) and minimum in Pusa 44 (33.74%). Similar trend was also observed for sugar recovery from rice variety treated with hot water.

Saccharification following microwave assisted alkali pretreatment showed higher sugar recovery, followed by microwave assisted acid and hot water. Amongst the varieties, Taraori basmati showed the highest sugar recovery, followed by Pusa basmati 1121, in both pretreatment and saccharification. The result is consistent with the findings of many researchers [Singh *et al.* (2010), Zhu *et al.* (2005), Sun and Cheng (2002)], who also reported that microwave assisted alkali treatment is the best pretreatment for enhanced biomass saccharification and fermentable sugar recovery. Microwave pretreatment has also been reported to remove up to 7.5% of acid-insoluble ash (silica content) in rice straw, which improved straw digestibility, as Si deposition in cell walls acts as another physical barrier, protecting the plant from enzymatic hydrolysis (Rezanka and Sigler, 2008).

5.3.1.3. Weight loss of pretreated rice straw after saccharification

The result observed for total weight loss after microwave assisted acid, alkali and hot water (HW) pretreatment and saccharification of the different rice varieties, is given in Table 7. The total weight loss was higher in Taraori basmati (36.15 to 39.07%). It ranged from 22.5 to 54.62% with Pusa basmati 1121 and minimum in Pusa 44 (28.9 to 31.6%). The results show that the pretreated rice straw by acid, alkali and hot water had weight loss varied from 28.9 to 57.9%. Maximum weight loss was observed in alkali pretreated and saccharified rice straw, while it was minimum in control. Similar

Table 7. Weight loss after saccharification

Pretreated with	Weight loss %				
	Pusa 44	IR 36	Pusa Basmati 1121	PRH-10	Taraori basmati
H₂SO₄	31.59	34.7	36.08	35.48	39.07
NaOH	43.48	44.85	54.50	46.11	57.98
Hot water	28.99	30.63	33.05	34.08	36.15
Control	13.31	15.21	19.3	16.4	22.5

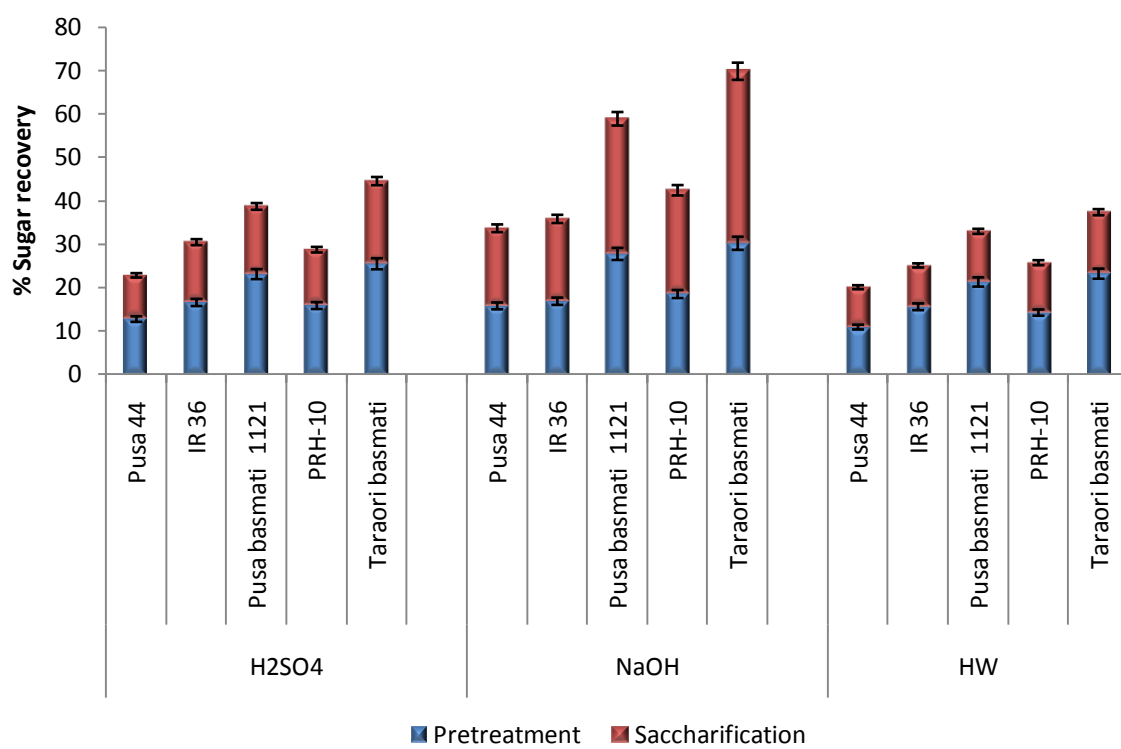


Fig. 6. Total fermentable sugars obtained after pretreatment and saccharification

results for weight loss of 30-50% after autohydrolysis or steam explosion and enzymatic saccharification were also reported by Tsubaki and Azuma (2010).

