BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC MATERIAL OF KANS GRASS

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **BIOETHANOL PRODUCTION FROM LIGNOCELLULOSIC MATERIAL OF KANS GRASS** , in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2006 to November 2011 under the supervision of Dr. sanjoy Ghosh, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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Abstract

World's energy demand has been increasing constantly with increasing in population and better life standard that resulted in the more use of energy in form of oil, coal and petroleum gas. This is causing depletion of natural fossil fuel reserves that result in increase of crude oil prices as well as emission of green house gasses. Bioethanol is one of the promising unconventional sources of bioenergy that can be produced from agricultural and other feedstocks including plant material, crop residue and industrial lignocellulosic waste

Kans grass (*Saccharum sponteneum*), a variety of switch grass is a perennial energy crop growing in waste land and has good potential to be used for energy production. Kans grass grows fast, does not require any maintenance and once planted it can supply biomass through out the year. Therefore, in the present study efforts were made to utilize Kans grass biomass for ethanol production by using different steps including pretreatment, hydrolysis and fermentation. Two pretreatment procedures: dilute acid and alkali were utilized for hemicellulosic sugars as well as lignin removal respectively. An effort was also done for the crude cellulase enzyme production from *Trichoderma reesei* and it was further used for the depolymerization of total reducing sugars of pretreated Kans grass biomass. The solubilized sugars obtained after acid pretreatment as well as enzymatic hydrolysis were further used for the ethanol production by *Pichia stipitis* as well as *Saccharomyces cerevisiae*. An adaptive strain to toxic compounds was also developed.

Kans grass, a novel substrate, was evaluated for composition analysis such as holocellulose, lignin, ash, moisture and other contents. A high holocellulose (cellulose and hemicellulose together) content (64.73%) make it suitable to utilize as sugars source The Moisture and ash content of Kans grass biomass were calculated to be 4.70±0.55 and 2.10±0.2% respectively while lignin content was estimated as 25.15±0.55%. The other components were found to be 3.32±0.25 % which may contain different extractives such as proteins and /or fatty acids. Hence Kans grass may be used as a potential substrate due to its availability throughout the year with out making burden on the food land.

Kans grass (5% w/v) was treated with different concentrations of H_2SO_4 (0.5-2% v/v) at moderate temperatures (100-120°C) for various length of reaction time (30-120 minutes). The total reducing sugars concentration in liquid residue was found to be in the range of 2.60-8.15 g/l at 100°C. When the pretreatment temperature was increased to 120°C the total reducing sugars concentration was also enhanced and was found to be in

the range of 7.23-11.66 g/l. Different toxic compounds (furfural and acetic acid) concentrations were also determined (which may inhibit the microbial growth during fermentation) in acid hydrolysate which were observed to be in the range of 1.17-2.34 and 0.12-1.8 g/l for furfural and acetic acid respectively at all pretreatment conditions. This range of toxic compounds were minimized to 0.32-1.23 g/l (furfural) and 0.05-0.87 g/l (acetic acid) after detoxification process by using $Ca(OH)_2$.

Pre-treatment condition corresponding to 2% H_2SO_4 concentration at 120°C and reaction time of 90 minutes was found to be the optimum condition that gives maximum total reducing sugar (TRS) concentration (11.66 g/l) in the acid-hydrolysate. However the sugar yield% was observed to be 17.2%. The Kans grass biomass after acid pretreatment was also analyzed for composition change as well as configurational change (SEM analysis). Further this hydrolysate which is composed of mixture of sugars (majorly pentose) was utilized as carbon source for hexose as well as pentose utilizing yeast to produce ethanol.

NaOH pretreatment of Kans grass (5% w/v) was carried out with low concentrations of NaOH (0.5- 2%) for different duration of time (30-120°C) at variable temperatures (100-120). At 120°C this range of lignin removal was observed to be 14.25-79.30%. It was also observed that at 120°C with reaction time of 120 minutes for all concentrations of NaOH (0.5-2%), more than 70% (70.13-73.23%) delignification was observed. The maximum lignin removal (79.31%) was observed at 90 minutes of duration at 120°C with 2% NaOH. In comparison of the H_2SO_4 pretreatment, NaOH assisted pretreatment was found to be more effective in terms of removal of the lignin which was observed to be 79.31%, however it was found to be 28.03% after H_2SO_4 pretreatment. Thus, NaOH pretreatment favors more lignin removal whereas H_2SO_4 pretreatment may be more suitable for hemicellulosic sugars liberation. In some studies including sorghum straw and cotton stalks 65-77% of lignin removal was observed with NaOH pretreatment. Hence, NaOH pretreatment with Kans grass improved the delignification.

T. reesei (NCIM 1052) was used for CMCase production with different carbon sources (cellulose, glucose, lactose and xylose), pH (4, 5 and 6), and temperatures (25, 28 and 30°C) and for variable duration of time (0-8 days). 28°C was found to be the optimum temperature with for maximum CMCase production (1.46 U/ml) with cellulose as substrate at pH 5. This crude preparation of enzymes also composed of xylanase (6.6 U/ml) activity with total cellulase activity of 1.14 U/ml.

Saccharification of H_2SO_4 treated biomass with 20 FPU/gdb crude enzymes was studied with the biomass loading of 1, 2, 2.5, 5 and 6% (w/v) in 0.05 M citrate buffer at

50°C. The best condition for saccharification was observed at 2% biomass (dilute acid pretreated) loading after 84 hours with 69.08 mg/gdb TRS formation. The maximum recovery of xylose sugars (14.13 mg/gdb) was also observed at 2% biomass loading. When same experiment was conducted with pure (commercial) cellulase preparation on acid pretreated biomass (20 FPU/gdb with biomass loading in the range of (1-6%) the TRS released was found to be maximum at 2% biomass loading (TRS, 63.21 mg/gdb). When untreated biomass (prior to acid treatment) was directly treated with enzyme preparation, the corresponding maximum TRS released was found to be 34.67 and 31.82 mg/gdb under same condition with crude and pure enzyme respectively with negligible amount of xylose released. It was observed that TRS released for untreated biomass was found to be much less as compared to acid pretreated biomass, that may be due to presence of lignin component in untreated biomass which acts as obstacle in enzymatic action, however this lignin was observed to be minimized after H_2SO_4 pretreatment and that resulted comparatively more TRS after enzymatic hydrolysis.

As NaOH pretreatment of Kans grass biomass, at 120°C (all NaOH concentration and residence time) resulted in removal of more than approximately 50% of lignin, hence NaOH pretreated biomass from these conditions were utilized for further saccharification with 20 FPU/ gdb of crude enzyme mixture (derived from *T. reesei* with 1.14 FPU total cellulase activity, 1.46 U/ml of CMCase and 6.6 U/ml of xylanase activity) in citrate buffer for 96 hours released 350mg/gdb (63% w/w) TRS under 5% biomass loading for NaOH pretreated biomass (0.5% for 120 minutes). Hence, in comparison of dilute H_2SO_4 pretreatment, NaOH pretreated biomass was observed to be more efficient for enzymatic hydrolysis.

An adaptive strain of *P. stipitis* was developed, adapted against inhibitors by sequentially transferring and growing the cell in the media containing increase concentration of non detoxified acid hydrolysate (20, 40, 60 and 80%) with supplementation of other media components. An adaptive strain that tolerated 60% non detoxified acid hydrolysate showed better ethanol yield (0.28 g/g) as comparison to wild strain (0.23 g/g) in detoxified acid hydrolysate media. The hydrolysate (11.66 g/l) obtained after optimized condition of dilute acid pretreatment was evaluated for the bioethanol production by increasing the sugars concentration to 60 and 90 g/l using adaptive *P. stipitis*. At 60 g/l the maximum ethanol yield was found to be 0.41 g/g after 72 hours whereas at 90 g/l it was 0.38 for same duration of time.

The sugars hydrolysate obtained after enzymatic hydrolysis (with crude enzyme) of dilute acid pretreated biomass was supplied for fermentation by *S. cerevisiae* and *P. stipitis*,

the ethanol yield was found to be 0.46 g/g and 0.43 g/g respectively after 28 hours. An ethanol yield of 0.44 g/g was also observed after 24 hours when hydrolysate obtained (after enzymatic hydrolysis) from NaOH pretreated biomass was fermented by using *P. stipitis*. Additionally, after fermentation with *S. cerevisiae* the ethanol yield was observed to be 0.38 g/g after 32 hours. This yield is comparable to the other studies carried out with bioethanol formation.

From the above study it can be concluded that Kans grass biomass which is a weedy plant, may be used as substrate for renewable energy production due to higher carbohydrate content. Dilute H_2SO_4 pretreatment of Kans grass biomass is more favourable for liberation of maximum soluble sugars. An improved delignification was observed during NaOH pretreatment and after enzymatic saccharification it resulted the liberation of more sugars in comparison of dilute H_2SO_4 pretreated biomass. Further, crude cellulase enzyme produced from *T. reesei* in present study is equally efficient that of commercial enzyme. As enzyme production cost contributes 40% of total cost of bioethanol synthesis process. This higher cost may be due to involved enzyme purification steps. Thus, utilization of crude enzyme (as produced in the present study) may cut down this cost in some extend. A toxic compound tolerate strain was also developed which showed much improved bioethanol yield as well as shorter fermentation of time in comparison to that of wild strain.

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List of abbreviations

Abbreviation	Full form
TRS	Total reducing sugars
FPU	Filter paper units
FPA	Filter paper assay
gdb	Gram of dry biomass
DAH	Detoxified acid hydrolysate
U/ml	Units/ml
P. stipitis	*Pichia stipitis*
S. cerevisiae	*Saccharomyces cerevisiae*
T. reesei	*Trichoderma reesei*

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Chapter 1
Introduction

1.1 Background

In 1895, a famous Swedish chemist, Svante Arrhenius, presented a paper titled "The Influence of Carbonic Acid in the Air upon the Temperature of the Ground" in Stockholm Physical Society in which he argued that the use of fossil fuel for combustion would lead to global warming [1, 2]. Although he was right, the widespread availability of inexpensive petroleum during the 20th century seemed to ignore this argument. At present, world's energy demand has been on rise with increasing in population and better life standard that in turn resulted in more use of energy in form of oil, coal and petroleum gas. This is causing the depletion of natural fossil fuel reserves thereby increase in crude oil price. Today the earth must deal with the consequences of global climate change as well as emission of green house gasses. In the newly released report of International Energy Outlook 2007 [3], scientists project continuous robust growth of worldwide energy demand over the period from 2004 to 2030. The demand of fuel (gasoline and diesel) has been estimated about 80 Mt for the year 2011–2012, which is about 1.3 times higher than that of current demand [4], while it is expected that crude oil production will start declining from 2010 [5]. Consequently, fossil fuel should be replaced with non- or less-emitted CO_2 fuels.

An alternative to reduce greenhouse gases is by replacing fossil fuels with fuels generated from renewable resources. Therefore, increase in research initiatives on alternative routes to produce chemicals and transportation fuels. In order to maintain high energy consuming lifestyles that people have grown accustomed to, the prospect of converting renewable biomass resources into biofuels such as ethanol, methanol, and biodiesel [6, 7] must thus be investigated. Biodiesel is most commonly produced through trans-esterification of vegetable oils, animal fats or energy crops like Jatropha [8] and has the potential to be economically competitive with conventional diesel if made from waste vegetable oil [6]. The technology for bioethanol production from corn or similar food crops are well defined, however the land use for food or energy production remains a crucial argument. Thus, production from other feedstocks like agro-forest residues that is lignocellulosic biomass still requires extensive research to develop a feasible production method. For this reason, this study is focused on technology to convert lignocellulosic biomass to ethanol.

Ethanol is produced from fermentation of sugars and it is a renewable fuel which can be used as sole fuel or blended with gasoline. Its 10 and 22% blends are being used in

the US and Brazil, respectively [9]. It is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NOx emission from combustion. It may be used directly (95% ethanol and 5% water) as a fuel, such nearly pure ethanol fuel provides a number of environment benefits, due to their low pressure and reduced emission of ethanol in to the atmosphere along with their clean burning characteristics [10]. The use of ethanol as an alternative motor fuel has been steadily increasing around the world for several reasons. Domestic production and use of ethanol for fuel can decrease dependence on foreign oil, reduce trade deficits, create jobs in rural area and reduce air pollution and carbon dioxide build-up [11].

Currently, the major feedstocks for ethanol production are corn kernels (starch) in US and sugarcane (sucrose) in Brazil [12, 13]. However, this raw material base, which is also used for animal feed and human needs, will not be sufficient to meet the increasing demand for fuel ethanol and the reduction of greenhouse gases resulting from use of sugar or starch-based ethanol is not as high as desirable [14]. There are many advantages for utilizing lignocellulosic material as cultivation of energy crops but there is also a great challenge, Concern and responsibility that cultivation of energy crop might reduce land availability for feed and food production. Thus there should not be any competition between cultivation of energy and traditional crops. The planting of trees or other energy crops in the areas like degraded and deforested lands has been suggested [15]. Hence, these factors call for the exploitation of lignocellulosic feedstocks, such as agricultural and forest residues as well as dedicated energy crops, for the production of ethanol.

Lignocellulose is a more complex substrate than starch. It is mainly composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrate polymers are tightly bound to lignin majorly by hydrogen bonds but also by some covalent bonds. The biological process for converting the lignocellulosic materials to fuel ethanol requires: 1) delignification to liberate cellulose and hemicellulose from their complex with lignin; (2) hydrolysis of the carbohydrate polymers to produce free sugars; and (3) fermentation of mixed hexose and pentose sugars to produce ethanol.

Natural lignocellulosic biomass is resistant to hydrolysis because of the crystallinity of cellulose, protection of cellulose by lignin, cellulose sheathing by hemicellulose and the limited accessible surface area of the biomass matrix [16]. Therefore, pretreatment, the most challenging step is essential to break down the close knit structure of lignocellulosic biomass thus providing hydrolysis agents with a more direct access to the feedstocks [17, 18]. Different pretreatment approaches (including physical, chemical, biological and their combinations) have been extensively investigated over the past decade and tailoring of an

appropriate pretreatment for specific biomass feedstocks is of great interest to researchers [19].

After pretreatment, the next step in the production of ethanol from lignocellulosic biomass is the hydrolysis or the break down of the sugar polymers into monomers. This degradation is usually done by enzymes or acid. The enzyme based technology (mainly cellulase) is advantageous over acid based treatment (conc.H_2SO_4) due to higher conversion efficiency, absence of substrate loss due to chemical modification, lack of inhibitory compounds production, low cost, no need of recycling of acid and the use of more moderate and non-corrosive conditions like low temperatures, neutral pH [20].

The Cellulase enzyme production accounts for 40% of total cost in bioethanol synthesis [21]. To reduce cost of production, on site production of crude enzyme is more viable than commercial cellulase due to their reasonable cost (exclusion of enzyme purification step), high enzyme production capacity etc. The reduction in cost paves a cost effective way for ethanol production [22]. In a typical cellulose degrading ecosystem, a variety of cellulolytic bacteria (e.g., *Clostridium*, *Bacillus*) and fungi (e.g., *Penicillium*, *Aspergillus*, *Trichoderma*) work in concert with related microorganisms to convert insoluble cellulosic substrates to soluble sugars, primarily glucose, which is then assimilated by the cell [23]. Due to its excellent ability to produce and secrete a complete set of cellulose degrading enzymes in such amounts, that makes their production viable at industrial scale, the soft rot fungus *Trichoderma reesei* has been in the focus of cellulase research for decades [24].

S. cerevisiae can ferment only certain mono- and disaccharides like glucose, fructose, maltose and sucrose. This microorganism is not able to assimilate cellulose and hemicellulose directly. In addition, pentoses obtained during hemicellulose hydrolysis (mainly xylose) cannot be assimilated by this yeast. D-Xylose is a major component of the hydrolyzate of hemicellulose from biomass. Therefore, ethanol production from xylose is essential for successful utilization of lignocellulosic materials [25]. Many bacteria, yeast, and fungi assimilate xylose, but only a few metabolize it to ethanol [26]. Xylose-fermenting yeasts, such as *P. stipitis, Pachysolen tannophilus* and *Candida shehatae* are widly used for hemicellulosic hydrolysate conversion to ethanol.

The biomass feedstocks used in the present study was Kans grass; (*Saccharum sponteneum)* also known as Switch grass variety, one of the novel and potential substrate for bioethanol production [15]. It is a perennial energy crop growing in marginal land and has a good potential to be used for energy production [27]. Kans grass grows fast, captures lots of solar energy and as it belongs to C_4 plant group, fixes more CO_2 than a C_3 plant

does, thereby, it actively and most efficiently takes part in carbon sequestration process [28], its roots reach deep into the soil for water and use it very efficiently. This does not require any maintenance and once it is planted can supply biomass over many years. It is widely available in Asia including India. Its ability to quickly colonize makes it special for its plantation in waste and marginal lands. Other advantages, it gives more biomass about 10 ton per hectare compared to raw crops which gives only 5-6 ton per hectare per year [29].

1.2 Objectives

The current work is dedicated to finding out the possibilities for bioethanol production from a novel lignocellulosic material Kans grass. Two pretreatment methods including dilute H_2SO_4 and NaOH were targeted to achieve the optimal condition of monomer sugars liberation as well as lignin removal. Further Investigation includes studies of cellulase production and enzymatic hydrolysis of biomass as well as fermentation for bioethanol production.

The objectives of current investigation were:

(1) Characterization of novel lignocellulosic material Kans grass (*Saccharum spontaneum*)

(2) To investigate effect of dilute H_2SO_4 and NaOH pretreatment on Kans grass biomass

(3) Production of cellulases from *Trichoderma reesei*

(4) To examine suitable conditions for enzymatic saccharification of dilute H_2SO_4 and NaOH pretreated biomass

(5) Production of ethanol from hydrolysate obtained after dilute H_2SO_4 pretreatment and enzymatic saccharification of pretreated biomass

Chapter 2
Literature review

2.1 Ethanol

Ethanol or ethyl alcohol is an important organic chemical because of its unique properties and it's wide use for various purposes. Ethanol is a straight-chain alcohol with molecular formula C_2H_5OH. Its empirical formula is C_2H_6O. An alternative notation of ethanol is $CH_3–CH_2–OH$, which indicates that the carbon of a methyl group ($CH_3–$) is attached to the carbon of a methylene group ($–CH_2–$), which is attached to the oxygen of a hydroxyl group (–OH). It is a constitutional isomer of dimethyl ether. Under ordinary conditions, ethanol is a volatile, flammable, clear, colourless liquid, miscible in both water and non-polar solvents. The production of ethanol has two routes: chemical synthesis and biochemical production. The chemical route of ethanol production is commonly carried out by a catalytic hydration of ethylene in vapour phase and often as a by-product of certain manufacturing processes [30]. The ethanol produced from this process is mostly used as a solvent (60%) and chemical intermediate (40%).

Ethanol has widespread use as a solvent of substances intended for human contact or consumption, including scents, flavourings, colourings, and medicines. In chemistry, it is both an essential solvent and a feedstock for the synthesis of other products. It has a long history as a fuel for heat and light, and more recently as a fuel for internal combustion engines.

2.2 Bioethanol as fuel

The biochemical route for ethanol production includes sugars fermentation and an account for 93% of the total ethanol production in the world is referred to as bioethanol. Bioethanol is an alcohol made by fermentation, mostly from carbohydrates produced in sugar or starch crops such as corn or sugarcane. Cellulosic biomass, derived from non-food sources such as trees and grasses, is also being developed as a feedstock for ethanol production. Ethanol can be used as a fuel for vehicles in its pure form, but it is usually used as a gasoline additive to increase octane and improve vehicle emissions. Bioethanol is widely used in the USA and in Brazil. Current plant design does not provide for converting the lignin portion of plant raw materials to fuel components by fermentation.

The ethanol produced is mostly used as fuels (92%); industrial solvents and chemicals (4%), and beverages (4%) [30]. Nowadays, crops are main feedstock used for ethanol production. Brazil is the largest ethanol producer with a capacity of 15.11 Giga-

L/year and uses sugar cane as feedstock, while the USA in second place (13.39 Giga-L/year) uses corn as feedstock [31].The other producer of bioethanol from various sugars sources are listed in Table 2.1. However, these crops are also food for human and animals, thus the expansion of production capacity, especially as ethanol becomes a worldwide alternative fuel, is limited by supply of the feedstock. In contrast, cheap abundant lignocellulosic materials in the form of agricultural and forestry wastes as well as municipal and industrial wastes are available as alternative feedstock for ethanol production. Therefore, the use of lignocellulosic materials for ethanol production is very promising.