5.3.2. Ethanol yield by fermentation

The sugar obtained after pretreatment and saccharification (Plate 8) was subjected to ethanol fermentation by *Pichia stipitis* NCIM 3498 in 250 mL flasks. The results for ethanol yield, fermentation efficiency and ethanol yield coefficient obtained after fermentation of the saccharified straw is given in Table 8. The ethanol yield varies from 2.3 to 4.3% (v/v) from rice varieties after pretreatment with microwave assisted acid and saccharification, while it ranged between 2.3 and 5.16% in microwave assisted alkali pretreated and saccharified straw. The yield varied from 2.1 to 4.19% after saccharification of microwave assisted hot water pretreated straw. The ethanol yield was found highest from Taraori basmati (4.19 to 5.16%), followed by Pusa basmati 1121 (3.72 to 4.12%). It was minimum in Pusa 44 (2.1 to 2.33%).

The straw that was pretreated with microwave assisted alkali and further saccharified showed higher ethanol yield, compared to the other treatments given. Zhu *et al.* (2005) also reported that enzymatic hydrolysis of rice straw treated by microwave assisted alkali had a higher rate and produced a hydrolysate with higher glucose content and lower xylose content, which is more suitable for subsequent fermentation. The high value of ethanol yield conversion and fermentation efficiency also proves the efficiency of the yeast *Pichia stipitis* to ferment biomass, which contains pentose sugars also. An adapted strain of *P. stipitis* with enhanced inhibitor tolerance was able to give a good ethanol yield of 0.44 g g^{-1} , which is equivalent to 87% of the maximum possible ethanol conversion, when used with NaOH-neutralized rice straw hydrolysate under weakly-acidic conditions of pH 5.0 (Huang *et al.*, 2009). Fermentation of dilute acid treated rice straw was reported to give ethanol yield of 74% using *R. oryzae*, 68% by *M. indicus* and 61% with *S. cerevisiae* (Karimi *et al.*, 2006).

5.3.3. Batch fermentation in bioreactor

The hydrolysed and saccharified sugar rich hydrolysate from rice straw of Taraori basmati (highest ethanol yield from previous sets of study), was subjected to kinetic study in a 7-litre capacity Aplikon fermentor (Plate 9). The results are given in

Table 8: Ethanol production by fermentation with *P. stipitis* 3498

Varieties	H ₂ SO ₄				NaOH				HW			
	Sugar (%)	Ethanol % (v/v)	EYC (gg ⁻¹)	FE (%)	Sugar (%)	Ethanol % (v/v)	EYC (gg ⁻¹)	FE (%)	Sugar (%)	Ethanol % (v/v)	EYC (gg ⁻¹)	FE (%)
Pusa 44	5.9	2.3	0.39	77.04	5.62	2.33	0.41	81.90	5.04	2.1	0.42	82.35
IR 36	7.55	3.2	0.42	83.76	7.98	3.49	0.44	86.46	6.30	2.86	0.45	89.79
Pusa basmati 1121	9.9	4.1	0.414	81.85	9.83	4.12	0.42	82.87	8.26	3.72	0.45	89.00
PRH-10	7.3	2.8	0.38	75.80	7.40	3.11	0.42	83.00	6.45	2.69	0.42	82.39
Taraori basmati	11.15	4.3	0.386	76.22	11.76	5.16	0.44	86.79	9.36	4.19	0.45	88.47



Plate 8. Hydrolysed and saccharified filtrates with enzymes



Plate 9. Fermentation in fermentor

Table 9. It was found that saccharified sugar rich hydrolysate from rice straw fermented by *Pichia stipitis* NCIM 3498, was able to produced 6.63% (v/v) ethanol, equivalent to 92.9% of the theoretical yield (based on the theoretical yield of 0.51 g ethanol/g glucose utilized) (Fig.7). The ethanol yield coefficient (Y_{ps}) was found to be 0.47 g ethanol g⁻¹sugar utilized. The kinetic parameters were in agreement with those reported by Laopaiboon *et al.* (2009); Prasad *et al.* (2009). In terms of ethanol yield (EY), fermentation efficiency (FE) and residual sugar (RS) or sugar utilization, rice straw has good potential for ethanol production.

5.4. Ethanol production potential of rice varieties

From the results obtained during the experiment, it can be concluded that microwave assisted alkali pretreated rice straw had maximum ethanol yield (%) with higher fermentation efficiency and EYC in all the studied varieties. The result of ethanol production potential of rice varieties is given in Table 10. The current study shows rice straw to have an ethanol production potential ranging from 141 to 360 L per ton of the different varieties of rice straw. Maximum ethanol production potential was found to be from rice variety Taraori basmati, followed by Pusa basmati 1121.

Zhu *et al.* (2005b) reported an ethanol concentration of 25.8gl⁻¹ and ethanol yield of 57.5%, when microwave assisted alkali pretreated rice straw was subjected to SSF using cellulase from *Trichoderma reesei* and *Saccharomyces cerevisiae*. The results obtained by Ibrahim *et al.* (2011) indicated that 28.9–58.4 g/L of glucose can be produced from rice straw depending on the pretreatment method.

5.5. Conclusion

The ethanol production potential of different varieties was studied. Saccharification of the microwave assisted chemical and water treated straw was found to yield high amount of sugars. The highest sugar was recovered from microwave assisted alkali pretreated straw of Taraori basmati. Fermentation of the saccharified straw also gave ethanol yield coefficients ranging from 0.38 to 0.45 g/g of sugar. Thus microwave assisted alkali pretreatment followed by saccharification and fermentation was found to be the best process among the methods compared in this study. Also, *Pichia stipitis* was seen to have high lignocellulose fermenting efficiency. Taraori

Table 9. Kinetics of ethanol production in batch fermentation

Time (Hrs)	Ethanol Yield % (v/v)	Residual sugars (%)	Fermentation Efficiency (%)
0	0.12	14.1	1.7
5	1.16	12.1	16.3
10	2.61	10.68	36.6
15	3.47	7.71	48.6
20	4.20	3.68	58.9
25	5.63	1.33	79.0
30	6.63	1.28	92.9
40	6.62	0.28	92.8
50	6.55	0.28	91.8
60	6.63	0.28	92.9
72	6.63	0.28	92.9

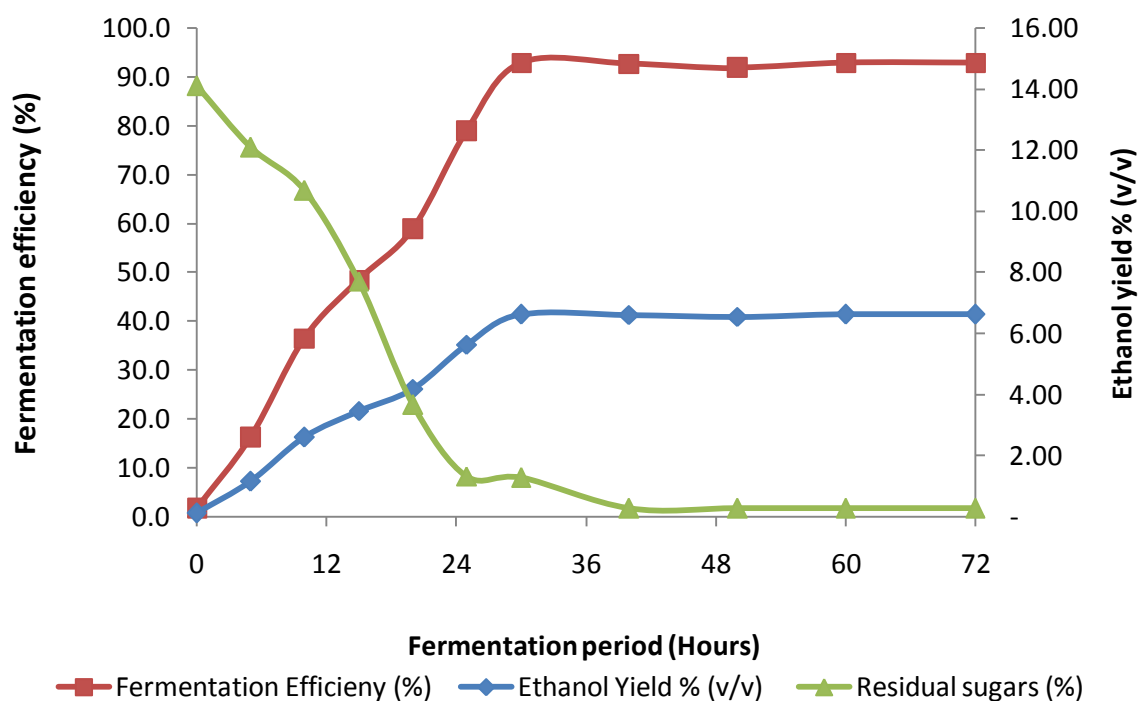


Fig. 7. Kinetics of ethanol production in batch fermentation

Table 10. Ethanol production potential of rice straw pretreated with NaOH

Variety	Yield (t)	Straw: grain ratio	Sugar %	EYC (gg⁻¹)	Ethanol potential per ton
Pusa 44	7	1.02	33.74	0.41	141.1
IR 36	5	1.02	35.9	0.44	161.1
Pusa basmati 1121	4	1.05	58.99	0.42	260.1
PRH-10	6.5	0.95	42.51	0.42	169.6
Taraori basmati	3.4	1.17	69.94	0.44	360.1

basmati showed highest yields for ethanol production also, and hence was selected for further kinetic studies. Its results further confirmed the potential of the rice straw. Maximum ethanol obtained was 6.63% (v/v), equivalent to 92.9% of the theoretical yield. The ethanol yield coefficient (Y_{ps}) was found to be 0.47 g ethanol g^{-1} sugar utilized. Taraori basmati was found the best, in terms of sugar and ethanol yield. The potential ethanol yield from the rice varieties ranged from 141 to 360 L per ton of straw. The ethanol yield coefficient of the rice varieties also indicate the high potential of rice as potential feedstock for fuel ethanol production.