The use of ethanol as fuel goes back to the origin of the use of vehicles itself. As Henry Ford's Model T, built in 1908, ran on ethanol. It was continued until the availability of cheap petrol replaced ethanol as a major transport fuel in the early part of the 20th century. The energy crisis of the 1970s renewed interest in ethanol production for fuels and chemicals. Although the interest waned in the following decade due to oil price abatement, the environmental issue of reducing greenhouse gas, rising vehicle fuel demand, and the security of energy supply sustain the development of ethanol production from renewable resources. Ethanol is used in vehicles either as a sole fuel or blended with gasoline. As an oxygenated compound, ethanol provides additional oxygen in combustion, and hence obtains better combustion efficiency. The physicochemical properties of some oxygenated high-octane additives are shown in Table 2.1. Since the completeness of combustion is increased by the present of oxygenated fuels, the emission of carbon monoxide can be reduced by 32.5% whereas the emission of hydrocarbons is decreased by 14.5% [32].

Table 2.1 The major Bioethanol producers in the world.

Country	Amount (million liters)	Share of the world production (%)	Primary feed stocks
Brazil	15,110	37	Sugarcane
USA	13,390	33	Maize
China	3,650	9	Maize, cassava and cereal grains
India	1750	4	Sugarcane, molasses, cassava
France	830	2	Sugar beets, wheat

Additionally, emission of nitrogen oxides is reduced by using ethanol as additive. A flow

diagram for carbon cycle in conventional/fossil fuel as well as biofuel (e.g. bioethanol) is shown in Fig.2.1

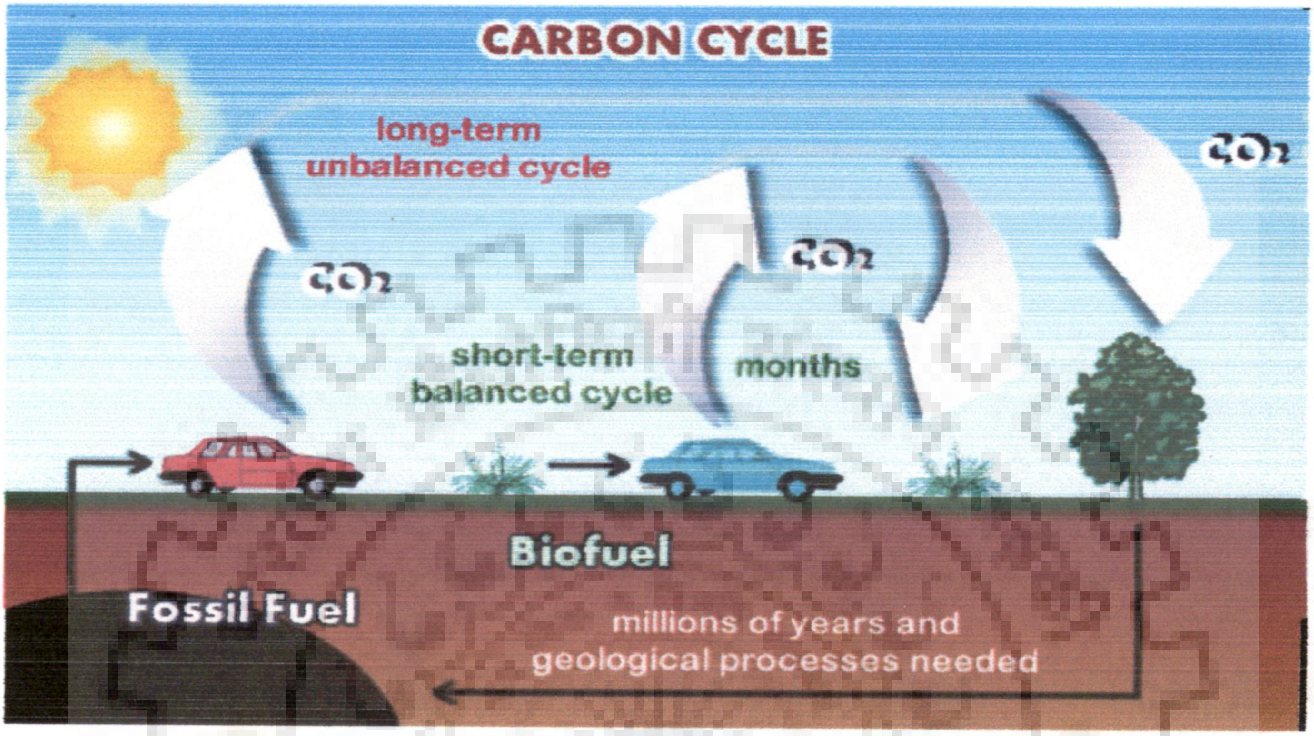


Fig. 2.1 Carbon cycle for fossile fuel and biofuel [33]

However, Ethanol has become more competitive as an oxygenated fuel especially because ethanol is produced from renewable resources by fermentation, resulting in less dependency on fossil fuel. Moreover, ethanol is less hygroscopic, contains a reasonable heat of combustion, has lower evaporation heat and, most importantly, is not toxic like methanol. In addition, acetaldehyde as a product of partial oxidation of ethanol in the exhaust gas of vehicles is much less toxic than formaldehyde, which is formed while using methanol.

Although a high-octane additive, ethanol has several drawbacks including emitting acetaldehyde of 2-4 times as much as does gasoline; highly corrosive in nature, which is a function of water content; a negative effect on rubber and plastic; and the blend with gasoline tends to separate in the presence of traces of water [32]. Fortunately, these drawbacks have been overcome. An additional 5% of water in a blend of ethanol and gasoline can minimize the production of acetaldehyde, also different stabilizers including higher alcohols, fossil oil, aromatic amines, ethers or ketones are useful to prevent separation. Some corrosion inhibitor such as hydroxyethylated alkylphenols and alkyl

imidazolines can act as anticorrosion. Additionally, different polymer industries have developed special materials that are resistant to penetration of alcohols [32].

Table 2.2 The physicochemical properties of some oxygenated high-octane additives to gasoline

Properties	Gasoline	Oxygenates fuel		
		Methanol	Ethanol	MTBE
Density at 15.56°C, kg/m3	719-779	794	792	742
Heat, kJ/kg				
Combustion (lower)	41,800-44,200	19,934	26,749	35,123
Evaporation	~ 349	1104	839	326
Flash point, °C	-42.8	6.5	12	-28
Octane number				
Research (RON)	90-100	107	108	116
Motor (MON)	81-90	92	92	101
Reid vapor pressure, kPa	55-103	32	16	54

2.3 Scope of ethanol production from alternative materials

Unlike fossil fuels, bioethanol is a renewable source of energy which is produced through fermentation of sugars. An enhanced production of ethanol by using the current corn starch, sugar cane or cereal based technology may not be practical because these crop productions for ethanol will compete for the limited agricultural land needed for food and feed production. A potential source for low cost ethanol production is to utilize lignocellulosic materials such as crop residues, grasses, Sawdust, wood chips, solid animal waste and industrial wastes [34].

Straw, a low-density residue, is the dominant residue. Rice husk, a by product of rice milling and it accounts for 20% of paddy. Unlike the cereals, crops such as red gram, cotton, rapeseed, mustard, mulberry and plantation crops produce woody (ligneous) residues. Residue production for mulberry, coconut and sugarcane were estimated based on field studies [35, 36]. The woody (ligneous) crop residues, rice husk and bagasse are considered for energy production [37]. Municipal solid waste (MSW) is normally collected, transported and dumped in the outskirts of towns and cities. Though sorting out

for the recyclable materials by the rag pickers is common, other ways of handling, like composting, incineration, ethanol production, etc., also take place to some extent. The quantity of wastes generated per family in a week has also increased substantially from 7 kg during 1980s to 20–30 kg at present. In India, based on 1991 census data, the estimated quantity of MSW generated in 10 major cities is more than 10 Mt annually. The disposal of such huge quantities has become a major problem. Thus, the utilization of MSW for energy production would mean a solution of this problem. In addition to MSW, large quantity of wastewater is generated in certain industrial plants like breweries, sugar mills, distilleries, food-processing industries, tanneries, and paper and pulp industries.

Out of this, food products and agro based industries together account for 65–70% of the total industrial wastewater in terms of organic load [38]. It was estimated that about 1056,730 Mm^3 wastewater generated in India by industries [37]. Hence, agricultural, urban and industrial residue is present in huge amount and increasing day by day, which could be utilized for ethanol production to reduce its demand and it also be reduced disposal problem of wastes and will help to make clean environment.

2.4 Lignocellulosic materials

Lignocellulosic plant material consists of mainly three different types of polymers (Fig 2.2), namely cellulose, hemicellulose and lignin, which are associated with each other [39]. Corn stover, switchgrass, miscanthus and woodchips are some of the more popular cellulosic materials for ethanol production. Starch based materials including corn, molasses [40] as well as sugar beet are also used for the production of different metabolites and products. Production of ethanol from lignocelluloses has the advantage of abundant and diverse raw material compared to starch based sources but requires a greater amount of processing to make the sugar monomers available to the microorganisms for fermentation.

In recent years, lignocellulosic biomass, especially agricultural crop residue, is being investigated for ethanol production because they are cheap, easily available, and require in time removal from the field. Extensive research has been done on the conversion of lignocellulosic materials to ethanol production in the last two decades [41, 42, 43, 44, 45, 46]. This conversion includes three major steps: (i) Pretreatment of lignocellulosic material for removal of lignin (ii) hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars and (iii) fermentation of the sugars to ethanol.

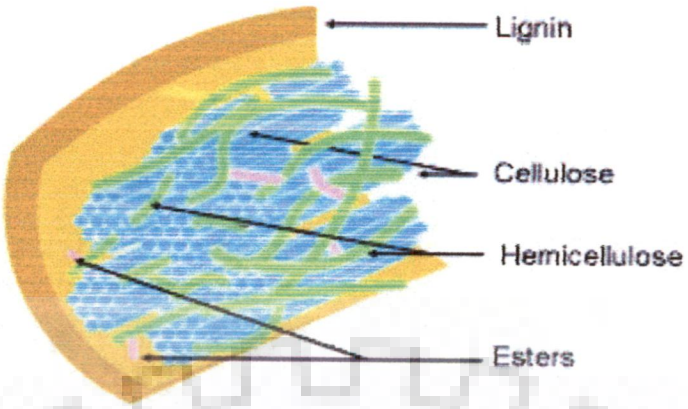


Fig. 2.2 Cellulose, hemicellulose and lignin content in common agricultural residues and wastes [47]

Removal of lignin and hemicellulose, reduction of cellulose crystallinity and increase of porosity in pretreatment processes can significantly improve the hydrolysis [48]. The hydrolysis is usually, catalyzed by cellulase enzymes or acid hydrolysis and the fermentation is carried out by yeast or bacteria. The factors that have been identified to affect the hydrolysis of cellulose include porosity, i.e., accessible surface area of the waste materials, cellulose fiber crystallinity and lignin and hemicellulose content [48]. The presence of lignin and hemicellulose makes the access of cellulose enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis. The contents of cellulose, hemicellulose and lignin in common agricultural residues and wastes are presented in Table 2.3.

A Schematic picture for the conversion of lignocellulosic biomass to ethanol, including the major steps is given in Fig. 2.3. Hydrolysis and fermentation can be performed separately (SHF, indicated by broken arrows) or as simultaneous saccharification and fermentation (SSF). In consolidated bio-processing (CBP) however, all bioconversion steps are minimized to one step in a single reactor using one or more microorganisms. Different techniques such as mutagenesis, co-culturing and heterologous gene expression have been used to improve sugars utilization of the microbial biocatalyst as well as activity and/or stability of hydrolytic fungal-derived enzymes in order to improve the overall yields. For reduction of production cost, ethanol production can be integrated with a combined heat and power plant using lignin.

Table 2.3 Distribution of cellulose, hemicellulose, and lignin in different Lignocellulosic materials

Lignocellulosic material	**Cellulose**	**Hemicellulose**	**Lignin**
Sugarcane baggages	33	30	29
Rice straw	32	24	13
Corn Cobe	42	39	14
Rice hulls	36	15	19
Costal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0
Grasses	25-40	35-50	10-30
Leaves	15-20	80-85	0
News papers	40-55	25-40	18-30
Cotton seed hairs	80-90	5-20	6.4
Waste paper from chemicals pulp	60-70	10-20	5-10
Sunflower stalk	38.5	33.50	17.50
Hard wood stem	40-55	24-40	18-25
Soft wood stems	45-50	25-35	25-35

Source: [45, 49, 50, 51, 52].

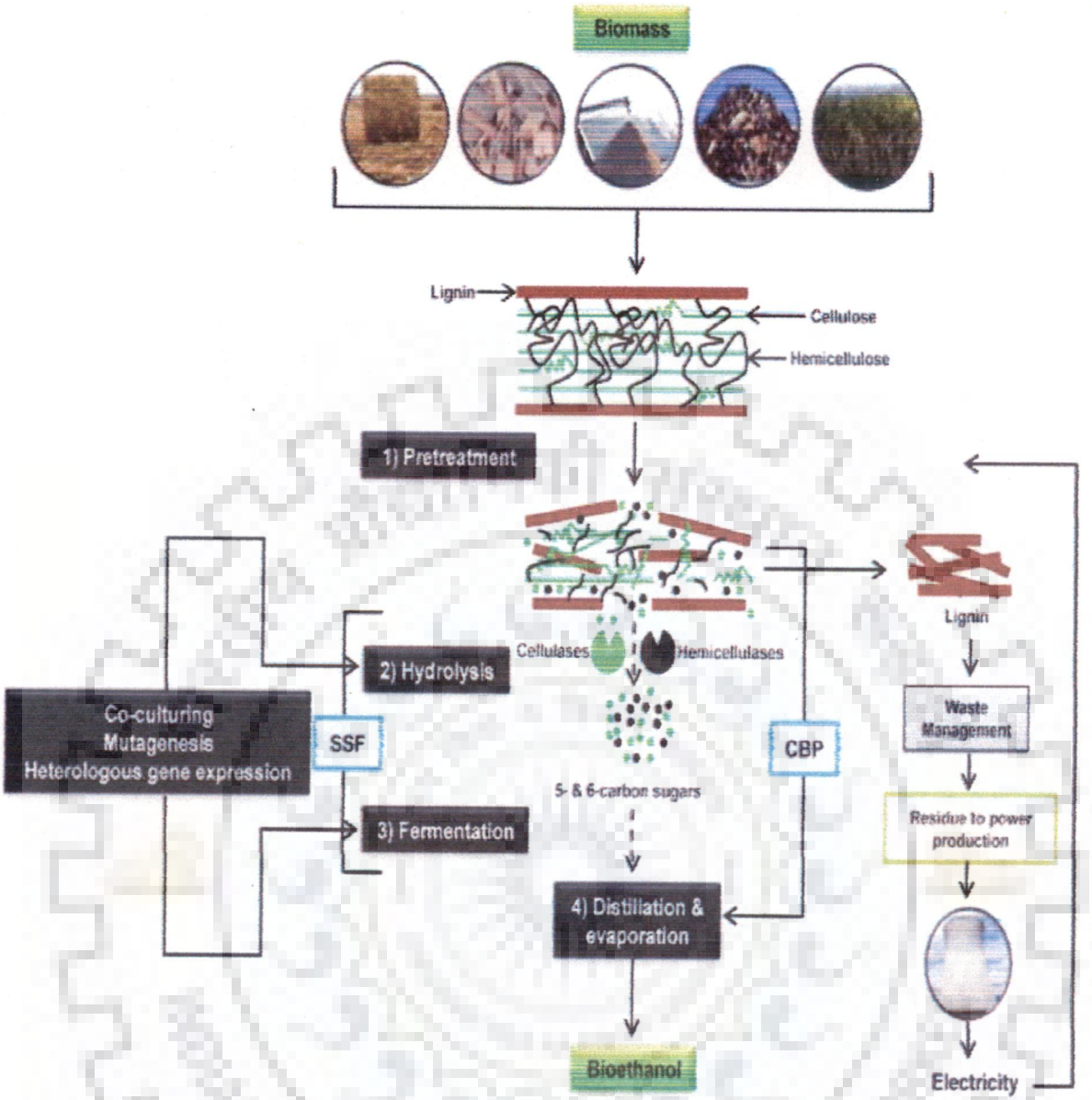


Fig. 2.3 Conversion of lignocellulosic biomass to ethanol

2.4.1 Cellulose

Cellulose is an organic compound with the formula $(C_6H_{10}O_5)_n$ (Fig. 2.4). Since Plants are able to make their own carbohydrates and is utilized for energy and to build their cell walls. Glucose is the simplest and most common carbohydrates in plants. Plants make glucose by using photosynthesis in presence of sunlight, which is used for energy or stored as starch for later use. A plant uses glucose to make cellulose when it links many simple units of glucose together to form long chains. Cellulose exists as D-glucose subunits, linked by β-1,4 glycosidic bonds [53]. The cellulose in a plant consists of parts with a crystalline structure. The cellulose strains are 'bundled' together and form so called

cellulose fibrils or cellulose bundles. These cellulose fibrils are mostly independent and weakly bounded through hydrogen bonding [54]. Cellulose is the structural component of the primary cell wall of green plants. Being abundant and outside the human food chain makes cellulosic materials relatively inexpensive feedstock for ethanol production.

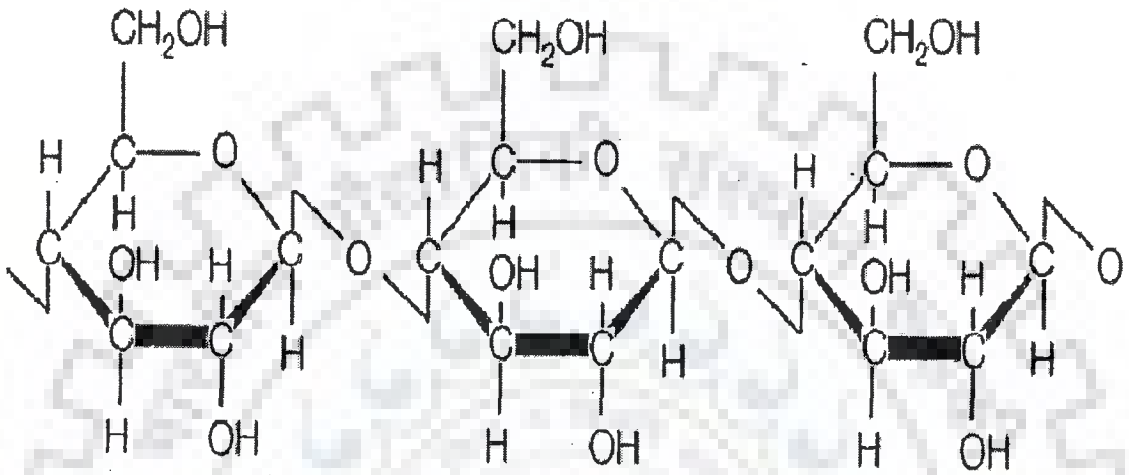


Fig. 2.4 Structure of cellulose with polymeric arrangement of glucose molecule [55]

2.4.2 Hemicellulose

Hemicellulose is a complex carbohydrate structure that composed of different polymers like pentoses (like xylose and arabinose), hexoses (like mannose, glucose and galactose), and sugar acids (Fig. 2.5). Xylan is the dominant component of hemicellulose from hardwood and agricultural plants, like grasses and straw, while this is glucomannan for softwood [53, 56]. Hemicellulose has a lower molecular weight than cellulose, and branches with short lateral chains that consist of different sugars, which are easy hydrolyzable polymers [53]. Hemicellulose acts as a connection between the lignin and the cellulose fibers and gives the whole cellulose–hemicellulose– lignin network more rigidity [54]. The solubility of the different hemicellulose compounds in descending order is: mannose, xylose, glucose, arabinose, and galactose. The solubility of the each component increases with increasing temperature. The xylan of hemicellulose can be extracted quite well in an acid or alkaline environment, while glucomannan can hardly be extracted in an acid environment and needs a stronger alkaline environment than xylan to be extracted [53, 57, 58]. Xylan appears to be the part that can be extracted the most easily.