Acknowledgement

The authors are thankful to the Post Graduate School and Director, Indian Agricultural Research Institute, New Delhi, India for providing for fellowship towards M.Sc. programme of the first author. We are also grateful to the Division of Environmental Sciences, IARI, New Delhi for providing necessary facilities for undertaking this study.

General discussion



With increasing gap between the energy requirement of the world and inability to replenish such needs from the limited sources of energy like fossil fuels and ever increasing levels of pollution, there has been an increased interest in the development of a sustainable energy source such as biofuels. Biomass, such as agricultural residues, wood and energy crops, are attractive materials for bio-ethanol fuel production since it is the most abundant reproducible resources on earth. Biofuel produced from lignocellulosic biomass, so-called second generation bioethanol shows energetic, economic and environmental advantages in comparison to bioethanol from starch or sugar (Alvira *et al.*, 2009). Although large-scale ethanol production is not a new concept, converting lignocellulosic biomass such as rice straw to ethanol is not a trivial matter. Due to the structure of the lignocellulosic complex, pretreatment is required for its degradation, the removal of lignin, partial or total hydrolysis of the hemicellulose, and the decrease in the fraction of crystalline cellulose, for increasing the efficiency of subsequent hydrolysis and fermentation. Each pretreatment has its own effect on cellulose, lignin and hemicellulose and the overall goal is to improve digestibility of the lignocellulosic material (Hendriks and Zeeman, 2009).

The present study emphasized the characteristics of different varieties of rice straw and the effect of autoclave and microwave irradiation assisted chemical pretreatment on the effective fermentable sugar recovery from rice straw, improvement in enzymatic hydrolysis and further fermentation using *Pichia stipitis* NCIM 3498 strain for ethanol production from the hydrolysate. The potential of the different varieties to produce ethanol was also approximated based on the results obtained. The characteristics of different varieties of rice straw showed variations in total and reducing sugar, fibres (cellulose, hemicellulose, lignin), silica, etc; thereby indicating variations in their response to various treatments and subsequently the ethanol production potential. Microwave assisted chemical treatment gave higher sugar recovery than autoclave treatment. The higher efficiency of microwave assisted chemical pretreatment may be due to the disruption of the silicified waxy surface, break down of

the lignin–hemicellulose complex and partially removal of silicon and lignin by microwave radiation, as reported by researchers such as Ma *et al.* (2009) and Phutela *et al.* (2011).

Microwave assisted alkali (NaOH) pretreatment gave significantly higher sugar recovery in all varieties, under all combinations of temperature and irradiation time, than either acid (sulphuric acid) or hot water, under similar conditions. This may be due to the delignification and solubilization of hemicellulose in the straw. Combination of microwave and alkali treatment at lower temperature has been viewed as an alternative for the high temperature ($>160^{\circ}\text{C}$) treatment of rice straw, which leads to production of inhibitors and other harmful decomposition products, and also the loss of sugars. The soluble sugar in hydrolysate was found to increase with the increase in temperature and irradiation time. The best yields were obtained on treatment at 160°C for 15 min.

The higher yield of sugars by saccharification of microwave assisted alkali pretreatment straw over microwave assisted acid and hot water treatment may be due to the effect of microwaves and/or alkali on the structure of straw. Microwave irradiation may have increased the enzymatic susceptibility of rice straw by removing more lignin and hemicellulose, disruption of bonds between the lignocellulosic components and expose more surface area of cellulose to cellulase increasing its accessibility to hydrolytic enzymes. These results also indicate the ability of microwave pretreatment to disrupt the lignin structure. Furthermore, lignin removal could also have reduced the unproductive binding of cellulase to lignin (Lu *et al.*, 2002). Microwave treatment has also been observed to remove up to 7.5% silica (Ma *et al.*, 2009), exposing the plant to enzymatic hydrolysis. Pretreatment with NaOH has been reported to give a higher enzymatic conversion ratio of cellulose compared to H_2SO_4 pretreatment as reported by Zhao *et al.* (2007).