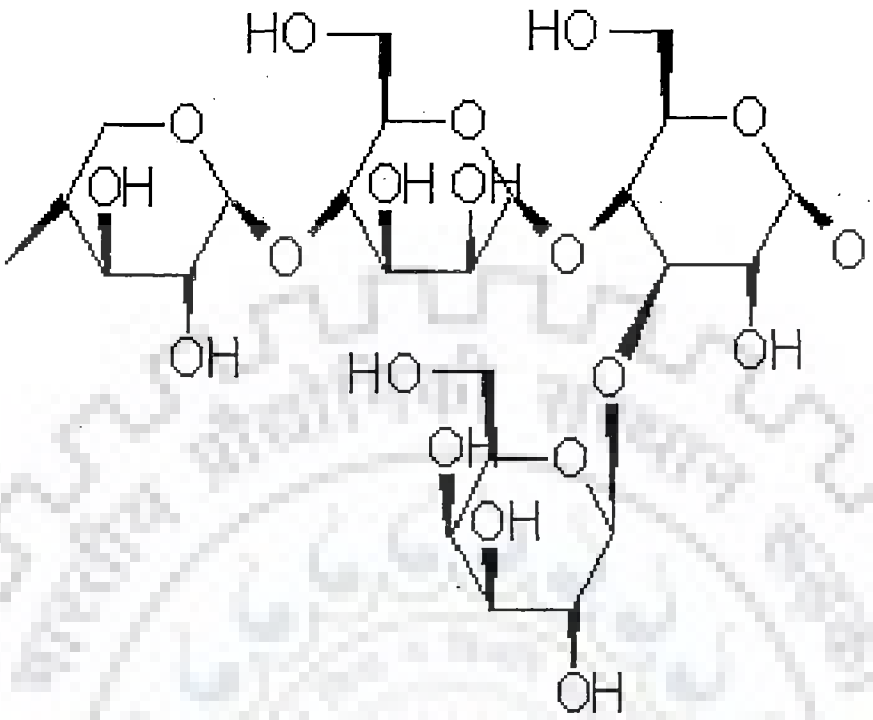


Fig. 2.5 Structure of hemicellulose [59]

2.4.3 Lignin

Lignin strengthens structures by stiffening and holding the cellulose and hemicellulose fibre together, therefore posing as an obstacle during hydrolysis of the lignocellulosic biomass [52]. The amorphous heteropolymer is water insoluble and optically inactive; all this makes the degradation of lignin very tough [39]. Lignin, after cellulose and hemicellulose is one of the most abundant polymers in nature and is present in the cellular wall. It is an amorphous heteropolymer consisting of three different phenylpropane units (p-coumaryl, coniferyl and sinapyl alcohol) that are held together by different kind of linkages (Fig.2.6). The main purpose of lignin is to give the plant structural support, impermeability, and resistance against microbial attack as well as oxidative stress. Based on the specific lignocellulosic structure, the common bioethanol process pretreatment step is generally involved. Pretreatment reduces the size and breaks down the lignin and crystalline structure of lignocellulosic biomass, thus facilitating the subsequent hydrolysis of cellulose and hemicellulose.

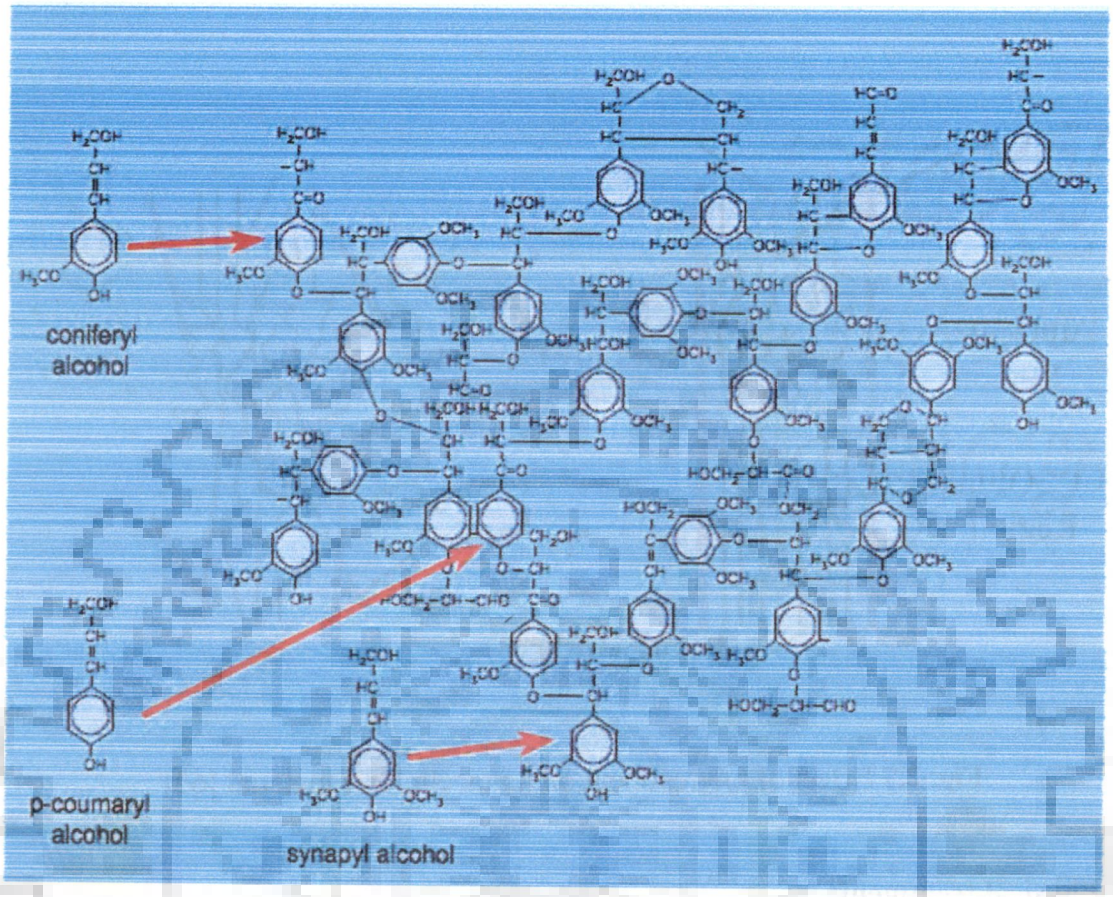


Fig. 2.6 A typical structure of Lignin [60]

2.5 Pretreatment

Pretreatment is one of the important step required to liberate cellulose hemicellulose and lignin from lignocellulosic materials. The mechanisms of pretreatment for the digestion of lignocelluloses are however not clearly known [61]. A one of the important goal of pretreatment is to increase the surface area of lignocellulosic material, so that polysaccharide gets susceptible to hydrolysis. Along with an increase in surface area, pretreatment effectiveness and hydrolysis improvement has been correlated with removal of hemicellulose and lignin and the reduction of cellulose crystallinity [48]. An overview of pretreatment process is shown in Fig. 2.7. A typical pretreatment must meet the following requirements: (1) To improve formation of sugars (2) Low or no degradation of sugars; (3) avoid the formation of toxic by products which cause inhibition during lateral steps of ethanol production (hydrolysis and fermentation) (4) Economical [34]. The large number of pretreatment used for lignocellulosic materials can be classified into groups as

physical, physico-chemical, chemical and biological and enzymatic treatment processes (Table 2.4).

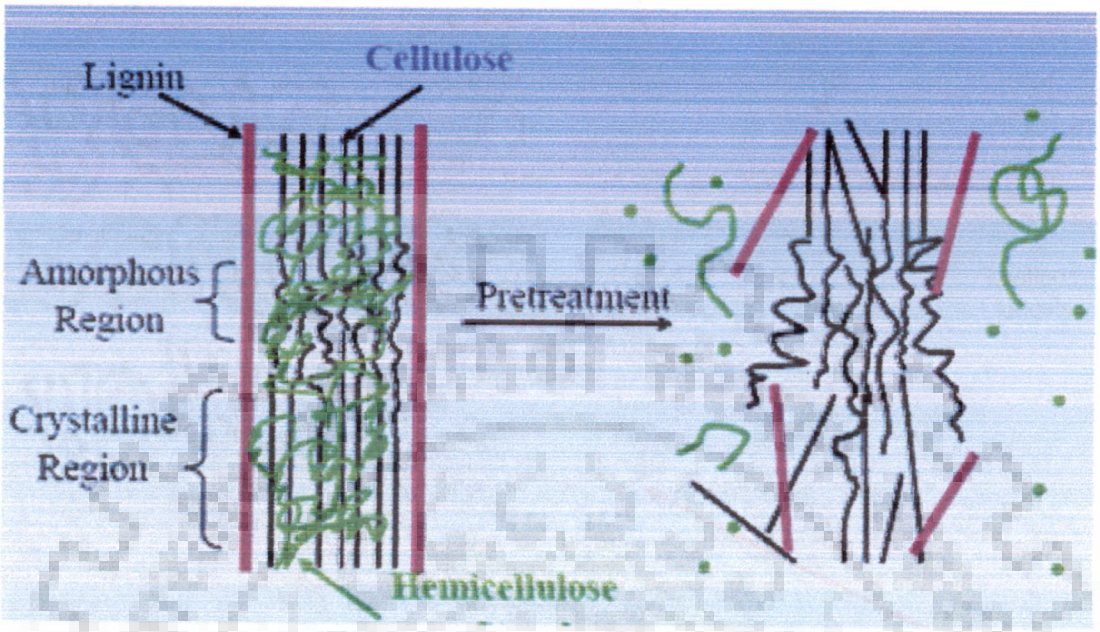


Fig. 2.7 Effect of pretreatment on lignocellulosic material [62]

2.5.1 Physical treatment

Mechanical comminution and extrusion is one of the important methods which are commonly utilized before other pretreatment techniques for the effective hydrolysis. The comminution of the lignocellulosic material is done by a combination of chipping, grinding, and milling. The size of the materials is usually 10 to 30 mm after chipping and 0.2 to 2 mm after milling or grinding [34] and the size reduction of the particle results the increase the more exposed area [63]. The increase in surface area, reduction of depolymerization, and the shearing, are all factors that increase the total hydrolysis yield of lignocellulose in most cases by 5–25% (depends on kind of biomass, kind of milling, and duration of the milling), but also reduces the digestion time by 23–59% (thus an increase in hydrolysis rate) [64, 65]. Extrusion is one of the novel and promising physical pretreatment methods for biomass conversion to ethanol production. In extrusion, the materials are subjected to heating, mixing and shearing which results in physical and chemical modifications during the passage through the extruder. It is assumed that Screw speed and barrel temperature disrupt the lignocellulosic structure results defibrillation, fibrillation and reduction of fibers size and increasing accessibility of carbohydrates for the enzymatic action for further depolymerization into monomer or simple sugars [66].

2.5.2 Physiochemical pretreatment

2.5.2.1 Steam explosion: SO_2-steam explosion

Steam explosion is the most widely employed hydrothermal physico-chemical pretreatment technology for lignocellulosic biomass. In this pretreatment, biomass is subjected to supply with pressurised steam for different duration of time from several second to several minutes and then suddenly depressurised. This pretreatment combines both mechanical forces and chemical effects during hydrolysis (autohydrolysis) of acetyl groups present in hemicellulose. Autohydrolysis occurs when high temperatures results in the formation of acetic acid from acetyl groups; as well as water can also act as an acid at high temperatures. The mechanical effects are caused because of the pressure is suddenly reduced and fibres are separated owing to the explosive decompression. In combination with the partial hemicellulose hydrolysis and solubilization, the lignin is removed in some extent from the material [67]. Removal of hemicelluloses exposes the cellulosic portion and increases enzyme accessibility to the cellulose microfibrils. A way to minimize the hemicellulose degradation during steam pretreatment is by separating the biomass from the condensate during the pretreatment [68], by keeping the pH between 5 and 7 during the pretreatment and by the addition of alkali [69, 70], or by applying a two-step steam pretreatment.

2.5.2.2. Liquid hot water

Liquid hot water is another hydrothermal treatment which does not require rapid decompression and does not employ any catalyst or added chemicals externally. Pressure is applied to maintain water in the liquid state at elevated temperatures (160–240°C) and provoke alterations in the structure of the lignocellulose. The objective of the liquid hot water is to solubilize mainly the hemicellulose, to make the cellulose more accessible and to avoid the formation of inhibitors. The slurry generated after pretreatment can be filtered to obtain two fractions: one solid cellulose-enriched fraction and a liquid fraction rich in hemicellulose derived sugars. To avoid the formation of inhibitors, the pH should be kept between 4 and 7 during the pretreatment because at this pH hemicellulosic sugars are retained in oligomeric form and monomers formation is minimized. Therefore the formation of degradation products is also lower [62]. Liquid hot water has been shown to remove up to 80% of the hemicellulose and to enhance the enzymatic digestibility of pretreated material in herbaceous feedstocks, such as corn stover [62], sugarcane bagasses [71] and wheat straw [72].

2.5.2.3. Ammonia fibre explosion (AFEX)

In the AFEX process, biomass is treated with liquid anhydrous ammonia at temperatures between 60 and 100°C and high pressure for a variable period of time. The pressure is then released, resulting in a rapid expansion of the ammonia gas that causes swelling and physical disruption of biomass fibers and partial decrystallization of cellulose. While some other pretreatments such as steam explosion produce a slurry that can be separated in a solid and a liquid fractions, AFEX produces only a pretreated solid material. AFEX has been reported to decrease cellulose crystallinity and disrupt lignin–carbohydrates linkages [54]. During the pretreatment only a small amount of the solid material is solubilized; little hemicellulose and lignin is removed [73]. Deacetylation of hemicellulose is also observed. AFEX removes the least acetyl groups from certain lignocellulosic materials [74]. Digestibility of biomass is increased after AFEX pretreatment [75] and therefore the enzymatic hydrolysis results in greater yields.

2.5.2.4 Wet oxidation

Wet oxidation is an oxidative pretreatment method which employs oxygen or air as catalyst. It allows reactor operation at relatively low temperatures and short reactor times [76]. The oxidation is performed for 10–15 min at temperatures from 170- 200°C and at pressures from 10 to 12 bar [77]. The addition of oxygen at temperatures above 170°C makes the process exothermic reducing the total energy demand. The main reactions in wet oxidation are the formation of acids from hydrolytic processes and oxidative reactions. It has been proven to be an efficient method for solubilization of hemicelluloses and lignin and to increase digestibility of cellulose and it has been widely used for ethanol production followed by SSF [78]. Also the production of furfural and HMF was low during wet-oxidation, but part of the hemicellulose was lost by reaction to carbon dioxide and water [79].

2.5.2.5 Microwave pretreatment

Microwave-based pretreatment can be considered as physicochemical process since both thermal and non-thermal effects are often involved. Pretreatments were carried out by immersing biomass in dilute chemical reagents and exposing the slurry to microwave radiation for residence times ranging from 5 to 20 min [80]. Preliminary experiments identified alkalis as suitable chemical reagents for microwave-based pretreatment [81].

radiation for residence times ranging from 5 to 20 min [80]. Preliminary experiments identified alkalis as suitable chemical reagents for microwave-based pretreatment [81].

2.5.2.6 Ultrasound pretreatment

The effect of ultrasound on lignocellulosic biomass have been employed for extracting hemicelluloses, cellulose and lignin but less research has been addressed to study the susceptibility of lignocellulosic materials to hydrolysis [82]. In spite of the minor research on ultrasound pretreatment from lignocellulose, some researchers have also shown that Saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment [83]. Different researchers have studied ultrasound pretreatment for different lignocellulosic materials. The frequency of 40 kHz carried out in ultrasonic bath in triticale [84], rice straw was pretreated in a sonicator with 20 kHz [85], however in corn meal [86] 40 kHz frequency was maintained for different duration of time (0.5-30 minutes).

Higher enzymatic hydrolysis yields after ultrasound pretreatment could be explained because cavitations effects caused by introduction of ultrasound field into the enzyme processing solution greatly enhance the transport of enzyme macromolecules toward the substrate surface.

2.5.2.7 CO_2 explosion

Carbon dioxide explosion is also used for lignocellulosic biomass pretreatment. The method is based on the utilization of CO_2 as a supercritical fluid, which refers to a fluid that remains in a gaseous form but is compressed at temperatures above its critical point to a liquid like density. Supercritical pretreatment conditions can effectively remove lignin increasing substrate digestibility. Addition of co-solvents such ethanol can improve delignification. Supercritical carbon dioxide (SC-CO_2) has been mostly used as an extraction solvent but it is being considered for non-extractive purposes due to its many advantages [87]. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. The yield was relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment [88, 89] compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse and repulping waste of recycled paper and found that CO_2 explosion was more cost effective than ammonia explosion and did not cause the formation of inhibitory compounds that could occur in steam explosion.

2.5.3 Chemical pretreatments

2.5.3.1 Alkali pretreatments

The effect of bases on lignocellulosic biomass is the basis of alkaline pretreatments, which are effective depending on the lignin content of the biomass [90]. Alkali pretreatments increase cellulose digestibility and they are more effective for lignin solubilization, exhibiting minor cellulose and hemicellulose solubilization than acid or hydrothermal processes [91]. Alkali pretreatment can be performed at room temperature and times ranging from seconds to days. It is described to cause less sugar degradation than acid pretreatment and was shown to be more effective on agricultural residues than on wood materials [92]. Nevertheless, possible loss of fermentable sugars and production of inhibitory compounds must be taken into consideration to optimize the pretreatment conditions.

Sodium, potassium, calcium and ammonium hydroxides are suitable alkaline pretreatments. NaOH causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and crystallinity, which provokes lignin structure disruption [93]. The bulk of the lignin removal occurs during the alkaline extraction stage [94]. NaOH has been reported to increase hardwood digestibility from 14% to 55% by lowering lignin content 24 and 55% [95]. Addition of an oxidative agent (oxygen/H_2O_2) to alkaline pretreatment (NaOH/$Ca(OH)_2$) can improve the performance by favouring lignin removal [96].

2.5.3.2 Acid pretreatment

The main objective of the acid pretreatment is to solubalize the hemicellulosic fraction of the biomass and to make the cellulose more accessible to enzymes. This type of pretreatments can be performed with concentrated or diluted acid but utilization of concentrated acid is less attractive for ethanol production due to the formation of inhibiting compounds. Furthermore, equipment corrosion problems and acid recovery are important drawbacks when using concentrated acid pretreatment. The high operational and maintenance costs reduce the interest of applying the concentrated acid pretreatment at commercial scale [97]. High hydrolysis yields have been reported when pretreating lignocellulosic materials with diluted H_2SO_4 which is the most studied acid. Hydrochloric acid, phosphoric acid and nitric acid have also been tested [98]. Saccharification yield as high as 74% was shown when wheat straw was subjected to 0.75% v/v of H_2SO_4 at 121°C for 1 h [99]. Olive tree biomass was pretreated with 1.4% H_2SO_4 at 210°C resulting in 76.5% of hydrolysis yields [100]. Recently, ethanol yield as high as 0.47 g/g glucose was

achieved in fermentation tests with cashew apple bagasse pretreated with diluted H_2SO_4 at 121°C for 15 min [101].

2.5.3.3 Ozonolysis

Ozone is a powerful oxidant that shows high delignification efficiency [34]. This lignin removal increases the yield in following enzymatic hydrolysis. The pretreatment is usually performed at room temperature and normal pressure and does not lead to the formation of inhibitory compounds that can affect the subsequent hydrolysis and fermentation. Ozone has been used to degrade lignin and hemicellulose in lignocellulosic materials such as cotton stalks, wheat straw, bagasses, and poplar sawdust [102, 103, 104, 105, 106].

Organosolv or Organosolvation method is a promising pretreatment strategy, since it has demonstrated its potential for lignocellulosic materials [107]. Numerous organic or aqueous solvent mixtures can be utilized, including methanol, ethanol, acetone, ethylene glycol and tetrahydrofurfuryl alcohol, in order to solubilize lignin and renders treated biomass suitable for enzymatic hydrolysis [108]. Comparing to other chemical pretreatments the main advantage of organosolv process is the recovery of relatively pure lignin as a by-product [108]. In some studies these mixtures are combined with acid catalysts (HCl, H_2SO_4, oxalic or salicylic) to break bonds in hemicellulose. A high yield of xylose can usually be obtained with the addition of acid. However, this acid addition can be avoided for a satisfactory delignification by increasing process temperature (above 185°C). High lignin removal (70%) and minimum cellulose loss (less than 2%) are achieved [107].

2.5.3.4 Ionic liquids (ILs) pretreatment

The use of ILs as solvents for pretreatment of cellulosic biomass has recently received much attention. ILs are salts, typically composed of large organic cations and small inorganic anions, which exist as liquids at relatively low temperatures; often at room temperature. Their solvent properties can be varied by adjusting the anion and the alkyl constituents of the cation. These interesting properties include chemical and thermal stability, non-flammability, low vapour pressures and a tendency to remain liquid in a wide range of temperatures [109]. Since no toxic or explosive gases are formed, ILs are called "green" solvents. Carbohydrates and lignin can be simultaneously dissolved in ILs with anion activity (e.g. the 1-butyl-3 methylimidazolium cation [C4mim]+) because ILs form

hydrogen bonds between the non-hydrated chloride ions of the IL and the sugar hydroxyl protons in a 1:1 stoichiometry. As a result, the intricate network of non-covalent interactions among biomass polymers of cellulose, hemicellulose, and lignin is effectively disrupted while minimizing formation of degradation products.

2.5.4 Biological pretreatments

Biological pretreatments employ microorganisms mainly brown, white and soft-rot fungi which degrade lignin and hemicellulose and very little of cellulose, more resistant than the other components [110]. Lignin degradation by white-rot fungi, the most effective for biological pretreatment of lignocellulosic materials, occurs through the action of lignin-degrading enzymes such as peroxidases and laccases [111]. Several white-rot fungi such as *Phanerochaete chrysosporium*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass showing high delignification efficiency [111, 112]. Biological pretreatment by white-rot fungi has been combined with organosolv pretreatment in an ethanol production process by simultaneous saccharification and fermentation (SSF) from beech wood chips [113]. In general, such processes offer advantages such as low-capital cost, low energy, no chemicals requirement, and mild environmental conditions. However, the main drawback to develop biological methods is the low hydrolysis rate obtained in most biological materials compared to other technologies [34]. To move forward a cost-competitive biological pretreatment of lignocellulose, and improve the hydrolysis to eventually improve ethanol yields, there is a need to keep on studying and testing more basidiomycetes fungi for their ability to delignifying the plant material quickly and efficiently.

Table 2.4 Different pretreatment methods

Pretreatment	Condition	Biomass	References
Physical method	Chipping, grinding, milling (final size: 0.2–2 mm)	Wood and forestry wastes (hardwood, straw)	[34, 114, 115]
Steam explosion	Saturated steam at 160–290 °C, pressure (P) = 0,69–4,85 MPa for several sec or min, then decompression upto atm. pressure	Poplar, aspen, eucalyptus, Softwood (Douglas fir)	[34, 116-127]
Liquid hot water	Pressurized hot water, P > 5 MPa,T = 170–230 °C, 1– 46 min; solids loading <20%	Bagasse, olive pulp, corn stover, alfa alfa, wheat straw, sugarcane baggases	[62, 72, 120, 128, 129, 123, 125, 130,131]
Ammonia fiber explosion (AFEX)	1–2 kg ammonia/kg dry biomass, 90 °C, 30 min, P = 1.12–1.36 MPa	Switchgrass, coastal, Bermudagrass, alfalfa, Aspen wood chips, Bagasse, wheat straw, barley straw, rice hulls, corn stover	[34, 132, 133]
Wet oxidation	1.2 MPa oxygen pressure, 195°C, 15 min; addition of water and small amounts of Na_2CO3/H_2SO_4	Corn stover, wheat straw	[133, 134]
Microwave assisted pretreatment	600 W for 4 min	sugarcane bagasse, sorghum	[135, 136]
Ultrasound	10 sec- 10 min;80-320 amplitute; 24-kHz; 15-400 W	Cassava, sugarcane bagasse, cellulase	[137, 138, 139]
CO_2 explosion	4 kg CO_2/kg fiber, P	Bagasse, Alfalfa,	[34]

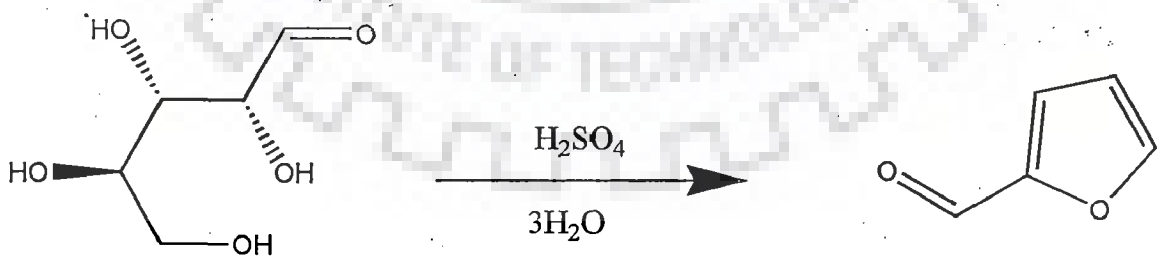
	= 5,62 MPa	Recycled paper	
Alkaline hydrolysis	Dilute NaOH, 24 h, 60°C; $Ca(OH)_2$, 4 h, 120°C; complemented by adding H_2O_2 (0.5–2.15 vol.%) at lower temperature (35°C	Bagasse, corn stover, straws with low lignin content (10–18%), cane leaves, sorghum straw	[34, 139,123, 140- 143,
Dilute-acid hydrolysis	0,75–5% H_2SO_4, HCl, or HNO_3, P=1MPa; low solids loads (5–10 wt% dry substrate/mixture): T = 160–200°C; batch process for high solids loads (10–40 wt% dry substrate/mixture): T = 120–160°C	Bagasse, corn stover, wheat straw, rye straw, rice hulls, Switchgrass, Bermudagrass	[139, 144-149]
Ozonolysis	Ozone, room temperature and pressure	Poplar sawdust, Pine, Bagasse, wheat straw, cotton straw, green hay, peanut	[32, 102, 103, 104, 105, 106, 108]
Biological methods	Brown-, white- and soft-rot fungi	Corn stover, wheat straw	[34, 150]

2.6 Fermentation inhibitors

Pretreatment of lignocellulosic biomass may produce degradation products with an inhibitory effect on the fermentation process. These inhibitory compounds may have toxic effects on the fermenting organisms, consequently reducing the ethanol yield and productivity. The level of toxicity depends in part of fermentation variables including cell physiological conditions, dissolved oxygen concentration and pH of the medium. Also, the fermenting organisms may be resistant to inhibitors or may become gradually adapted to their presence. However, the optimal approach is to prevent the formation of inhibitors as much as possible during the pretreatment processes. The inhibitory effect of these compounds is higher when they are present together due to a synergistic effect [151, 152]. During pretreatment the major inhibitory compounds may be classified as: sugar degrading product, lignin degrading product, Compounds derived from lignocellulosic structure and heavy metal ion.

2.6.1 Sugar degradation products

During hydrolysis, pentose sugars can degrade to furfural (Fig.2.8), a toxic compound that, depending on its concentration in the fermentation medium, can inhibit cells and affect the specific growth rate and cell-mass yield per ATP (Adenosine-5'-triphosphate, transports chemical energy within cells for metabolism) [153]. When studying ethanol production by the yeast *Pichia stipitis*, [154] it was observed that furfural concentrations lower than 0.5 g/l had a positive effect on cell growth, whereas concentrations above 2 g/l inhibited cell growth almost completely. The furfural concentrations of 0.5, 1.0 and 2.0 g/l reduced *P. stipitis* growth by 25%, 47% and 99% respectively [155]. It was found that a furfural concentration of 0.25 g/l in fermentation medium does not reduce the ethanol yield and productivity to a great extent, but a concentration to 1.5 g/l interfere in respiration and growth of the microorganism [156]. Ethanol yield and productivity were found to be decreased by 90.4% and 85.1% respectively. Regarding hydroxymethylfurfural (HMF), a toxic compound originating from hexose degradation (Fig.2.9), it was also reported that its inhibitory effect is similar to that of furfural, causing a longer lag phase during growth [153]. However, HMF is considered less toxic than furfural, and its concentration in hemicellulose hydrolyzates is normally low due to three factors: (1) the low quantity of hexose in hemicellulose, (2) the conditions employed in the hydrolytic processes, which normally do not degrade hexoses in large quantities, and (3) the high reactivity of this compound.



xylose **furfural**

Fig. 2.8 Formation of furfural after xylose degradation

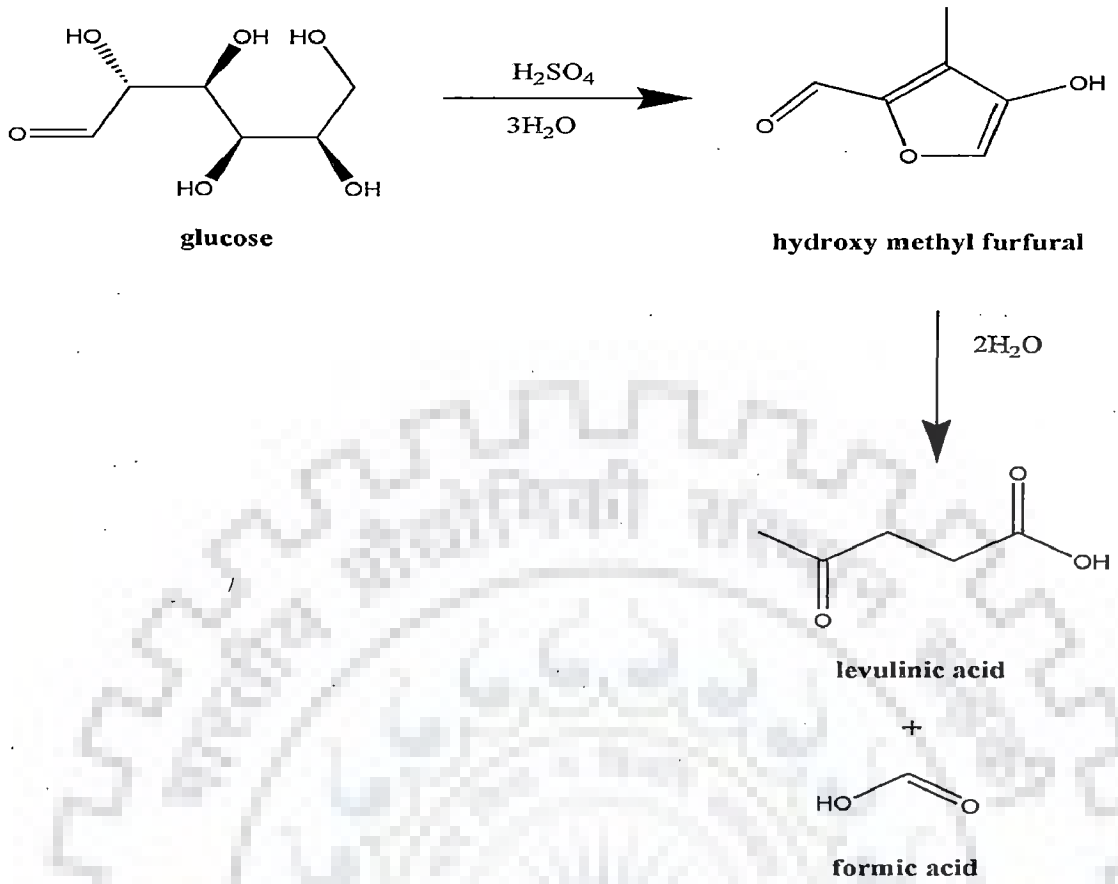


Fig. 2.9 Degradation of glucose to formic acid and levulinic acid

2.6.2 Lignin degradation products

A large variety of compounds (aromatic, polyaromatic, phenolic, and aldehydic) are released from lignin during hydrolysis of lignocellulosic materials. Phenolic compounds have a considerable inhibitory effect on the fermentation of lignocellulosic hydrolyzates, and those with low molecular weight are the most toxic. Phenolic compounds cause a partition and loss of integrity of biological membranes, thereby affecting their ability to serve as selective barriers and enzyme matrices [157]. The cell growth and sugar assimilation are reduced [153]. It was reported that lignin degradation products are more toxic to microorganisms than furfural and hydroxymethylfurfural, even at low concentrations [158]. They also reported that xylose metabolism of *S. cerevisiae* from wood hydrolyzate was totally or half inhibited by vanillin at concentrations of 5 or 3.7 g/l, respectively. In an investigating the effects of different concentrations of phenols (from 0.1 to 4 g/l) on xyloseto- xylitol bioconversion by *Candida guilliermondii*, it was

observed that phenol at concentrations lower than 0.1 g/l, does not affect xylose consumption or cell growth nor it xylitol production, but at higher concentrations it was strongly inhibitory [159].

2.6.3 Compounds derived from lignocellulosic structure

Several inhibitory products, such as raw material extractives (acidic resins, taninic, and terpene acids) and acetic acid derived from acetyl groups present in the hemicellulose are discharged into the hydrolyzate during the hydrolytic process. According to McMillan these extractives produce less inhibition of microbial growth than lignin derivatives or acetic acid [48]. When the pH of the medium is low, acetic acid appears in the undissociated form and diffuses across the plasma membrane [160]. Once in the cell interior, where pH is 7.4, this acid dissociates and accumulates in the cytoplasm, discharging protons. As a consequence, the internal pH drops inhibiting cell activity, and even causing cell death.

2.6.4 Heavy metal ions

Heavy metal ions (iron, chromium, nickel and copper) can originate from corrosion of hydrolysis equipment, and their toxicity inhibits enzymes in the microorganism's metabolic pathways. Watson et al. evaluated the effects of metal cations on the cellular growth of *P. tannophilus* and on the activity of the enzymes involved in xylose metabolism [161]. They employed synthetic media containing concentrations of metal cations similar to those normally found in hydrolyzates. Microbial activity was slightly reduced when copper, nickel, chromium, and iron ions were present in the media in quantities below 4, 5, 100 and 150 mg/l, respectively. On the other hand, a 60% reduction was produced by nickel ions at a concentration of 100 mg/l.

The conclusion is that especially formation of phenolic compounds from lignin degradation should be prevented and secondly, the formation of furfural and HMF by keeping the process temperature and residence time as low and as short as possible.

2.7 Detoxification

Detoxification is the specific removal of inhibitors from acid hydrolysate prior to fermentation. Different methods have been employed for detoxification from lignocellulosic hydrolysate including: Biological, physical, and chemical methods [162].

Lignocellulosic hydrolyzates vary in their degree of inhibition, and also different microorganisms have different levels of inhibitor tolerances.

2.7.1 Biological detoxification methods

Treatment with the enzymes peroxidase and laccase, obtained from the lignolytic fungus *Trametes versicolor*, has been shown to increase the maximum ethanol productivity in a hemicellulose hydrolysate of willow two to three times [163]. The laccase treatment led to selective and virtually complete removal of phenolic monomers (2.6 g/l in the crude hydrolysate) and phenolic acids. The detoxifying mechanism was suggested to be oxidative polymerisation of low molecular weight phenolic compounds. The filamentous soft-rot fungus *Trichoderma reesei* has been reported to degrade inhibitors in a hemicellulose hydrolysate obtained after steam pretreatment of willow, resulting in around three times increased maximum ethanol productivity and four times increased ethanol yield [164]. In contrast to the treatment with laccase, treatment with *T. reesei* resulted in a 30% decrease in absorbance at 280 nm, indicating that the mechanisms of detoxification were different. Acetic acid, furfural and benzoic acid derivatives were removed from the hydrolysate by the treatment with *T. reesei*.

2.7.2 Physical methods

The acid hydrolysate concentration by vacuum evaporation is a physical detoxification method for reducing the contents of volatile compounds such as acetic acid, furfural and vanillin, present in the hydrolysate. However, this method also moderately increases the concentration of non-volatile toxic compounds (extractives and lignin derivatives) and consequently some degree of fermentation inhibition was observed [165]. The volume of hydrolysate was reduced to about 1/3 and the fermentation time necessary for the yeast to consume about 90% xylose increased from 24 to 94 h attained a total removal of furfural from wood hemicellulosic hydrolysate by reducing its volume by 90% through vacuum evaporation [166]. On the other hand, the hydroxymethylfurfural concentration decreased only by 4%. Silva and Roberto utilized vacuum-evaporated with rice straw hydrolyzate as a substrate for microbial conversion of xylose (90 g/l) into xylitol, and noticed that the production process was drastically hindered by the increase in concentration of non-volatile compounds, which are toxic to the microorganism and strongly interfere with fermentation [167]. It was reported that evaporation is suitable to remove acetic acid, furfural and other volatile compounds from hemicellulose hydrolyzates, improving the fermentative process for xylitol production [168]. The

vacuum-evaporation method was employed either before or after treating sugarcane bagasse hemicellulose hydrolysate with activated charcoal [169]. The result was that 98% of furfural was removed whereas acetic acid was only partially eliminated, because this compound is volatile in its undissociated form.

2.7.3 Chemical methods

Detoxification of lignocellulosic hydrolyzates by alkali treatment, by increasing the pH to 9±10 with $Ca(OH)_2$ (overliming) and readjustment to 5.5 with H_2SO_4, has been described as early as 1945 by Leonard and Hajny. $Ca(OH)_2$ adjustment of pH has been reported to result in better fermentability than NaOH adjustment due to the precipitation of toxic compounds [159]. This has been demonstrated by the fact that pre-adjustment to pH 10 with NaOH of a strongly inhibiting dilute-acid hydrolysate of spruce prior to fermentation resulted in twice as high ethanol yield (and comparable to the yield in a reference fermentation containing glucose and nutrients) as after only adjustment to fermentation pH (5.5) [170]. Pre-adjustment to pH 10 with NaOH and $Ca(OH)_2$ has been reported to decrease the concentration of ketones in a dilute acid hydrolysate of spruce from 203 to 158 (22% decrease) and to 143 mg/l (30% decrease), respectively, and the concentration of both furfural and HMF by 20%. In contrast to what has been reported previously [171, 159] the concentration of acetic acid was not affected by either treatment in the referred study. Similarly, adjustment to pH 10 with KOH, readjustment to pH 6.5 with HCl and addition of 1% sodium sulphite at room temperature has been found to be the most efficient method of improving fermentation of a hemicellulose hydrolysate of sugar cane bagasse with *P. stipitis* [159]. The effect of the combined treatment was probably due to decreased concentrations of ketones and aldehydes, and the removal of volatile compounds when a heat treatment was employed

2.7.4 Combination

Different combinations of treatment methods have been used to detoxify lignocellulosic hydrolyzates. In an experiments on xylose-to-xylitol bioconversion, different combinations of bases (CaO or $Ca(OH)_2$) and acids (H_2SO_4 or H_3PO_4) to change the initial pH of sugarcane bagasse hemicellulose hydrolyzate, with or without activated charcoal [172]. The best results were attained when the pH was increased from 0.5 to 7.0 with CaO and then decreased to 5.5 with H_3PO_4, before 2.4% activated charcoal was added to the hydrolyzate. In these conditions, the values of xylitol yield and productivity were 0.79 g/g and 0.47 g/l h, respectively, after 63 h of fermentation. On the other hand, the pH

increased from 0.5 to 7.0 with CaO and then decreased to 5.5 with H_2SO_4, without the addition of activated charcoal, was the worst condition for hydrolyzates treatment, resulting in a xylitol yield and productivity of 0.58 g/g and 0.35 g/l h respectively.

2.8 Inhibitor tolerate microorganisms

Harsh conditions used in pretreatment create a variety of toxic compounds that inhibit the fermentation performance. Inhibitors have been categorized by Olsson and Hahn-Hägerdal [162]. Specifically, acetic acid is released from acetylxylan decomposition, furan derivatives result from sugar dehydration, and phenolic compounds are derived from lignin. Furan derivatives include 2-furaldehyde (furfural) and 5-hydroxymethylfurfural (HMF), which result from pentose and hexose dehydration, respectively [173]. Ethanologenic *E. coli* also has higher tolerance to lignocellulosic inhibitors than its fermentative counterparts [174, 175, 176]. It was compared that ethanol production between these three microorganisms, determining that *E. coli* is comparable with or surpasses other reported production levels, despite its low membrane tolerance to ethanol [177]. The fermentation performance of six selected strains of *Saccharomyces cerevisiae* in dilute-acid spruce hydrolysate was compared using two different modes of fermentation [178]. Yeast strains Y1, application patent no. 200710175715.0, China, CGMCC 2160, Y4 application patent no. 200710175714.6, China, CGMCC 2159, and Y7 application patent no. 200810223301.5, China, CGMCC 2661 demonstrated high conversion efficiencies for sugars and high abilities to tolerate or metabolize inhibitors in dilute-acid lignocellulosic hydrolysates [179].