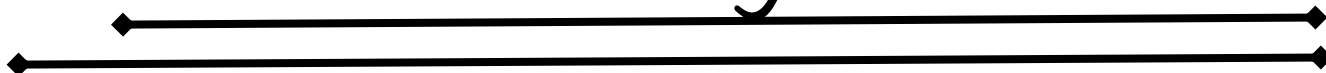
In the treatments with microwave and subsequent saccharification, Taraori Basmati was found to be the best variety, followed by Pusa Basmati 1121 with respect to the release of soluble sugars. The low silica and lignin content in Taraori Basmati may be responsible for the higher sugar yields after treatment, as silica and lignin have been reported to affect the resistance of the lignocellulosic biomass to disruption (Rezanka and Sigler, 2008).

Fermentation of pretreated and saccharified straw using *Pichia stipitis* also yielded high quantities of ethanol. Ethanol conversion of 0.39 to 0.45 gg⁻¹ were obtained, indicating high efficiency. This may be due to the use of *Pichia* sp., which is able to utilise not only the hexoses but also the pentose sugars in the hydrolysate, leading to high conversion efficiency from the sugars recovered by treatment. Microwave irradiation is also reported to enhance the pentosans in NaOH aqueous solution (Zhu *et al.*, 2005), hence justifying the use of pentose fermenting yeast such as *Pichia*. Fermentation of rice straw hydrolysate to ethanol by other pentose fermenting yeasts like *Candida shehatae* and *Zymomonas mobilis* have also reported higher yields of 0.37–0.50 g/g and 0.46 g/g of ethanol per sugars utilized [Abbi *et al.*, (1996); Krishnan *et al.*, (2000)], compared to traditional forms like *S. cerevisiae* which ferment only hexoses. The ethanol yield was highest in the alkali pretreated and saccharified straw hydrolysate and also from the variety Taraori basmati. Further confirmation of the ethanol production kinetics in fermentor under controlled conditions gave an yield of 0.47 g ethanol g⁻¹ sugar utilized with a fermentation efficiency of 92.9%. This indicates the possibility of higher yields under controlled conditions.

The estimation of potential of different varieties to produce ethanol by microwave assisted alkali pretreatment, saccharification and fermentation using *Pichia stipitis* showed good results. The yields ranged from 141 to 360 liters per ton of straw. Thus, rice straw is having very high potential to be used as a biofuel substrate.

Rice straw is one major agricultural residue and one potential lignocellulosic substrate for fuel ethanol production. Until now, the work on ethanol production from rice straw was mainly restricted to the analysis of effect of various methods of pretreatment and saccharification on solubilization of sugars, and its fermentation and no detailed study has been reported on the varietal variation in ethanol production potential. The use of pretreatment methods according to the characters of specific variety will be a more economic and effective approach in the path forward. The development of an appropriate conversion technology will go a long way forward in making this biomass waste into a potential source of economically sound and environment-friendly fuel, in order to meet the increasing demand of our growing economy.

Summary and Conclusion



7. SUMMARY & CONCLUSION

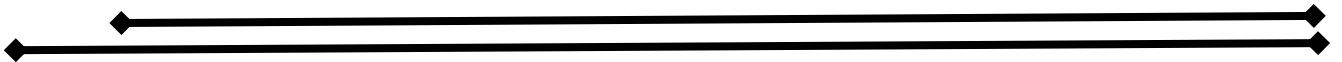
The lignocellulosic biomass represents the largest renewable reservoir of potentially fermentable carbohydrates on earth and hence offers huge possibilities for the production of fuel ethanol at large scale. But conversion of lignocelluloses into biomass presents a different set of challenge compared to cane, beet or corn, due to its highly recalcitrant structure. Consequently, the involved technologies of pretreatment, enzymatic saccharification and fermentation are more complex. Rice straw is an attractive lignocellulosic material for bioethanol production, since it is one of the most abundant renewable resources. The variation in the proportions of fibres, silica etc present between different varieties, necessitates its screening for maximum ethanol production. The present study was taken to screen a suitable variety of paddy straw from among five selected varieties including Pusa 44, PRH 10, IR 36, Pusa basmati 1121 and Taraori basmati; to study the effect of autoclave and microwave assisted chemical pretreatment on increasing the efficiency of saccharification and ethanol production and hence find the potential of rice straw as a source of ethanol. The work can be summarized as follows:

- ✓ Characterization of straw showed highest concentration of silica in Pusa basmati 1121 followed by IR 36. Minimum amount of silica and lignin were in Taraori basmati, which is favorable for ethanol fermentation. Maximum cellulose and hemicellulose content was found in Pusa 44 and Taraori basmati, respectively. Total soluble sugar and reducing sugar was found highest in Taraori basmati.
- ✓ From all the pretreatments, microwave assisted alkali (sodium hydroxide) treatment was found superior to acid (sulfuric acid) at 160°C for 15 min, with respect to total sugars released, giving maximum recovery of 30.3 per cent.
- ✓ Among the rice varieties, Taraori basmati was gave the highest sugar recovery under all treatment conditions with acid, alkali and hot water, followed by Pusa Basmati 1121 with respect to the release of soluble sugars.
- ✓ Total sugar recovery after saccharification of rice straw pretreated with microwave assisted alkali was also found to be maximum, with highest in Taraori basmati (39.65 per cent) followed by Pusa basmati 1121 and PRH 10.
- ✓ Fermentation of pretreated and saccharified straw with *Pichia stipitis* NCIM 3498 gave highest ethanol yield from Taraori basmati (5.16 per cent) followed by Pusa basmati 1121.