2.9 Cellulase enzyme production

The production of bioethanol from lignocellulosic material is one of the most important strategies due to continue depletion of fossile fuel and environmental concerns. A lignocellulosic material that includes forest waste, agricultural residue, lignocellulosic waste or grasses is supplied for enzymatic hydrolysis or saccharification after pretreatment for bioethanol production. The enzymes (cellulases) production cost accounts 40% of the overall process of bioethanol production. To reduce the commercial enzyme the on site produced crude enzyme is more viable due to reasonable cost and high enzyme production [180, 181]. A cellulosic enzyme system consists of three major components [182, 183]: endo-ß-glucanase (EC 3.2.1.4), exo-ß-glucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21). The mode of action of each of these being:

(1) Endo-p-glucanase, 1,4-ß-D-glucan glucanohydrolase, CMCase, Cx: "random" scission of cellulose chains yielding glucose and cello-oligo saccharides.

(2) Exo-P-glucanase, 1,4-ß - D-glucan cellobiohydrolase, Avicelase, C1: exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure.

(3) ß-glucosidase, cellobiase: hydrolysis of cellobiose to glucose.

Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases [184]. *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, they do not produce high enzyme titres [185]. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production is focused on fungi [185].

Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum*, *Penicillium*, *rhizopus* [187, 188, 189, 190, 191, 192]. Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production [188].

The genus *Trichoderma,* filamentous *ascomycetes* are widely used in industrial applications because of high secretory capacity and inducer promoting characteristics [193]. *T. reesei* was selected as the best cellulase producing strain. Morphology studies were carried out on *T. reesei* QM 9414 in submerged culture [194]. Hypercelluloytic mutant strains secrete large amounts of cellulases [196]. Biosynthesis of cellulase was made on *T. reesei* QM 9414 using cellulose as carbon source. The production of cellulase was carried out by culture using *T. reesei* Rut C-30 and *T. reesei* NG-14. The maximum growth of *T. reesei* C_5 and the production of cellulase enzyme were obtained with lactose as carbon source [197]. The detailed study was made on the production of cellulase using mutants of *T. reesei* [198]. Fermentative production of cellulase was made on substrates cellulose, xylose and lactose using *T. reesei* [199]. The inductive formation of cellulases and β-glucosidase is subject to catabolite repression by glucose and other readily

metabolizable substrates [200]. Growth of cellulolytic fungi in the presence of an abundant quantity of easily degraded cellulose can result in glucose accumulation and repression of cellulase synthesis. Derepression of enzyme synthesis occurs upon exhaustion of the accumulated glucose. Maximum cellulase synthesis can be achieved by growing *T reesei* on slightly limiting concentrations of cellulose.

The synthesis of cellulase using *T. reesei* QM 9414 [201] increased using a mixture of cellulose with lactose. Cellulase production was improved in the mixture substrates of cellulose with xylose using *T. reesei*. Cellulase protein has also been produced from sugarcane bagasse by *Aspergillus niger* and *T. reesei* [199, 202]. Biosynthesis of cellulase was also made on lignocellulosic materials such as agro residue [203], sugar cane baggases [201], Ocimum gratissimum seed [204], sugar beet pulp and alkaline extracted sugar beet pulp and cellulose [166]. Cellulose and hemi cellulose-degrading enzyme was produced on wet-oxidized wheat straw using various filamentous fungi [204]. In this study, cellulase protein production was made on substrates glucose, xylose, lactose, cellulose, sugarcane bagasse, rice straw and mixture of carbon sources using *T. reesei* QM 9414, 97.177 and Tm3. The enzymatic activity of exoglucanase, endoglucanse and β-glucosidase were determined as a function of time. Optimization of D.O. level, pH, temperature, agitator speed and medium composition were also made. The growth kinetics of *T. reesei* 97.177 and the product formation kinetics for cellulase protein production were investigated.

2.10 Hydrolysis

Most processes for the production of ethanol from lignocellulosic materials have similar designs based on feedstock handling, hydrolysis, fermentation and distillation. Big difference usually lies in how the hydrolysis step is performed. Therefore, the processes have been divided into two groups, according to the design of the hydrolysis steps, acid and enzymatic hydrolysis processes.

2.10.1 Chemical hydrolysis

Dilute acid hydrolysis is fast and easy to perform and it has the advantage of the relatively low acid consumption, but is hampered by non-selectivity and by-product formation. Namely, high temperatures are required to achieve acceptable rates of conversion of cellulose to glucose, but high temperatures increase also the rates of hemicellulose sugar decomposition and equipment corrosion. Under these conditions, xylose degrades to furfural and glucose degrades to 5-hydroxymethyl furfural (HMF), both of which is toxic to microorganisms and can also cause inhibition in the subsequent

fermentation stage. The maximum yield of glucose is obtained at high temperatures and shorter residence time, but even under these conditions the glucose yield is only between 50% and 60% of the theoretical. If dilute acids (H_2SO_4 and HCl) are employed, temperatures of 200–240°C at 1.5% acid concentrations are required to hydrolyze the crystalline cellulose, but the degradation of glucose into HMF and other non-desired products is unavoidable under these conditions. Similarly, xylose is degraded into furfural and other compounds. During two-stage regime, a first stage under mild conditions (190 °C, 0.7% acid, 3 min) was carried out to recover pentoses, while in the second stage, the remaining solids underwent harsher conditions (215°C, 0.4% acid, 3 min) to recover hexoses. In this way, 50% glucose yield was obtained [205]. One variant of the acid hydrolysis was the use of extremely low acid and high temperature conditions during batch processes (auto-hydrolysis approach) that had been applied to sawdust [206]. Concentrated acid process using 30–70% H_2SO_4 gave higher glucose yield (90%) and was relatively rapid (10–12 h) but the amount of used acid was a critical economic factor. By continuous ion-exchange, over 97% acid recovery was possible [206].

2.10.2 Enzymatic hydrolysis

Hydrolysis of cellulose is currently carried out using microbial cellulolytic enzymes. Enzymatic hydrolysis has demonstrated better results for the subsequent fermentation because no degradation components of glucose are formed although the process is slower. Most of the commercial cellulases are obtained from *Trichoderma reesei*, though a small portion is obtained from *A. niger*. *Aspergillus niger* is a filamentous ascomycete that predominantly grows on dead or decaying plant material which is composed of cellulose, xylan, pectin, xyloglucan, galactomannan and released the enzymes for degrading the same [107]. However, *T. reesei* releases a mixture of cellulases, among which at least two cellobiohydrolases, five endoglucanases, β-glucosidases and hemicellulases can be found [208]. The action of cellobiohydrolases causes a gradual decrease in the polymerization degree while endoglucanases cause the rupture of cellulose in smaller chains reducing rapidly the polymerization degree. Endoglucanases especially act on amorphous cellulose, whereas cellobiohydrolases can act on crystalline cellulose as well [209]. Although *T. reesei* produces some β-glucosidases, which are responsible of hydrolyzing formed cellobiose into two molecules of glucose, their activities are not very high. Unfortunately, cellobiohydrolases are inhibited by the cellobiose. For this reason, β-glucosidase from other source needs to be added in order to complement the action of the cellulases of this fungus. Factorial optimization techniques have been applied for the

design of cellulases mixtures from different sources including β-glucosidase in order to maximize the yield of produced glucose [210]. The development of a multicellulase plasmid in which the different cellulase genes could be expressed to produce cellulases with an optimum ratio from a single cultivation has been suggested. Cellulases should be adsorbed on the surface of substrate particles before hydrolysis of insoluble cellulose takes place [211, 212]. The three-dimensional structure of these particles in combination with their size and shape determines whether β-glucosidase linkages are or are not accessible to enzymatic attack [212]. This makes cellulose hydrolysis to be slower compared to the enzymatic degradation of other biopolymers. For instance, the hydrolysis rate of starch by amylases is 100 times faster than hydrolysis rate of cellulose by cellulases under industrial processing conditions.

2.11 Fermentation

Microorganisms are capable of growing on a wide range of substrates and can produce a remarkable spectrum of products. Hence the word fermentation means an energy-generating process in which organic compounds act as both electron donors and acceptors, that is in an anaerobic process energy is produced without the participation of oxygen or other inorganic electron acceptors. The growth of a microorganism may resulted in the production of a range of metabolites [213, 214] but to produce a particular metabolite the desired organism must be grown under precise cultural conditions for a reasonable growth rate.

2.11.1 Principles of ethanol fermentation

Ethanol fermentation begins with the completion of glycolysis, which is also termed the EMP (Embden-Meyerhoff-Parnas) pathway [215]. The EMP is the most common reaction for oxidizing glucose to pyruvate which is an important intermediate metabolite for most living organisms. The EMP pathway is composed of three stages, namely activation of glucose, hexose splitting and energy extraction. In the simplest form, production of ethanol from glucose can be expressed by the following equation:

$$C_6H_{12}O_6 + 2\,Pi + 2\,ADP \longrightarrow 2\,C_2H_5OH + 2\,CO_2 + 2\,ATP + 2\,H_2O$$

$$\text{Glucose} \longrightarrow \text{2 Etahnol+ 2 carbon dioxide + 2 Energy}$$

Ethanol is formed as an end product of the EMP pathway. First, acetaldehyde is produced from pyruvate by reducing a molecule of CO_2 out of pyruvate, and then acetaldehyde is reduced to ethanol along the redox reaction between NADH and NAD+. Besides ethanol, lactic acid is another end product of microbial fermentation.

Methods for C6 sugar fermentation were already known (at least) 6000 years ago, when Sumerians, Babylonians and Egyptians began to perfect and describe the process of making beer from grain (starch) and it became possible to free the C6 sugars in lignocellulosic crops (end 19th century). Only in the 1980s research on xylose fermentation began to bear fruit, when a number of wild type yeast were identified that could convert xylose to ethanol [216].

A variety of microorganisms, generally either bacteria, yeast, fungi, ferment carbohydrates to ethanol under oxygen-free conditions. They do so to obtain energy and to grow. According to the reactions, the theoretical maximum yield is 0.51 kg ethanol and 0.49 kg carbon dioxide per kg sugar:

$$\underset{\text{xylose}}{3C_5H_{10}O_5} \longrightarrow \underset{\text{ethanol}}{5C_2H_5OH} + 5CO_2 \qquad (1)$$

$$\underset{\text{glucose}}{C_6H_{12}O_6} \longrightarrow \underset{\text{ethanol}}{2C_2H_5OH} + 2CO_2 \qquad (2)$$

Since certain types of inhibitors are formed during acid pretreatment such as acetic acid, furfural, and lignin-derived compounds [217], commercial organisms capable of fermenting reducing sugars must be able to function normally in the large-scale environment and tolerate various toxic compounds contained in the hydrolysate [21]. By studying the natural yeast strains that can convert hexoses and pentoses to ethanol, researchers are trying to optimize the conditions for cell growth and fermentation, including hydrolysate composition, pH and acetate concentration.

Sreenath and Jeffries conducted a study that focused on the fermentation ability of *Candida shehatae* and *Pichia stipitis* strains on wood hydrolysate with 1:1 mixture of glucose and xylose [217]. It was reported that the optimum ethanol production rate was obtained for a pH range between 5.5 and 6.0, and an ethanol yield of 0.41-0.46 g/g glucose and xylose could be achieved within these optimized batches. Additionally, the loading of microbial cells and cell recycle were also of great importance in enhancing ethanol production (Table 2.5).

2.11.2 Ethanol production by bacteria

Ethanol-producing bacteria have attracted much attention in recent years because their growth rate is substantially higher than that of the *Saccharomyces*, presently used for production of fuel alcohol and, with the recent advances in biotechnology, they proved their potential to play a key role in making production of ethanol more economical [215]. Among such ethanol-producing bacteria, *Z. mobilis* is a well-known organism used historically in tropical areas to make alcoholic beverages from plant sap [218]. The advantages of *Z. mobilis* are its high growth rate and specific ethanol production; unfortunately, its fermentable carbohydrates are limited to glucose, fructose and sucrose. On the other hand, the Gram-negative strain *Zymobacter palmae*, which was isolated by Okamoto et al. (1993) using a broad range of carbohydrate substrates, is a facultative anaerobe that ferments hexoses, di and tri-saccharides, and sugar alcohols (fructose, galactose, glucose, mannose, maltose, melibiose, sucrose, raffinose, mannitol and sorbitol) [219]. This strain produces approximately 2 mol of ethanol per mole of glucose without accumulation of by products and shows productivity similar to that of *Z. mobilis* [220].

2.11.3 Ethanol production by yeasts

Metabolic pathway engineering is constrained by the thermodynamics and stoichiometric feasibility of enzymatic activities of introduced genes. Engineering of xylose metabolism in *S. cerevisiae* is focused on introducing genes for the initial xylose assimilation steps from *P. stipitis*, a xylose-fermenting yeast, into *S. cerevisiae*, a yeast traditionally used in ethanol production from hexose. However, recombinant *S. cerevisiae* created in several laboratories have used xylose oxidatively rather than in the fermentative manner that this yeast metabolizes glucose [221]. D-Xylose is a major component of the hydrolyzate of hemicellulose from biomass. Therefore, ethanol production from xylose is essential for successful utilization of lignocellulose [25]. Many bacteria, yeast, and fungi assimilate xylose, but only a few metabolize it to ethanol [26]. Xylose-fermenting yeasts, such as *P. stipitis, Pachysolen tannophilus* and *Candida shehatae* require precisely regulated oxygenation for maximal ethanol production [222, 223], and detoxification of the hydrolysate, because they withstand the inhibitory environment of lignocellulose hydrolysates poorly [224, 225, 226, 227].

Table 2.5 Bioethanol production by different microorganisms

Substrates	Organism	Sugar (g/l)	Ethanol (g/l)	Ethanol yield (g/g)	Reference
Corn stover	*P. stipitis*	40	15.92	0.4	228
Prosopis juliflora	*P. stipitis*	18	7.1	0.39	229
Rice straw	*P. stipitis*	33	14.9	0.45	230
Sun flower seed hull	*P. stipitis*	34	11	0.32	231
Sugar cane bagasse	*Pachysolen tannophilus*	63.5	19	0.34	232
Sugar maple	*P. stipitis*	35	12.4	0.35	233
Corn stover	*P. stipitis*	40	15	0.37	234
Sugar cane bagasse	*Pachysolen tannophilus*	30	8.67	0.29	235
Corn stover	*P. stipitis*	60	25	0.42	236
Red oak wood chips	*P. stipitis*	36	14.5	0.40	237
Red oak spent sulfite liquor	*P. stipitis*	49	20.2	0.41	238
Wheat straw	*P. stipitis*	52	22.3	0.43	239
Corn cob	*P. stipitis*	30	10.4	0.34	240
Poplar	*P. stipitis*	39	12	0.31	240
Switch grass	*Candida shehatae*	39	14	0.36	240
Rice straw	*Candida shehatae*	20	9	0.45	241
Rice straw	*P. stipitis*	15	6	0.40	243
L. camara	*P. stipitis*	16.8	5.16	0.33	244
Molasses	*S. cerevisiae*	1.6–5.0	5–18.4	-	245
Corn fiber	*Escherichia coli*			0.39–0.41	246
Corn stover	*E. coli* FBR5			0.46	246
Spruce	*S. cerevisiae*			0.43	246
Sugar mixture	*S. cerevisiae* 1400	58	22	-	247
Sugar mixture	*S. cerevisiae* BH42	100	28	-	248
Sugar mixture	*Z. mobilis*	80	33.5	-	249

2.11.4 Separate hydrolysis and fermentation (SHF)

When sequential process is utilized, solid fraction of pretreated lignocellulosic material undergoes hydrolysis (saccharification). This fraction contains cellulose. Once hydrolysis is completed, the resulting cellulose hydrolyzate is fermented and converted into ethanol. One of the main features of the SHF process is that each step can be performed at its optimal operating conditions. The most important factors to be taken into account for saccharification step are reaction time, temperature, pH, enzyme dosage and substrate load. By testing lignocellulosic material from sugar cane leaves, It was found that the best values of all these parameters varying in each experimental series the value of one of the factors fixing the other ones [250]. Hydrolysis tests for steam-pretreated spruce also indicate the need of high enzyme loadings of both cellulases and β-glucosidase to achieve cellulose conversions greater than 70% due to the less degradability of the softwood [251]. The composition of lignocellulosic material has an important influence on the enzyme dosage as claimed in a patent [252]. In particular, the ratio of arabinan plus xylan to total non starch polysaccharides determines its relative cellulase requirement to convert the cellulose to glucose. Thus, higher this ratio, the less enzyme is required after the pretreatment, and hence the more economical the production of ethanol. 65–70% cellulose conversion was achieved at 50°C and pH of 4.5. Although enzyme doses of 100 FPU/g cellulose caused almost a 100% hydrolysis, this amount of cellulases is not economically justifiable.

2.11.5 Simultaneous saccharification and fermentation (SSF)

The SSF process shows more attractive indices than the SHF as higher ethanol yields and less energetic consumption. In this case, the cellulases and microorganisms are added to the same process unit allowing the glucose formed during the enzymatic hydrolysis of cellulose be immediately consumed by the microbial cells converting it into ethanol. Thus, the inhibition effect caused by the sugars over the cellulases is neutralized. However, the need of employing more dilute media to reach suitable rheological properties makes that final product concentration low. In addition, this process operates at non-optimal conditions for hydrolysis and requires higher enzyme dosage, which positively influences on substrate conversion, but negatively on process costs. SSF of pretreated spruce with a xylose fermenting recombinant strain of *Saccharomyces cerevisiae* was performed which showed up to 77% of xylose utilization and 85% of the theoretical yield

of ethanol [253]. Considering that the enzymes account for an important part of production costs, it is necessary to find methods reducing the cellulases doses. With this aim, addition of surfactants has been proposed. Alkasrawi et al. showed that the addition of the non-ionic surfactant Tween-20 to the steam exploded wood during a batch SSF using *S. cerevisiae* had some effects: 8% increase in ethanol yield, 50% reduction in cellulases dosage (from 44 FPU/g cellulose to 22 FPU/g cellulose), increase of enzyme activity at the end of the process, and decrease in the time required for reaching the highest ethanol concentration [254]. It is postulated that the surfactant avoids or diminishes the non-useful adsorption of cellulases to the lignin. However, Saha et al. obtained marginal increases (3.5%) in saccharification of rice hulls using 2.5 g/L of Tween 20 [255].

2.11.6 Fermentation of pentose

Currently, fermentation of a mixture of hexoses and pentoses is inefficient. This is one of the major restrictions for the commercial scale-up of bioethanol production from cellulosic feedstocks, since the hydrolysate of these feedstocks comprises of both hexoses and pentoses in the same broth rather than merely hexoses, as in corn and sugarcane. Yeast *S. cerevisiae* is only able to metabolize hexoses, such as glucose, galactose and mannose as substrates for fermentation. Pentoses, however, are not consumed by *S. cerevisiae* to yield ethanol. Therefore, the presence of pentose complicates the fermentation process [21].

The metabolism of D-xylose by some natural occurring bacteria and other yeasts (*Candida shehatae, Pichia stipitis* and *Pachysolen tannophilus*) has been extensively studied [155, 256, 257]. Bacteria can directly convert xylose to xylulose with xylose isomerase. However, yeasts that are capable of consuming pentose, first reduce xylose to xylitol with NADPH-dependent xylose reductase, and then convert xylitol to xylulose with NAD+-dependent xylitol dehydrogenase. Thereafter, xylulose is converted to xylulose-5-phosphate by xylulokinase. Through the pentose phosphate pathway (PPP), xylulose-5-phosphate, the central metabolite, can be converted to fructose-6-phosphate, then via glycolysis, pyruvate is produced as an intermediate in ethanol production. However, wild-type strains of *S. cerevisiae* are unable to ferment D-xylose directly and can only act on xylulose to produce ethanol when exogenous xylose isomerase is introduced to the D-xylose containing system [256]. Various approaches have been extensively tested to genetically modify the gene of *S. cerevisiae* to endow it with the ability to ferment both 5C and 6C sugars. Since the production of ethanol from mixed sugar hydrolyzates has not reached a commercially acceptable yield as of now [21], more research is needed for further improvement. Ho and Tsao constructed a *Saccharomyces cerevisiae* strain that

contained several categories of enzymes needed; the recombinant strain was reported to have the ability to effectively ferment both glucose and xylose, which helped to make cellulosic biomass-to-ethanol technology closer to commercialization [258].

2.12 Product recovery

Ethanol from the fermentation broth is recovered by distillation followed by a dehydration step. Ethanol and water forms an azeotropic mixture of 95 percent ethanol and 5 percent water by volume. In the past, ternary azeotropic distillation using an agent such as benzene, cyclohexane, diethyl ether, and *n*-pentane was employed to produce anhydrous ethanol .46 Molecular sieves currently are used for dehydration of 95 percent ethanol. Molecular sieves are synthetic zeolite adsorbents having cylindrical or spherical shapes manufactured from materials such as potassium aluminosilicates.