- ✓ Batch fermentation in bioreactor of the saccharified hydrolysate of Taraori basmati straw, produced 6.63 per cent (v/v) ethanol, equivalent to 92.9 per cent of the theoretical yield. The ethanol yield coefficient (Y_{ps}) was found to be 0.47 g ethanol g⁻¹ sugar utilized.
- ✓ The current study shows rice straw to have an ethanol production potential ranging from 141 to 360 L per ton of the different varieties of rice straw. Maximum ethanol production potential was found to be from rice variety Taraori basmati, followed by Pusa basmati 1121.

This study indicates that there are varietal variations in the chemical characteristics, which affect sugar recovery and the utilization of rice straw as a substrate for ethanol. Microwave assisted dilute alkali has been observed to be an efficient method of pretreatment of rice straw for maximizing sugar recovery. Production of ethanol from rice straw has been shown to have the potential to meet the needs of fuel as well as to account for the environmental pollution caused by burning the waste straw.

Abstract



Ethanol production potential of rice straw

Abstract

Ethanol is one of the most important renewable fuels that can be utilized for the reduction of negative environmental impacts generated by the worldwide utilization of fossil fuels. The lignocellulosic wastes represent an abundant and potential source of ethanol production. Rice is the third most important grain crop in the world behind wheat and maize in terms of production. According to FAO, India acquires significant position in the global scenario as it is the second largest producer of rice accounting for 26%. Rice straw, one of the cheap lignocellulosic biomass, is composed of heterogeneous complex of carbohydrate polymers, especially cellulose and hemicelluloses, which can be readily hydrolyzed into fermentable sugars by pretreatment and enzymatic saccharification. Ethanol fermenting microorganisms can convert these hydrolyzed sugars into ethanol. In the present study, straw of rice varieties Pusa 44, PRH 10, IR 36, Pusa basmati 1121 and Taraori basmati, collected from the experimental field of IARI, were analyzed and characterized on the basis of chemical constituents. Samples were processed for autoclave and microwave assisted chemical pretreatment, subsequent enzymatic saccharification and ethanol fermentation. The kinetics of ethanol fermentation was carried out in Applikon fermentor.

Characterization of the rice varieties on the basis of fibre, sugar content and other chemical constituents, showed it as a potential feedstock for ethanol production. The efficiency of autoclave and microwave assisted chemical pretreatment was compared. Sugar recovery was found maximum by microwave assisted alkali (NaOH) treatment, followed by acid (H_2SO_4) and minimum in hot water. The results showed microwave assisted alkali pretreatment to be a promising approach to enhance sugar yields. Enzymatic saccharification of pretreated straw gave sugar ranging from 9 to 39%. Taraori Basmati had higher sugar recovery (69.9%), followed by Pusa Basmati 1121 (58.9%), after saccharification and microwave assisted alkali pretreatment. Fermentation of saccharified hydrolysates using *Pichia stipitis* NCIM 3498, gave ethanol yields of 2.1 to 5.16%(v/v), maximum from the rice varieties Taraori basmati, followed by Pusa basmati 1121. The kinetic study of ethanol fermentation from Taraori basmati in fermentor gave 6.63% (v/v) ethanol, equivalent to 92.9% of the theoretical yield. The ethanol yield coefficient was 0.47 g ethanol g^{-1} sugar utilized. The potential of ethanol production from the rice varieties, according to the study, ranged from 141 to 360 L per ton of microwave assisted-NaOH pretreated straw. These findings highlight the enormous potential of rice straw for the production of ethanol.