2.13 Residual solids/power production

The main solid residual from the bioethanol process from lignocellulosic material is lignin. Its amount and quality differs with feedstock and the applied process. Production of coproducts from lignin, such as high-octane hydrocarbon fuel additives, may be important to the competitiveness of the process. Lignin can replace phenol in the widely used phenol formaldehyde resins. Both production costs and market value of these products are complex. In corn based ethanol plants the stillage (20% protein) is very valuable as animal feed. In this study all residual solids (lignin, residual holocellulose compounds, and cell mass) are assumed to be deployed for production of heat and electricity.

The solids come available at 60% moisture and are dried (using steam) to 15%. To generate electricity and heat, at small scale (30 MWe) probably a boiler with steam turbine would be applied. At larger scale a gasifier combined cycle (higher efficiency) may become attractive.

Chapter 3
Materials and methods

3.1 Lignocellulosic material (Substrate)

The Kans grass (*Saccharum spontaneum*, a perennial grass, growing up to three meters in height with spreading rhizomatous roots that quickly colonize on disturbed soil and allowed it to become an invasive species over croplands, pasturelands as well as waste land) used in present study was obtained from various parts of Uttrakhand (India) (Fig 3.1). It was chopped in to small pieces of about up to 0.5-1.0 cm, washed thoroughly and dried at 60°C over night and stored at room temperature (Fig 3.2 (a) and (b)). All the steps involved in bioethanol production in present study are outlined in Fig 3.3.

Fig. 3.1 Kans grass (*Saccharum spontaneum*)

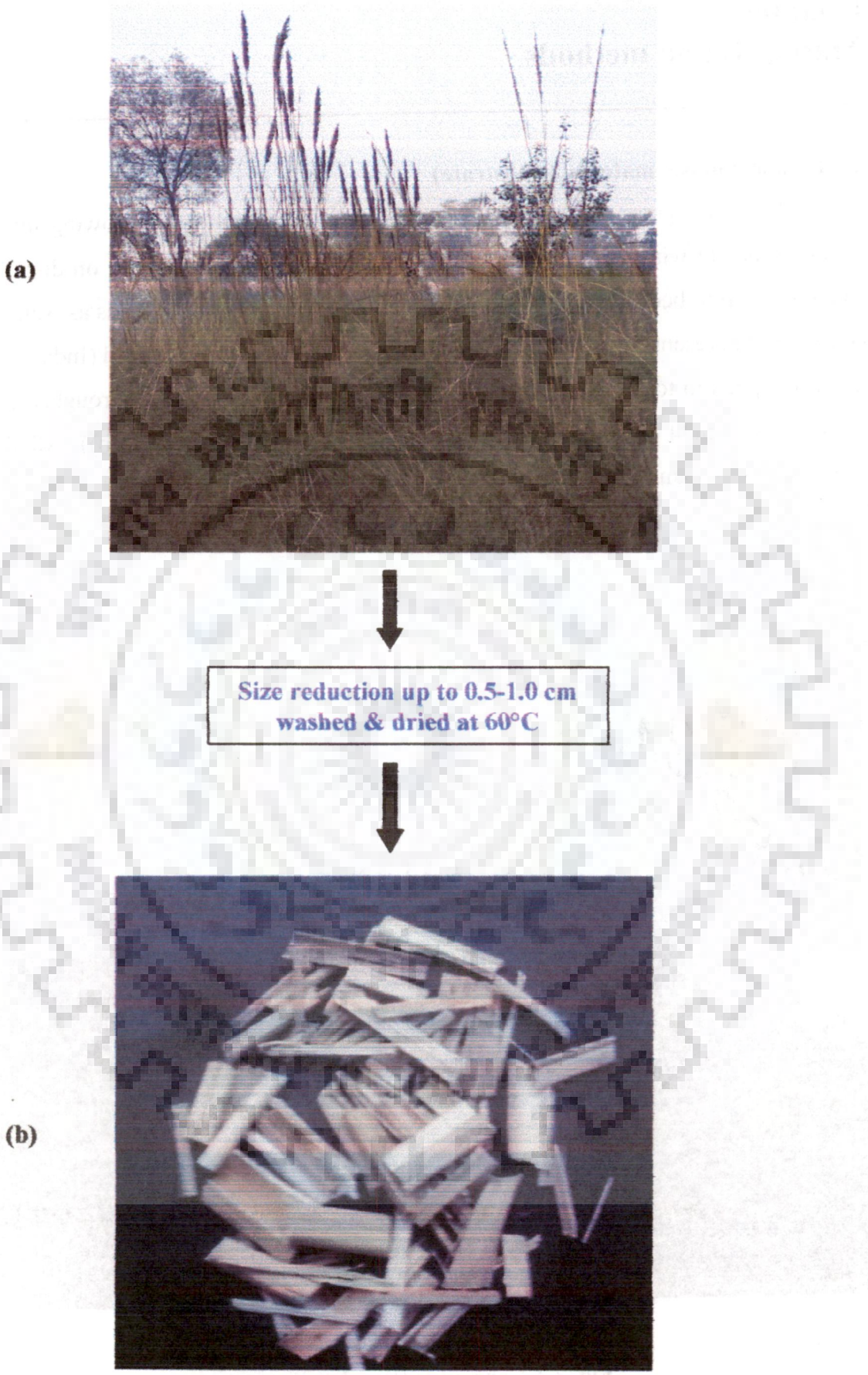


Fig. 3.2 Size reduction of Kans grass **(a)** Kans grass **(b)** Kans grass after washing and size reduction

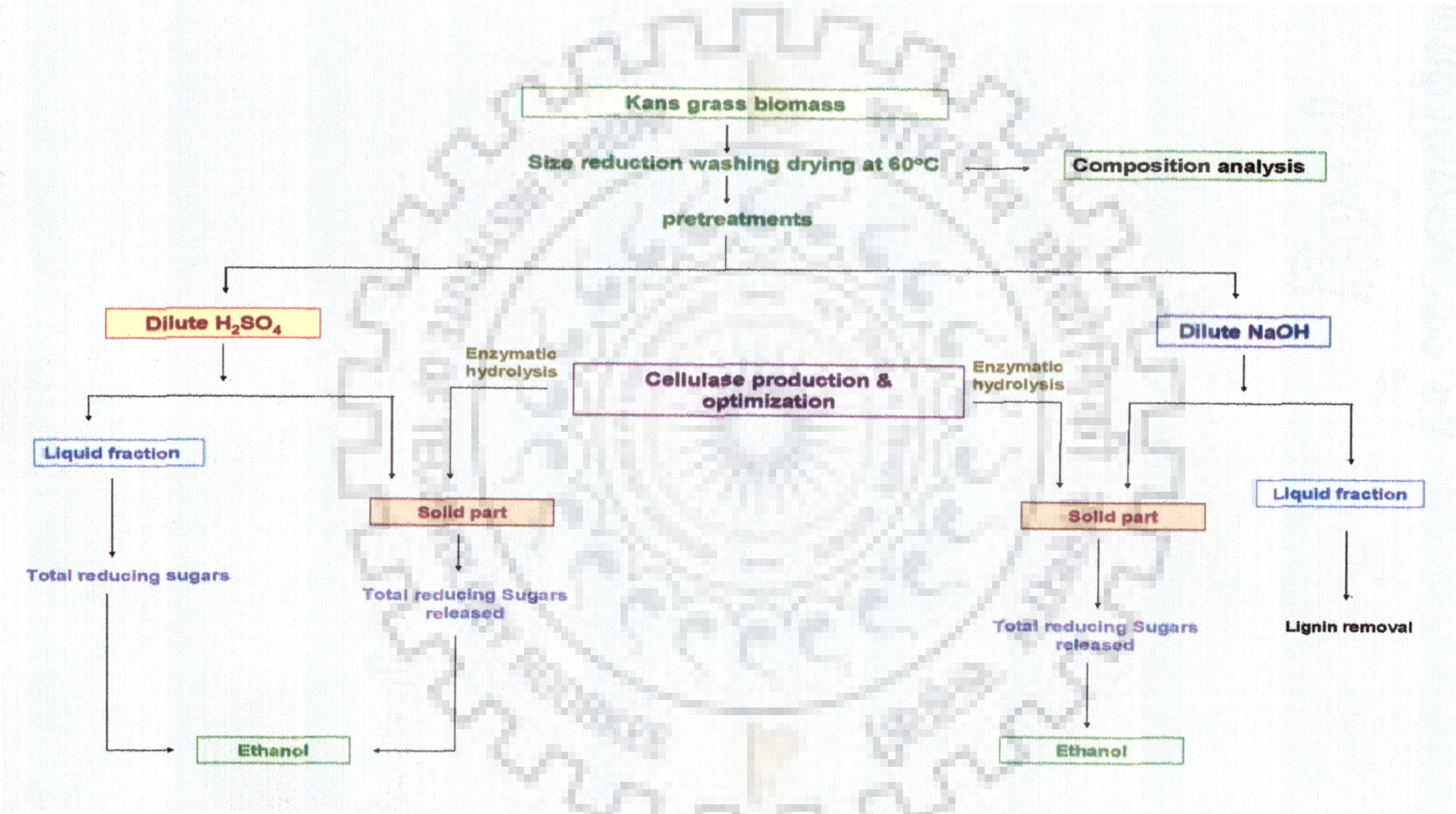


Fig. 3.3 The overall scheme of Bioethanol production from Kans grass biomass

3.2.3 Acid insoluble lignin estimation

Acid insoluble lignin (AIL) and acid soluble lignin (ASL) in biomass were measured according to the NREL [261]. For the analysis approximately 0.3 g moisture-free switch grass biomass was weighed and placed in 250 ml conical flask in triplicate. Three millilitres of 72% (v/v) sulphuric acid was added to each conical flask and the acid-soaked biomass was incubated for 1 h in water bath at 30°C. The mixture was stirred at the beginning and occasionally during the incubation process to ensure complete contact between acid and biomass particles. After completion of incubation, conical flasks were removed from the water bath and acid was diluted by adding 84 ml deionised water to reduce the concentration to 4%. All conical flasks were sealed and autoclaved for 1 h at 121°C/15 psi. Samples, allowed to cool to room temperature after autoclaving and were then filtered. The filtrate was saved separately in tubes at 4°C for acid soluble lignin. Solids residue was rinsed with deionised water and dried in a muffin furnance at 105°C for 16 h. The crucibles were transferred to the desiccator, weighed and placed in the furnace at 500°C for 24 h. The total weight of crucibles together with the solid residue ash was measured. Acid insoluble lignin was calculated using Equation:

$$\text{Percent Acid Insoluble Lignin} = \frac{W1 - W2}{W} \times 100$$

Where, W = weight of the moisture free sample determined by multiplying 40°C oven- dried sample weight (g) by '% total solids'

W1 = total weight of crucible and the solid residue after drying at 105°C in the oven (g);

W2 = total weight of crucible and ash after burning at 500°C in the furnace

3.2.4 Acid soluble lignin estimation

The filtrate saved during acid insoluble lignin analysis was used for acid soluble lignin measurement in a quartz cuvette with a spectrophotometer (Carry UV- Vis, Varians spectrophotometer). The wavelength was set at 205 nm [262] and the filtrate was diluted 10-fold to bring the absorbance between 0.1 and 1.0. Four percent sulphuric acid was also diluted 10-fold and used as blank in spectrophotometer measurement. Acid soluble lignin was calculated as follows:

$$\text{Percent Acid Soluble Lignin} = \frac{\text{UV abs} \times \text{FiltrateVolume} \times \text{Dilution} \times \frac{1L}{1000ml}}{W} \times 100$$

Where ,

$UV_{abs} = \frac{A}{b \times a}$, A = absorbance at 205 nm

b = cell path length (1 cm),
a = absorptivity which equals 110 L/(g·cm)

Filtrate volume = 87 ml

Dilution = 10

W = weight of the moisture free sample for the grass (g).

3.2.5 Total lignin

Total lignin was estimated as the summation of the acid soluble and acid insoluble lignin content and presented as Lignin content dry wt basis.

3.2.6 Holocellulose content determination

To 2.5 g of sample, 80 ml of hot distilled water was added with addition of 0.5 ml acetic acid, and 1 g of sodium chlorite in a 250 ml Erlenmeyer flask [263]. The mixture was heated on a water bath at 70°C. After 60 min, 0.5 ml of acetic acid and 1 g of sodium chlorite were added. After each succeeding hour, fresh portions of 0.5 ml acetic acid and 1 g sodium chlorite were added with shaking. Addition of 0.5 ml acetic acid and 1 g of sodium chlorite is repeated four times and the samples were left without further addition of acetic acid and sodium chlorite in the water bath overnight. After 24 hours of reaction samples were cooled and filtered the holocellulose on filter paper using a Buchner funnel to remove the yellow colour and the odour of chlorine dioxide was removed. Further the residue was washed with acetone and dried at 105°C for 24 hours, placed in a desiccator for an hour and weighed.

3.2.7 Hemicellulose and cellulose content estimation

Hemicellulose was estimated as per the method describe by Han [263]. Approximately 2 g of vacuum-oven dried holocellulose was weighted and place into a 250-mL glass beaker with a glass cover. 25 mL of 17.5% NaOH solution was prepared in a a graduated cylinder and maintain at 20°C. 10 mL of 17.5% NaOH solution was added to the holocellulose in the 250-mL beaker, covered with a watch glass, and maintain at 20°C. Holocellulose was mixed lightly with a glass rod with the flat end and After 2 minutes, manipulated the specimen with the glass rod and stirred until the particles were separated from one another. After the addition of the first portion of 17.5% NaOH solution to the specimen, 5 ml of NaOH solution was added at 5 minutes intervals for three times and

allowed the mixture to stand at 20°C for 30 min, making the total time for NaOH treatment 45 min. 33 ml of distilled water was added at 20°C to the mixture and allowed to stand at 20°C for 1 hour before filtering, transferred the entire holocellulose residue to the crucible, and washed with 100 ml of 8.3% NaOH solution at 20°C. After the NaOH wash solution has passed through the residue in the crucible, the washing was continue at 20°C with distilled water, till it is ensured that every thing has been transferred from the 250-ml beaker to the crucible. Poured 15ml of 10% acetic acid (at room temperature) to make neutral pH and finally the sample was washed by 250 mL of distilled water. The sample was placed in an oven to dry at 105°C. Cellulose content was estimated as difference between holocellulose and hemicellulose contents.

$$\alpha - cellulose(\%) = \frac{W2}{W1} \times 100$$

W2 = weight of the oven-dry hemi cellulose residue

W1 = weight of the original oven-dry holocellulose sample

3.3 Dilute H_2SO_4 pretreatment

Dilute acid (H_2SO_4) pretreatment of Kans grass biomass (5% w/v) was done by treated the biomass with different sulphuric acid concentrations (0.5, 1, 1.5 and 2% w/v) for variable reaction time (30, 60, 90 and 120 minutes) at different temperatures (100,110 and 120°C) in an autoclave with biomass size 0.5-1.0 cm. After pretreatment, solid residue obtained was separated from the liquid portion. The liquid fraction (acid hydrolysate) was kept at -20°C for the analysis of total reducing sugars, xylose, arabinose, glucose, furfural and acetic acid concentration. However, the collected solids were washed with distilled water to obtained neutral pH, dried at 60°C. These Portions of the solid residues were used for determination of total residual solid, holocellulose and lignin content. The reduction in lignin following pretreatment was calculated based on the initial dry-weight of lignin in the untreated sample and the dry-weight of lignin in the remaining solids after pretreatment. All the experiments performed in three sets of independent experiments.

3.4 Dilute NaOH pretreatment

Dilute alkali (NaOH) pretreatment of Kans grass biomass (5% w/v) was done by treated the biomass (0.5-1.0 cm) with different sodium hydroxide concentrations (0.5, 1, 1.5 and 2% w/v) for variable reaction time (30, 60, 90 and 120 minutes) at different

temperatures (100,110 and 120°C) in an autoclave. After pretreatment, solid residue obtained was separated from the liquid portion. The liquid fraction (hydrolysate) was kept at -20°C for the analysis of total reducing sugars, xylose. However, the collected solids were washed with distilled water to obtained neutral pH, dried at 60°C/. These Portions of the solid residues were used for determination of total residual solid, holocellulose and lignin content. The reduction in lignin following pretreatment was calculated based on the initial dry-weight of lignin in the untreated sample and the dry-weight of lignin in the remaining solids after pretreatment. All experiment were carried out in triplicates.

3.5 Estimation of toxic compounds (furfural and acetic acid)

Furfural determination was performed using molybdic acid complex method. added 5 ml of molybdic acid solution (250 mg in 5 ml of 10% NaOH + water to 100 ml) was added to 5 ml of the sample solution, shaked and kept in a boiling water bath for 1 h, after cooling transferred to a volumetric flask and make up to 25ml with water. The absorbance was measured against a reagent blank at 331 nm. A parallel control experiment with out molybdic acid solution addition (used distilled water) was also performed with the samples and difference between the working and this control absorbance was used in the calculation. A calibration curve was prepared and used to estimate the furfural concentration in the sample. This method of estimation of furfural was used by Shukla and Sharma [264]. However, Acetic acid was analyzed by GC (capillary column) at column temperature 100°C for 1 min and then enhanced to 180°C with a rate of 10°C/min. The injector and detector temperatures were set at 210°C with carrier gas as N_2 with a flow rate of 1ml/min.

3.6 Detoxification of acid hydrolysate

Acid-hydrolysate (obtained after acid pretreatment of biomass) may have several toxic compound which may inhibit the microbial growth during fermentation so detoxification of these compounds was performed that lowers the concentration of toxic compounds. The acid-hydrolysate was preheated to a temperature of 42°C and then the pH was adjusted to 10-10.5 by the addition of $Ca(OH)_2$. Temperature was increased to 50-52°C due to addition of lime, that temperature was maintained for 30 minutes. This process brings about detoxification by precipitating toxic compounds and also destabilise some inhibitors [206]. Insoluble residues were removed by filtration and centrifugation to get clear supernatant for further use after adjusting the pH to 5 by 10 N H_2SO_4. Estimation of inhibitors, furfural and acetic were done without detoxification (only neutralization of hydrolysate by adjusting pH between 5 to 5.5 by addition of NaOH and after detoxification.

3.7 *Trichoderma reesei* and maintenance

Fungal strain *Trichoderma reesei* NCIM 1052 was procured from NCIM Pune and was used as source of cellulase enzyme production, grown and maintained on potato dextrose agar (PDA) at 30°C for one week. The culture plate with spores was washed with autoclaved distilled water and the suspension form was further used as seed culture after the spore counting.

3.8 Cellulase production media

The composition of basal medium for production of cellulase [266] is as follows (g/l): KH_2PO_4, 2.0; Urea, 0.3; $MgSO_4.7H_2O$, 0.3; $CaCl_2.2H_2O$, 0.3 and mineral salt solution (mg/l): $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2.6H_2O$, 2.0. In addition peptone (0.1%), Tween 80 (polyoxyethylene sorbitan monoleate) 0.1% and 10g/l, carbon sources (cellulose, glucose, lactose and xylose) was added to the medium to induce cellulase production at different pH (4, 5 and 6) and temperatures (25, 28 and 30°C). The media was seeded with a suspension of *T. reesei* spores ($2x10^9$spores/ml) and submerged culture was incubated at 200 rpm at different temperatures. 5ml samples were taken out periodically centrifuged at 10,000 rpm for 15 minutes and supernatant was stored at -20°C for further analysis of enzyme activities (CMCase, FPA and xylanase), total protein and sugars consumption. The pallet was used to estimate dry cell biomass. Three sets of experiments were performed.

3.9 Enzymatic saccharification of acid pretreated Kans grass biomass

The culture supernatant with optimum CMCase activity obtained from *T. reesei* was used as source of cellulases enzyme mixture for the hydrolysis of the substrate (untreated Kans grass or acid pretreated). The acid pretreated Kans grass biomass (0.5-1cm particle size) obtained after optimized condition (2% v/v H_2SO_4, 120°C and 90 minutes reaction time) for maximum total reducing sugar removal which mainly composed of hemicellulosic portion was utilized in present study for enzymatic saccharification. The 250 ml conical flasks containing citrate buffer (0.05M, pH 4.8) with different biomass loading of pretreated or untreated Kans grass (1, 2, 2.5,5 and 6% (w/v)) were autoclaved and 20 FPU/g dry biomass (gdb) of filtered sterilized crude *T. reesei* cellulases was added aseptically after autoclaving and cooling to room temperature. The flasks were incubated at 50°C for 96 hours, at 200rpm. Samples collected at 12 hours of interval and analyzed for total reducing sugars (TRS) liberated. The pure commercial cellulase enzyme (Sigma

Aldrich, Germany) was used as control in this study. The results shown are the mean of three independent experiments.

3.10 Enzymatic saccharification of alkali (NaOH) pretreated Kans grass biomass

Dilute NaOH pre-treatment was carried out with biomass (0.5-1.0 cm particle size) different NaOH concentrations, time and temperatures and the condition were optimized for maximum removal of lignin. At 120°C the maximum removal of lignin (14-79.30%) was observed with solubilization of sugars. As more the lignin was removed the solubilization of total reducing sugars was also observed thereby the reduction of holocellulose content of the biomass. Hence the solid residue remained (NaOH concentration and pretreatment duration) after pretreatment were washed with distilled water and dried at 60°C over night and used for enzymatic saccharification.

The 250 ml conical flasks containing citrate buffer (0.05 M, pH 4.8) with different biomass loading of NaOH pretreated Kans grass (2, 4, 5 and 6% (w/v)) were autoclaved and cooled and then 20 FPU/g dry biomass (gdb) of filtered sterilized crude *T. reesei* cellulases was added aseptically. The flasks were incubated at 50°C for 96 hours, at 200rpm. Samples collected at 12 hours interval and analyzed for total reducing sugars (TRS) liberated. The results shown are the mean of three independent experiments.

3.11 Estimation of cellulases (Endoglucanase or CMCase, FPA and xylanase) activity

The supernatant of *T. reesei* production media obtained after regular intervals of time was analyzed for endoglucanase, cellulase and xylanase activity. Endoglucanase activity (CMCase) activity was estimated using the method described by Xiao [267], whereas Xylanase activity was measured using oat spelt xylan as substrate and method described by Bailey [268]. Cellulase activity (measured as filter paper hydrolyzing activity, using a 1x6 cm strip of Whatman no. 1 filter paper) was assayed according to the method recommended by Ghosh [269]. One unit of enzyme (CMC, FPU and xylanase) is defined as one micro mol production of glucose/xylose per ml per minute. All colorimetric observations were recorded using UV–Vis Spectrophotometer. The total reducing sugars were analysed by DNS method [270].

3.12 Estimation of total protein

Protein estimation was done by Bradford method using BSA as standard protein [271].

3.13 Sugars estimation

Total reducing sugars were estimated by DNS method [270], xylose estimation was done by phloroglucinol assay [272] however analysis of glucose was done by oxidase–peroxides method [273]. Arabinose was analyzed by HPLC (verians HPLC system) on RI detector, with mobile phase as 0.05mM H_2SO_4 at 0.8ml/min flow rate and calculated from corresponding standard.

3.14 Cell mass estimation of *T. reesei*

Dry cell mass of *T. reesei* was estimated by taking 40 ml sample at 24 hours interval the sample was centrifuged (15,000 rpm for 20 minutes) and the pallet was dried overnight at 60°C using the pre-weighted Filter paper and the cell mass was estimated as the difference between the filter paper with fungal biomass to that of blank paper. Supernatant was stored at -20°C for further analysis.

3.15 Maintenance and adaptation of *P. stipitis* to acid hydrolysate and fermentability test

Pichia stipitis strain (NCIM 3497), obtained from NCIM, Pune, India, was grown at 30°C and maintain at 4°C on MGYP agar plates containing (g/l): glucose, 10; yeast extract, 30; peptone, 5; agar 20 (pH 5.0). An adaptation for *P. stipitis* (wild strain) was done by sequentially transferring and growing the cell in the media containing increase concentration of non detoxified acid hydrolysate (20, 40, 60 and 80% v/v) obtained after optimization of acid pretreatment. The hydrolysate was supplemented with (g/l); xylose 50; yeast extract 5; protease peptone 10 (pH 5.0) and allowed to grow at 30°C at 150 rpm. An 'Acid adaptive strain' was identified, that tolerated up to 60% of acid hydrolysate concentration and no growth was observed above 60% of acid hydrolysate. This adaptive yeast strain was maintained at agar plates containing (g/l); xylose 30; yeast extract, 15; peptone and agar, 2 in 50% v/v hydrolysate. The resulted strain was sub cultured to ensure the stability of tolerance of hydrolysate and was monitored through estimation of cell biomass and ethanol concentration. The comparative study between wild and 'adaptive' strain was done by cultivating both the strains in detoxified (treatment with $Ca(OH)_2$ mentioned in chapter 3) as well as neutralised (only adjustment of pH to 5) acid hydrolysate media and estimation of ethanol, cell biomass and sugars consumption were done.

The fermentation media consists of the following composition: (g/l): yeast extract, 1; $(NH_4)HPO_4$, 2; $(NH_4)SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.25; and trace element solution 1 ml per litre. The trace element solution contained (g/l): $CuSO_4 \cdot H_2O$, 2.5; $FeCl_3 \cdot 6H_2O$, 2.7;

$MnSO_4 \cdot H_2O$, 1.7; $Na_2Mo_2O_4 \cdot 2H_2O$, 2.42; $ZnSO_4 \cdot 7H_2O$, 2.87; $CaCl_2 \cdot 6H_2O$, 2.4 and concentrated sulphuric acid (18N), 0.5 ml. Medium pH was adjusted to 5.0 [274]. Fermentation was carried out in conical flasks with 100 ml working media (DAH/neutralised hydrolysate and all above mentioned components) on a orbital shaker (150 rpm) with addition of 5% (v/v) of mid exponential phase (16 h) inoculum (wild /adaptive) at a temperature of 30°C, the initial pH was adjusted to 5.0. Aliquots of 5 ml were withdrawn periodically for the estimation of cell mass, ethanol and residual sugars in fermentation broth. All the experiments were performed in triplicates.

3.16 Maintenance and cultivation of Saccharomyces *cerevisiae*

The yeast strain *Saccharomyces cerevisiae* (MTCC 170), procured from MTCC, IMTECH, Chandigarh India, was grown at 30°C and kept at 4°C on MGYP agar plates containing ($g.L^{-1}$) glucose, 10; yeast extract, 3;malt extract,3; peptone, 5; agar 20 (pH 5.0). Fermentation was carried out with sugar hydrolysate by supplementation of other essential medium components [275] (g/l) yeast extract, 6.0; $CaCl_2.2H_2O$, 0.3; $(NH_4)2SO_4$, 4.0; $MgSO_4.7H_2O$, 1.0 and KH_2PO_4, 1.5 (pH 5.5) in conical flasks with 100 ml working media on a shaker (150 rpm) with addition of 5% (v/v) of mid exponential phase (12 h) inoculum at a temperature of 30°C, the initial pH was adjusted to 5.0. A parallel control experiment was also carried out by using additive synthetic sugars with all other media components. All experiments were performed in triplicates.

3.17 Production of ethanol from concentrated detoxified Acid hydrolysate (DAH) obtained after acid pretreatment

Kans grass biomass was pretreated with dilute H_2SO_4 (optimised condition) to obtained the maximum soluble sugars and after detoxification the hydrolysate was utilised for the ethanol production. The detoxified hydrolysate composed of 11.66 g/l of total reducing sugars. The sugars concentration of the detoxified acid hydrolysate (DAH) was increased to 60 and 90.5 g/l by lyophilization and fermentation experiments were carried out for ethanol production with *P. stipitis* (adaptive strain) in production media. A parallel experiment in media with synthetic sugar in place of DAH was also carried out. 5ml of sample were withdrawn at regular intervals of time for the quantification of ethanol, cell biomass and total reducing sugars. All the experiments were carried out in triplicates and the results shown are the mean of the three independent experiments with the corresponding standard deviation.

3.18 Ethanol production from hydrolysate obtained after enzymatic hydrolysis of acid (H_2SO_4) pretreated Kans grass

The solid residue remained after dilute H_2SO_4 pretreatment was hydrolysed with the crude enzyme and resulted soluble sugar was utilised for ethanol production by both *P. stipitis* and *S. cerevisiae* separately. The initial sugars concentration was adjusted to 10 g/l and also a control experiment was carried out with 10 g/l of pure glucose as carbon sources. 5ml of sample were withdrawn at regular intervals of time for estimation of ethanol concentration, cell biomass and total reducing sugars. All the experiments were performed in triplicates.

3.19 Ethanol production from hydrolysate obtained after enzymatic hydrolysis of NaOH pretreated Kans grass biomass

NaOH pretreatment condition were optimised for maximum removal of lignin from Kans grass biomass and again for the maximum released of the soluble sugars after enzymatic (crude) hydrolysis from NaOH pretreated solid residue were recovered. The sugar hydrolysate was further used for the ethanol production by using *P. stipitis* and *S. cerevisiae* respectively.

3.20 Biomass estimation for *P. stipitis* and *S. cerevceai*

To estimate dry cell biomass of the yeasts, a calibration curve between optical density and biomass concentration was prepared. The optical density of the samples was measured at 600 nm and dry cell biomass was further calculated from calibration curve.

3.21 Ethanol determination

Analysis of ethanol was carried out by using a capillary gas chromatograph (DANI GC) coupled with a flame ionization detector on a solgel wax column. The column temperature was initially adjusted at 100°C for 2 minutes; it was increased to 180°C for 1 minute at a rate of 10°C/min and was further increased to 220°C with a rate of 5°C/min for 5 minutes. The injection and detector temperatures were set at 210°C. The carrier gas (N_2) flow-rate was kept at 1 ml/min whereas auxiliary and hydrogen gas flow rates were maintained at 350 and 30 ml/min respectively.

3.22 SEM analysis

Untreated as well as pretreated Kans grass samples were scanned under SEM (model no. LEO 435VF, England) for the morphological structure analysis.

Chapter 4
Results and Discussion

4.1 Composition of Kans grass

Kans grass biomass which is known as one of the cultivar of switchgrass. The composition of Kans grass and other agricultural by products vary depending on the growing location, season, and harvesting methods. The composition of the Kans grass used in this study from Uttrakhand India is shown in Table 4.1. The holocellulose content was estimated to be 64.73% of dry wt. which composed of cellulose (43.68%) and hemicellulose (21.05%). The compositions of the other switch grass verities are given in Table 4.2 in which the holocellulose content is found to be in the range of 56.67-63.32% and thus it is evident from the Table 4.2 that the total carbohydrate content (holocellulose) is found to be highest among all other varieties of switch grass studied by different researcher. Moreover, the carbohydrate content of Kans grass is fairly comparable with other lignocellulosics like sugarcane baggase (67.15%), corn stover (58.29%), wheat straw (54%), and sorghum straw (61%) [277], and widely accepted switch grass (52.9%) [278]. Hence, total carbohydrate content (64.73%w/w) makes Kans grass as the potential biomass source for the production of ethanol. Glucan and xylan, with a ratio of about 3:2, are two of the most abundant polysaccharides synthesized from glucose and xylose respectively and in switch grass samples contribute to approximately 40–50% of the dry matter.

The moisture and ash content of Kans grass biomass were calculated to be 4.70 ± 0.055 and 2.10 ± 0.2% while lignin content was estimated to be 25.15 ± 0.55% (Table 4.1). Lignin is the main component of plant structure that provides strength by stiffening and holding the fibers of polysaccharides hence it acts as a sealing agents between cellulose and hemicellulose. In comparison to other switch grass varieties, [279] Kans grass has higher lignin content but due to its cultivation on waste land, low cultivation cost, less need of water, no extra pressure on cultivation land and availability of biomass throughout the year are the reasons to make it to utilise as sugars source [15]. The other components were found to be 3.32% which may contain different extractives such as proteins or fatty acids.

Table 4.1 Composition of Kans grass

Component	% dry weights basis
Holocellulose	64.73 ± 0.55
Cellulose	43.68±0.54
Hemicellulose	21.05±0.46
Lignin	25.15 ± 0.55
Moisture	4.70 ± 0.055
Ash	2.10 ± 0.2
Others	3.32± 0.25

Table 4.2 Composition (% dry basis) of different switchgrass varieties

Switchgrass varieties	Holocellulose	Cellulose	Hemicellulose	Lignin
Alamo (whole plant)	59.59	33.48	26.10	17.35
Blackwell (whole plant)	60.99	33.65	26.29	17.77
Trailblazer (whole plant)	58.30	32.06	26.24	18.14
Cave-in-Rock (whole plant)	59.07	32.11	26.96	17.47
Kanlow – leaves	56.70	31.66	25.04	17.29
Kanlow – stems	63.32	37.01	26.31	18.11
Kans grass (whole plant in Present study)	***64.73***	***43.68***	***21.05***	***25.15***

4.2 Dilute H_2SO_4 pretreatment

Kans grass biomass with 5% (w/v) biomass loading was supplied with different concentrations of H_2SO_4 (0.5, 1, 1.5 and 2% v/v) at different temperatures (100, 110 and 120°C) for various length of reaction time (30, 60, 90 and 120 minutes). After pretreatment the solid residue was separated from the liquid fraction, washed with distilled water and stored at room temperature after drying at 60°C overnight in an incubator for further compositional analysis. The liquid fraction was estimated for total reducing sugars (TRS), xylose, arabinose and glucose after detoxification (Fig. 4.1-4.4). Estimations of toxic compounds including furfural and acetic acid were also done before and after detoxification process and are shown in Table 4.3-4.5.

4.2.1 Effect of pretreatment on sugars solubilization

Dilute acid pretreatment is one of the most effective methods for lignocellulosic biomass pretreatment and has been investigated extensively in last decade. High temperature and acid concentration result in hydrolysis of hemicellulosic components into monomer sugars as well as exposure of cellulose for enzymatic digestibility. The hydrolysis at temperature range between 120°C-200°C with dil. HCl or H_2SO_4 was also studied by different authors [280, 281, 282]. However, this involves higher energy cost and release of toxic compounds along with soluble sugars. Hence, in the present study, the liberation of total reducing sugars was tried to achieve comparably at a lower temperature as well as acid concentration that make this process economically feasible.

After acid pretreatment, total reducing sugars concentration in liquid residue was found to be in the range of 2.60-8.15 g/l when pretreatment was performed at 100°C with variable concentrations of H_2SO_4 (0.5, 1.0, 1.5 and 2.5% v/v) and for different reaction time or pretreatment durations (30, 60, 90 and 120 minutes). However as the pretreatment temperature was increased to 110 and 120°C the total reducing sugars concentration in the liquid residue increased and found to be in the range of 7.0-10.32 and 7.23-11.66g/l respectively (Fig. 4.1).

Xylose, which is the major constituent of hemicellulose, was the major sugar component that released in higher concentrations in comparison to all other sugars released and was found to be in the range of 1.68-5.24 g/l at all pretreatment conditions (Fig. 4.2). The arabinose released (Fig. 4.3) for all pretreatment conditions was found to be in range of 0.33- 3.53 g/l. Glucose was released in least concentration and was in the range of 0.01-0.94 g/l for all the pretreatment condition (Fig. 4.4).

In a study switch grass was pretreated at 180°C with 0.9% sulphuric acid and obtained approximately 90% solubilization of xylan within the first minute of the reaction [283]. But high temperature cause higher of energy input. Moreover, other studies on dilute acid pretreatment have shown that hemicellulose in the lignocellulosic feedstock can be completely solubalize at very intense conditions. It has been demonstrated that hemicellulose was the only component in plant fibre that could be thoroughly removed by dilute acid pretreatment [284, 285]. A greater level of hemicellulose solubilization results in enhanced digestibility of cellulose in residual solids which is essential for the increase in enzymatic conversion efficiency [62].

Hence, temperature seems to be an important factor in acid pretreatment as the temperature of the pretreatment was increased the total reducing sugars concentration was also enhanced. Moderate temperature, less than 160°C, has been observed to be the best for the hemicellulose hydrolysis, however, temperature more than 160°C favours depolymerization of the cellulose to glucose as well as lignin decomposing products (toxic compounds) including furfural and acetic acid [286]. These inhibitory compounds may hinder the ethanol production as they affect the metabolism of microorganism that causes the adverse effect on the microbial growth as well as ethanol production. Sulphuric acid at concentrations usually below 4% v/v has been studied for the dilute acid pretreatments as it is inexpensive and effective [287]. Depending on the substrate and the conditions used, 70-95% of the hemicellulosic sugars can be recovered by dilute acid pretreatment from the lignocellulosic material [288, 289, 290]. Cara *et al.* reported hemi-cellulose recovery up to 83% from olive tree biomass at 170 °C with 1% sulphuric acid concentration [290].

4.2.2 Production of toxic compounds and Detoxification of acid hydrolysate

Toxic compounds which generated after dilute acid pretreatments can be divided in four groups: sugar degradation products, lignin degradation products, compounds derived from lignocellulosic structure, and heavy metal ions. These toxic compounds can cause stress to fermentative organisms to a point beyond which the efficient utilization of sugars is reduced and product formation decreases. During hydrolysis, pentose sugars can degrade to furfural, a toxic compound that, depending on its concentration in the fermentation medium, can inhibit cells and affect the specific growth rate and cell biomass yield per ATP [275]. The results of estimation of furfural and acetic acids are mentioned in Table 4.3-4.5. When the temperature was 100°C the furfural and acetic acid concentration were found to be in range of 1.10-1.40 g/l and 0.12-0.75 g/l respectively (Table 4.3), further when temperature was increased to 110°C (furfural 1.6-2.34 g/l and acetic acid 0.2-1.7 g/l)

and 120°C (furfural 1.31-2.04 g/l and acetic acid 0.21-2.26g/l) the increase in toxic compounds concentration was also observed (Table 4.4 and 4.5). It was observed that at certain conditions of pre-treatment furfural concentrations goes above that limit (Table 4.4 and 4.5) that essentially needs to be detoxified before proceeding for fermentation.

When studying ethanol production by the yeast *P. stipitis*, Roberto et al. [291] observed that furfural concentrations lower than 0.5 g/l had a positive effect on cell growth, whereas concentrations above 2 g/l inhibited cell growth almost completely. Acid concentration and temperature in pre-treatment of lignocellulosic biomass are crucial factors for of toxic compounds formation (furfural or acetic acid) which produce due to sugars degradation. Acetic acid, another toxic compound produced during pre-treatment process and it's concentration of 5 g/l causes a complete inhibition on ethanol production as well as growth of *P. stipitis* [291].

The toxic products (furfural and acetic acid) concentrations were minimized by the detoxification of acid hydrolysate by $Ca(OH)_2$ (overliming), the toxic compounds were precipitated and removed. The results of concentration of toxic compounds after detoxification process with corresponding hydrolysate before detoxification are listed in Table 4.3-4.5. The various pre-treatment process conditions that were applied in this study produced acetic acid and furfural in the range of 0.12-1.8 and 1.17-2.34 g/l respectively. Hence, these toxic compounds concentration was minimized by detoxification process which was further reduced to a range 0.05-0.87 (acetic acid) and 0.32-1.23g/l (furfural) (Table 4.3-4.5).

In similar study by adjusting the pH of sugarcane bagasses hemicellulosic hydrolysate first to 10 with $Ca(OH)_2$, and then to 6.5 with H_2SO_4, Roberto et al. obtained a partial removal of phenolic and other compounds [292].

4.2.3 Optimized condition for maximum depolymerization of total reducing sugars

Pre-treatment condition corresponding to 2% H_2SO_4 concentration, 120°C of temperature and the duration of 90 min (Fig. 4.1) was found to be the optimum condition that gives maximum total reducing sugar concentration (11.66 g/l) in the acid-hydrolysate. However xylose, arabinose and glucose concentration at this condition were estimated to be 5.13, 3.34 and 0.94 g/l respectively (Fig.4.2-4.4). The other sugars which may contain mannose and galactose were determined (at particular pretreatment condition) as the difference of all the above sugars from total reducing sugars and found to be 2.9 g/l (Table 4.6). As acid hydrolysate at this optimized condition composed of 2.26 g/l of furfural and 1.19 g/l of acetic acid so these compounds were minimized to 1.11g/l (furfural) and 0.33

g/l (acetic acid) after detoxification with $Ca(OH)_2$ (Table 4.5). This detoxified hydrolysate was further utilized for the ethanol production by pentose and hexose utilizing yeasts.