धान के पुआल की इथेनॉल उत्पादन क्षमता

सारांश

इथेनॉल एक महत्वपूर्ण अक्षय ईंधन संसाधन है जिसे जीवाश्म ईंधन के उपयोग से उत्पन्न नकारात्मक पर्यावरणीय प्रभावों को कम करने के लिए दुनिया भर में उपयोग किया जा सकता है। लिग्नोसेलुलोजिक पदार्थ इथेनॉल उत्पादन के लिए प्रचुर संभावित स्रोत का प्रतिनिधित्व करते हैं। उत्पादन की दृष्टि से अनाज वाली फसलों में धान, गेहूं और मक्का के बाद दुनिया की तीसरी सबसे महत्वपूर्ण फसल है। एफएओ के अनुसार, वैश्विक परिदृश्य में भारत, 26 प्रतिशत धान उत्पादन के साथ विश्व का दूसरा सबसे बड़ा उत्पादक एवं महत्वपूर्ण देश है। धान का पुआल, एक सस्ता लिग्नोसेलुलोजिक बायोमास है जो कार्बोहाइड्रेट पॉलिमर, विशेष रूप से सेलूलोज और हेमीसेलूलोज से बना होता है जिसे आसानी से पुर्व-उपचार और एंजाइमेटिक शर्कराकरण के द्वारा किण्वन योग्य शर्करा में परिवर्तित किया जा सकता है। जलयोजित किण्वन योग्य शर्करा को सूक्ष्मजीव इथेनॉल में किण्वित या परिवर्तित कर सकते हैं। वर्तमान अध्ययन में, आई. ए. आर. आई. प्रयोगात्मक क्षेत्र से एकत्रित की गयी धान की विभिन्न प्रजातियों पूसा 44, पीआरएच 10, आईआर 36, पूसा बासमती 1121 और तरावड़ी बासमती के पुआल को विश्लेषण करते हुए रासायनिक घटकों के आधार पर विशेषीकरण किया गया। संसाधित नमूनों को आटोक्लेव और माइक्रोवेव सहयोजित रासायनिक पुर्व-उपचार और एंजाइमेटिक शर्कराकरण के बाद इथेनॉल किण्वन के लिए उपयोग किया गया। इथेनॉल किण्वन अभिक्रिया अणुगतिकी अप्लीकान फरमेंटर में किया गया।

फाइबर, शर्करा और अन्य रासायनिक घटकों के आधार पर धान की प्रजातियों का लक्षण वर्णन, इन्हें इथेनॉल उत्पादन के लिए एक संभावित एवं संक्षम फीडस्टॉक के रूप में प्रदर्शित करता है। शर्करा उत्पादन के लिए आटोक्लेव एवं माइक्रोवेव सहयोजित रासायनिक पुर्व-उपचार की क्षमता की तुलना की गयी। अधिकतम शर्करा उत्पादन माइक्रोवेव सहयोजित सोडियम हाइड्राक्साइड (अलकली) पुर्व-उपचार में, इसके बाद एसिड पुर्व-उपचार तथा सबसे कम गर्म जल पुर्व-उपचार से प्राप्त की गयी। प्राप्त परिणामों से पता चलता है कि माइक्रोवेव सहयोजित अलकली पुर्व-उपचार से शर्करा की उत्पादन बढ़ाने के लिए एक आशाजनक दृष्टिकोण है। पुर्व-उपचारित पुआल के एंजाइमेटिक शर्कराकरण द्वारा 9 से 39 प्रतिशत शर्करा प्राप्त की गयी। शर्कराकरण और माइक्रोवेव सहयोजित क्षार पुर्व-उपचार के बाद अधिकतम शर्करा तरावड़ी बासमती से (69.9 प्रतिशत), इसके बाद पूसा बासमती 1121 (58.9 प्रतिशत) प्राप्त की गयी। धान की प्रजातियों के पुआल से प्राप्त शर्करा का किण्वन पिचिया स्टैप्टिस एनसीआईएम 3498 के उपयोग द्वारा 2.1 से लेकर 5.16 प्रतिशत (वी/वी) तक इथेनॉल प्राप्त की गयी। अधिकतम इथेनॉल धान की किस्म तरावड़ी बासमती से, तथा इसके बाद पूसा बासमती 1121 से प्राप्त की गयी। फरमेंटर द्वारा इथेनॉल किण्वन की अभिक्रिया अणुगतिकी अध्ययन में तरावड़ी बासमती से 6.63 प्रतिशत (वी/वी) इथेनॉल, सैद्धांतिक उपज 92.9 प्रतिशत के समतुल्य पायी गयी। इथेनॉल उपज गुणांक 0.47 ग्राम इथेनॉल/ग्राम शर्करा उपयोग, पायी गयी। अध्ययन के अनुसार, माइक्रोवेव सहयोजित सोडियम हाइड्राक्साइड (अलकली) पुर्व-उपचारित धान की किस्मों से इथेनॉल उत्पादन की क्षमता, पुआल द्वारा 141 से लेकर 360 लीटर प्रति टन पायी गयी। अध्ययन से प्राप्त निष्कर्षों ने धान के पुआल की विशाल इथेनॉल उत्पादन क्षमता को प्रदर्शित किया।

Annexure



ANNEXURE

Table 1. Total soluble sugars (%)recovery by autoclave pretreatment (120°C, 15min) from rice straw

Varieties	Sulphuric acid (H ₂ SO ₄)	Sodium hydroxide (NaOH)	Hot water (DW)
Pusa 44	18.34	6.42	4.97
IR 36	17.91	9.76	7.44
Pusa basmati 1121	28.68 ^a	15.82 ^a	11.31 ^a
PRH-10	20.7	12.04	8.28
Taraori basmati	27.13 ^a	14.51 ^{ab}	11.23 ^a
LSD (<i>P</i> =0.05)	2.9	2.6	1.23

Table 2. Total soluble sugars (%)recovery by microwave assisted H₂SO₄ pretreated rice straw

Varieties	120°C			140°C			160°C		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Pusa 44	3.96	7.04	8.54	4.17	7.86	10.04	4.87	8.79	12.81
IR 36	4.10	9.55	12.92	5.27	12.86	14.94	6.13	14.86	16.64
Pusa basmati 1121	5.62 ^b	17.71 ^b	21.03 ^a	7.78 ^b	20.93 ^b	22.00 ^a	9.24 ^b	23.03 ^a	23.18 ^b
PRH-10	4.54	8.76	10.73	5.37	10.26	11.64	5.64	12.44	15.92
Taraori basmati	8.37 ^a	20.79 ^a	21.16 ^a	10.52 ^a	22.35 ^a	23.11 ^a	13.13 ^a	23.43 ^a	25.57 ^a
LSD (<i>P</i> =0.05)	0.8637	2.12	0.72	0.86	0.78	1.63	0.64	1.69	0.91

Table 3. Total soluble sugars (%)recovery by microwave assisted NaOH pretreated rice straw

Varieties	120°C			140°C			160°C		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Pusa 44	4.24	10.44	11.97	4.43	11.03	14.04	5.59	11.91	15.85
IR 36	5.06	10.30	13.97	6.34	11.17	16.78	7.55	15.01	16.92
Pusa basmati 1121	8.69 ^b	19.80 ^b	22.88 ^b	9.18 ^b	21.81 ^a	24.68 ^b	10.46 ^b	24.34 ^a	27.84 ^b
PRH-10	5.92	10.30	11.52	6.02	13.96	16.83	6.55	15.81	18.58
Taraori basmati	10.20 ^a	22.21 ^a	25.56 ^a	12.83 ^a	23.37 ^a	27.18 ^a	13.77 ^a	25.73 ^a	30.29 ^a
LSD (<i>P</i> =0.05)	1.25	1.61	1.23	2.1	2.6	0.87	0.84	1.65	1.87

Table 4. Total soluble sugars (%)recovery by microwave assisted hot water pretreatment

Varieties	120°C			140°C			160°C		
	5 min	10 min	15 min	5 min	10 min	15 min	5 min	10 min	15 min
Pusa 44	3.32	6.86	8.10	4.62	7.76	10.76	4.82	8.47	10.97
IR 36	4.41	9.91	12.54	5.30	13.01	12.97	6.91	13.38	15.65
Pusa basmati 1121	5.67 ^b	15.81 ^b	19.18 ^b	6.87 ^b	20.23 ^a	20.51 ^b	8.01 ^b	22.72 ^a	21.37 ^b
PRH-10	5.31 ^b	8.82	12.01	5.69	10.56	12.66	6.17	11.42	14.31
Taraori basmati	7.24 ^a	19.73 ^a	21.97 ^a	9.18 ^a	20.65 ^a	22.37 ^a	12.58 ^a	22.97 ^a	23.28 ^a
LSD (<i>P</i> =0.05)	1.52	0.67	0.78	0.98	1.1	1.17	1.07	0.84	0.98

Table 5. Total soluble sugar recovery by enzymatic saccharification of pretreated rice straw

Pretreated with	Pusa 44	IR 36	Pusa basmati 1121	PRH-10	Taraori basmati
H ₂ SO ₄	10.09±1.70	13.9±0.80	15.6±2.11	12.89±0.64	19.05±0.39
NaOH	17.89±1.34	18.98±2.37	31.15±0.93	23.93±2.09	39.65±3.70
Hot water	9.19±0.85	9.53±0.57	11.67±0.36	11.5±0.98	14.17±2.52
Control	4.08±0.22	4.13±0.16	6.66±0.37	4.4±0.21	7.97±0.52

Table 6: Total fermentable sugars obtained after pretreatment and saccharification

Varieties	Sugar recovery %								
	H ₂ SO ₄			NaOH			HW		
	Pretreatment	Saccharification	Total	Pretreatment	Saccharification	Total	Pretreatment	Saccharification	Total
Pusa 44	12.81	10.09	22.9	15.85	17.89	33.74	10.97	9.19	20.16
IR 36	16.64	13.9	30.54	16.92	18.98	35.9	15.65	9.53	25.18
Pusa basmati 1121	23.18	15.6	38.78	27.84	31.15	58.99	21.37	11.67	33.04
PRH-10	15.92	12.89	28.81	18.58	23.93	42.51	14.31	11.5	25.81
Taraori basmati	25.57	19.05	44.62	30.29	39.65	69.94	23.28	14.17	37.45

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