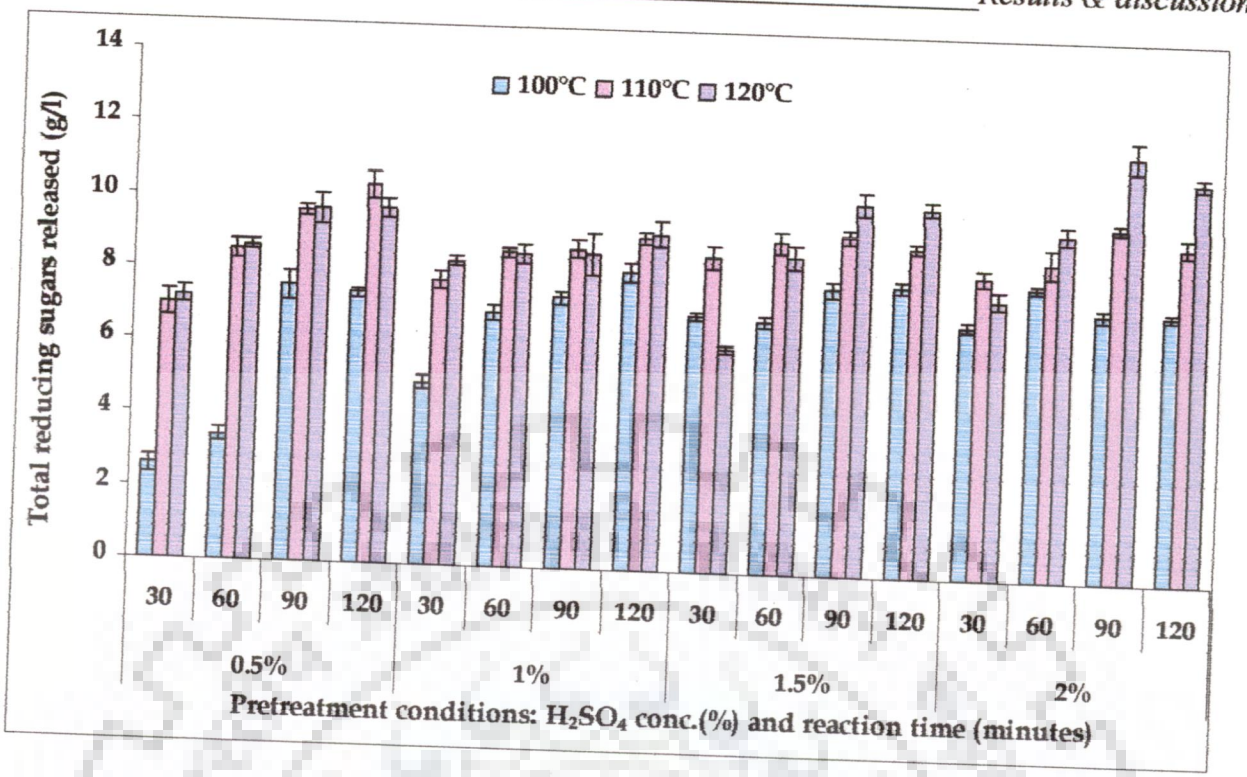


Fig. 4.1 Total reducing sugars formation (g/l) when 5% (w/v) biomass was pretreated with H_2SO_4 (0.5, 1, 1.5 and 2%) for different durations (30, 60, 90 and 120 minutes) at temperature (100,110 and 120°C)

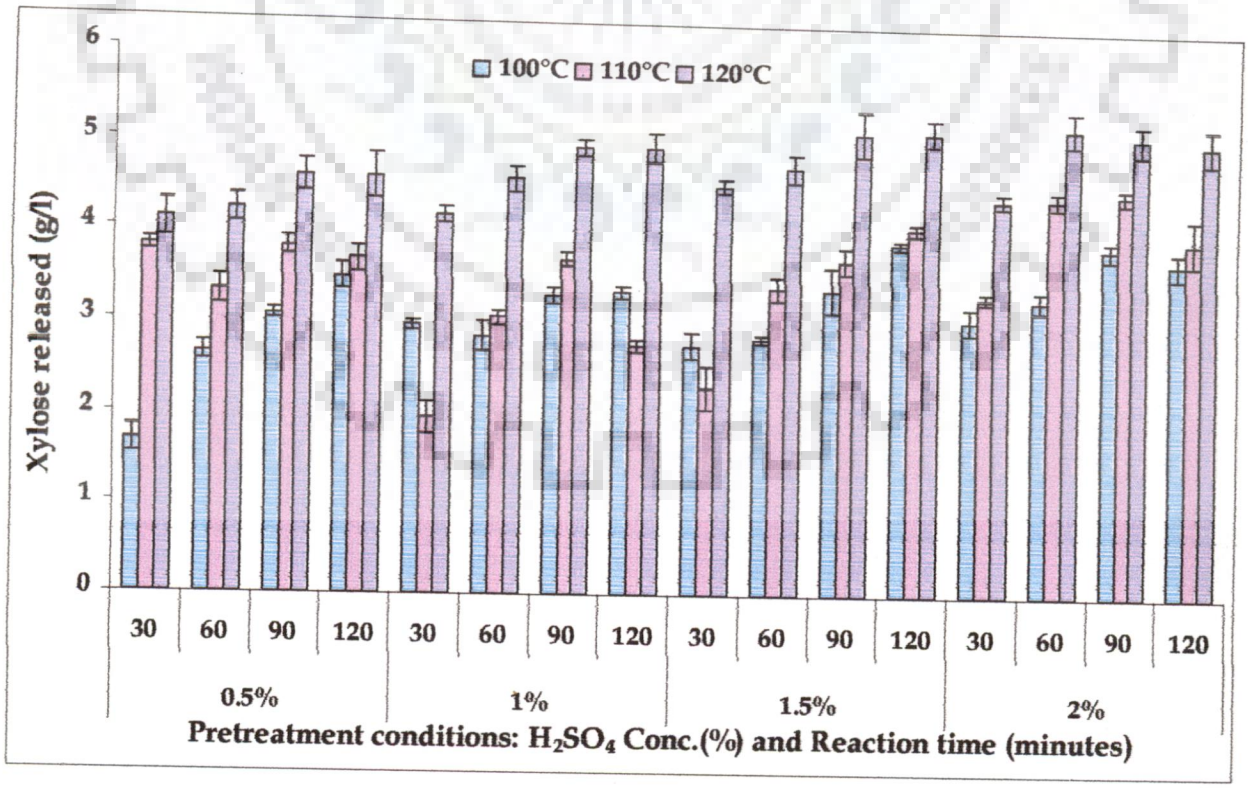


Fig. 4.2 Xylose formation (g/l) when 5% (w/v) biomass was pretreated with H_2SO_4 (0.5, 1, 1.5 and 2%) for different durations (30, 60, 90 and 120 minutes) at temperature (100,110 and 120°C)

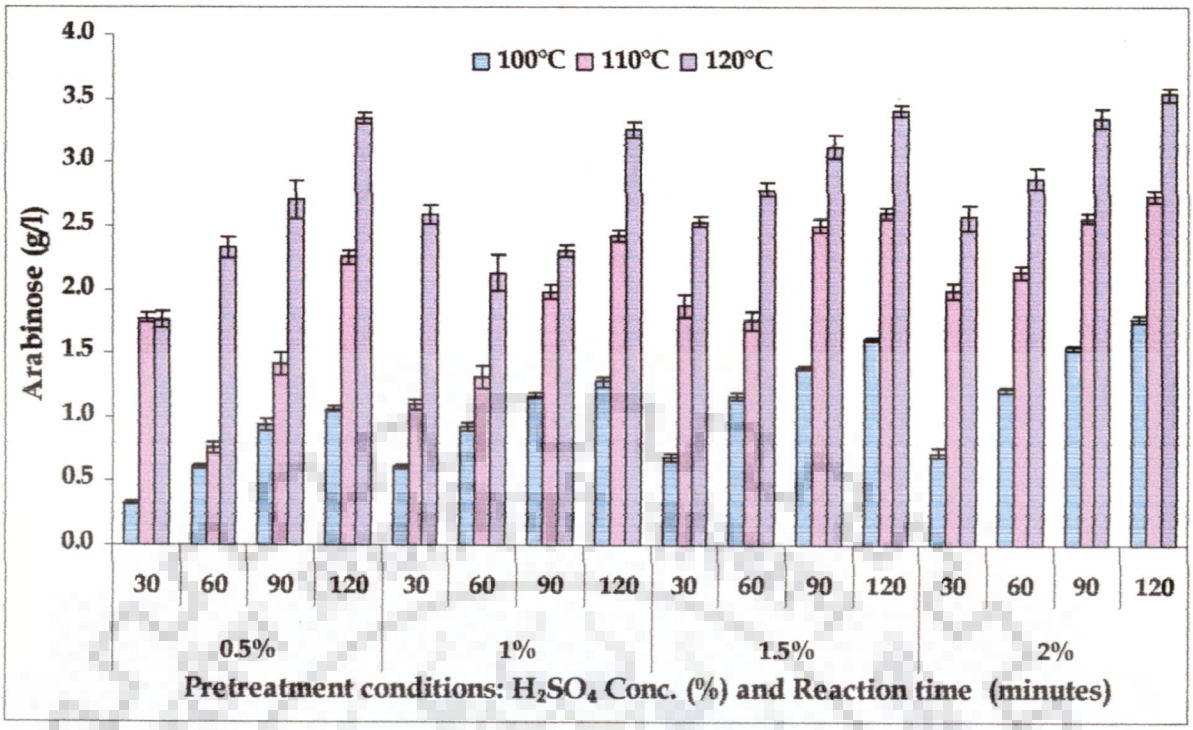


Fig. 4.3 Arabinose formation (g/l) when 5% (w/v) biomass was pretreated with H_2SO_4 (0.5, 1, 1.5 and 2%) for different durations (30, 60, 90 and 120 minutes) at temperature (100,110 and 120°C).

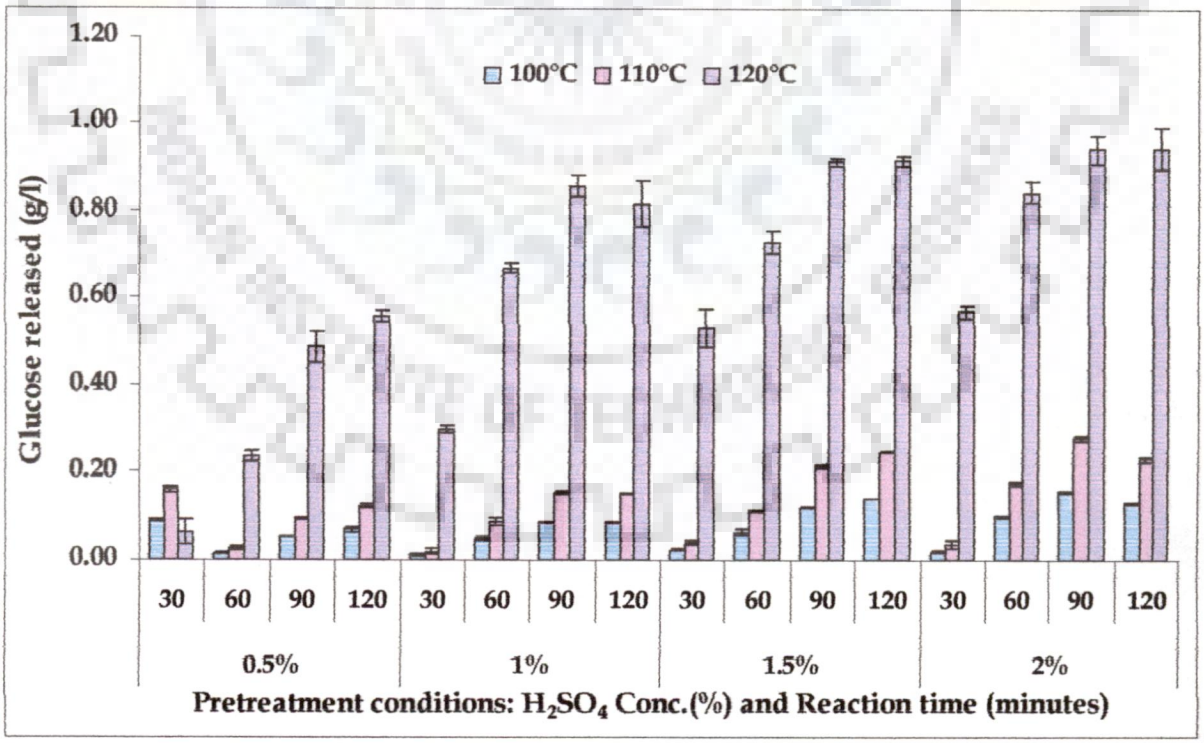


Fig. 4.4 Glucose formation (g/l) when 5% (w/v) biomass was pretreated with H_2SO_4 (0.5, 1, 1.5 and 2%) for different durations (30, 60, 90 and 120 minutes) at temperature (100,110 and 120°C).

Table 4.3 Inhibitors (Furfural and Acetic acid) concentrations in acid hydrolysate after neutralization and detoxification, produced after acid pretreatment at 100°C

Acid conc. & Time of pretreatment	**Furfural (g/l)**		**Acetic acid (g/l)**	
	Without Detoxi-fication	After Detoxi-fication	Without Detoxi-fication	After Detoxi-fication
0.5% 30	1.17 ±0.054	0.32 ±0.036	0.12 ±0.018	0.05 ±0.0012
0.5% 60	1.20 ±0.045	0.34 ±0.025	0.19 ±0.021	0.06 ±0.0032
0.5% 90	1.40 ±0.075	0.5 ±0.024	0.23 ±0.025	0.05 ±0.0021
0.5% 120	1.45 ±0.065	0.51 ±0.035	0.37 ±0.016	0.06 ±0.0034
1% 30	1.23 ±0.036	0.36 ±0.023	0.15 ±0.019	0.03 ±0.0026
1% 60	1.27 ±0.086	0.39 ±0.044	0.30 ±0.026	0.04 ± 0.0036
1% 90	1.33 ±0.058	0.50 ±0.023	0.75± 0.016	0.04 ± 0.0032
1% 120	1.34 ±0.079	0.52 ±0.046	0.72 ±0.023	0.02 ±0.0025
1.5% 30	1.22 ±0.048	0.36 ±0.045	0.33 ±0.013	0.05 ±0.0025
1.5% 60	1.23 ± 0.079	0.38 ±0.032	0.74 ±0.026	0.03 ±0.0046
1.5% 90	1.27 ±0.054	0.4 ±0.044	0.60 ±0.029	0.04 ±0.0029
1.5% 120	1.28 ±0.097	0.4 ±0.068	0.46 ±0.038	0.04 ±0.0025
2% 30	1.16 ±0.068	0.34 ±0.046	0.49 ±0.016	0.05 ±0.0027
2% 60	1.37 ±0.065	0.34 ±0.075	0.74 ±0.017	0.06 ±0.0037
2% 90	1.39 ±0.068	0.38 ±0.025	0.53 ±0.014	0.04 ±0.0028
2% 120	1.40 ±0.089	0.47.±033	0.15 ±0.024	0.04 0.0036

Table 4.4 Inhibitors (Furfural and Acetic acid) concentrations in acid hydrolysate after neutralization and detoxification, produced after acid pretreatment at 110°C

Acid conc. & Time of pretreatment	Furfural (g/l)		Acetic acid (g/l)	
	Without Detoxi-fication	After Detoxi fication	Without Detoxi -fication	After Detoxi -fication
0.5% 30	1.45 ±0.075	0.68 ±0.054	0.53 ±0.055	0.49 ±0.045
0.5% 60	1.64 ±0.067	0.78 ±0.025	1.67 ±0.098	0.78 ±0.057
0.5% 90	1.75 ±0.067	0.82 ±0.014	0.93±0.076	0.80±0.023
0.5% 120	1.88 ±0.076	0.84 ±0.043	1.38 ±0.027	0.98 ±0.087
1% 30	1.76 ±0.086	0.88 ±0.024	1.11 ±0.068	0.33 ±0.057
1% 60	1.81 ±0.065	0.94 ±0.013	1.23 ±0.045	1.14 ±0.074
1% 90	1.93 ±0.075	0.96 ±0.042	1.26 ±0.052	1.02 ±0.12
1% 120	2.04 ±0.057	1.03 ±0.023	1.35 ±0.046	0.95 ±0.054
1.5% 30	1.31 ±0.065	0.86 ±0.043	1.0 ±0.046	0.34 ±0.036
1.5% 60	1.73 ±0.046	0.84 ±0.014	1.41 ±0.076	0.9 ±0.068
1.5% 90	1.78 ±0.075	0.88 ±0.024	1.25 ±0.046	1.06 ±0.036
1.5% 120	2.02 ±0.096	0.96 ±0.034	1.37 ±0.056	1.22 ±0.068
2% 30	1.41 ±0.025	0.84 ±0.023	1.41 ±0.085	0.63 ±0.047
2% 60	1.9 ±0.057	0.86 ±0.044	1.25 ±0.033	0.81 ±0.075
2% 90	1.97 ±0.064	0.97 ±0.053	1.88 ±0.024	0.95± 0.036
2% 120	1.99 ±0.037	1.08 ±0.032	1.83 ±0.062	1.43 ±0.058

Table 4.5 Inhibitors (Furfural and Acetic acid) concentrations in acid hydrolysate after neutralization and detoxification, produced after acid pretreatment at 120°C

Acid conc. & Time of pretreat-ment	Furfural (g/l)		Acetic acid (g/l)	
	Without Detoxifi -cation	After Detoxifi- Cation	Without Detoxifi -cation	After Detoxifi- cation
0.5% 30	1.66 ±0.094	0.78 ±0.045	0.24 ±0.057	0.18 ±0.023
0.5% 60	1.88 ±0.084	0.89 ±0.068	0.28 ±0.047	0.22 ±0.024
0.5% 90	2.01 ±0.054	0.93 ±0.053	0.30 ±0.053	0.22 ±0.016
0.5% 120	2.15 ±0.083	0.96 ±0.087	0.61 ±0.094	0.19 ±0.045
1% 30	2.01 ±0.034	1.00 ±0.035	0.21 ±0.065	0.14 ±0.025
1% 60	2.07 ±0.097	1.07 ±0.026	0.42 ±0.046	0.19 ±0.078
1% 90	2.21 ±0.057	1.10 ±0.096	1.1 ±0.057	0.26 ±0.065
1% 120	2.34 ±0.074	1.18 ±0.046	0.58 ±0.084	0.40 ±0.056
1.5% 30	1.50 ±0.035	0.98 ±0.15	0.90 ±0.034	0.24 ±0.036
1.5% 60	1.98 ±0.068	0.96 ±0.043	1.75 ±0.078	0.42 ±0.064
1.5% 90	2.04 ±0.034	1.01 ±0.084	2.26 ±0.097	0.43 ±0.046
1.5% 120	2.31 ±0.086	1.10 ±0.024	1.43 ±0.040	0.41 ±0.024
2% 30	1.62 ±0.032	0.96 ±0.057	0.94 ±0.036	0.18 ±0.057
2% 60	2.18 ±0.057	0.99 ±0.078	1.198 ±0.076	0.22 ±0.036
2% 90	2.26 ±0.049	1.11 ±0.057	1.194 ±0.056	0.33 ±0.064
2% 120	2.27 ±0.083	1.23 ±0.052	0.95 ±0.057	0.32 ±0.037

4.2.4 Compositional and Configuration change in Kans grass biomass after dilute Acid pretreatment

The Kans grass biomass after acid pretreatment (2% v/v H_2SO_4, 90 minutes and 120°C) was analyzed for composition change (Table 4.7). There was removal of lignin (28.04% of total lignin) along with hemicellulose (77.06% of total hemicellulose) and cellulose (19.72% of total cellulose) was also observed on the dry wt. basis (Fig. 4.5). The compositional change in Kans grass shown in Fig. 4.5, and the physical change is shown in Fig. 4.6. The configurational changes were observed under SEM analysis (Fig. 4.7). Fig. 4.7 (a) showed the intact Kans grass structure whereas Fig. 4.7 (b) shows the structure after acid treatment under optimum condition. The untreated Kans grass biomass have smooth and continuous surface, whereas the dilute H_2SO_4 pretreated Kans grass biomass have a rough surface. This indicates the removal of external fibers that in turn increase surface area so that cellulose becomes more accessible to enzymes (cellulases). Similar structural changes were also earlier reported for Sugarcane Bagasse pretreated with formic acid [293], rice straw pretreatment with electron beam irradiation [294] and for rice straw pretreated with aqueous ammonia soaking pretreatment [295].

However it is important to note that lignin removal was not that significant (only 28.04% of total lignin was removed). The sugar released (mainly hemicellulose) was used for ethanol production.

Table 4.6 Composition of Sugars in dilute acid hydrolysate obtained after optimized condition

Component	Values in g/l
Total reducing sugars	11.66±0.32
Xylose	5.13±0.25
Arabinose	3.34±0.21
Glucose	0.94±0.089
Other fermentable sugars	2.19±0.17

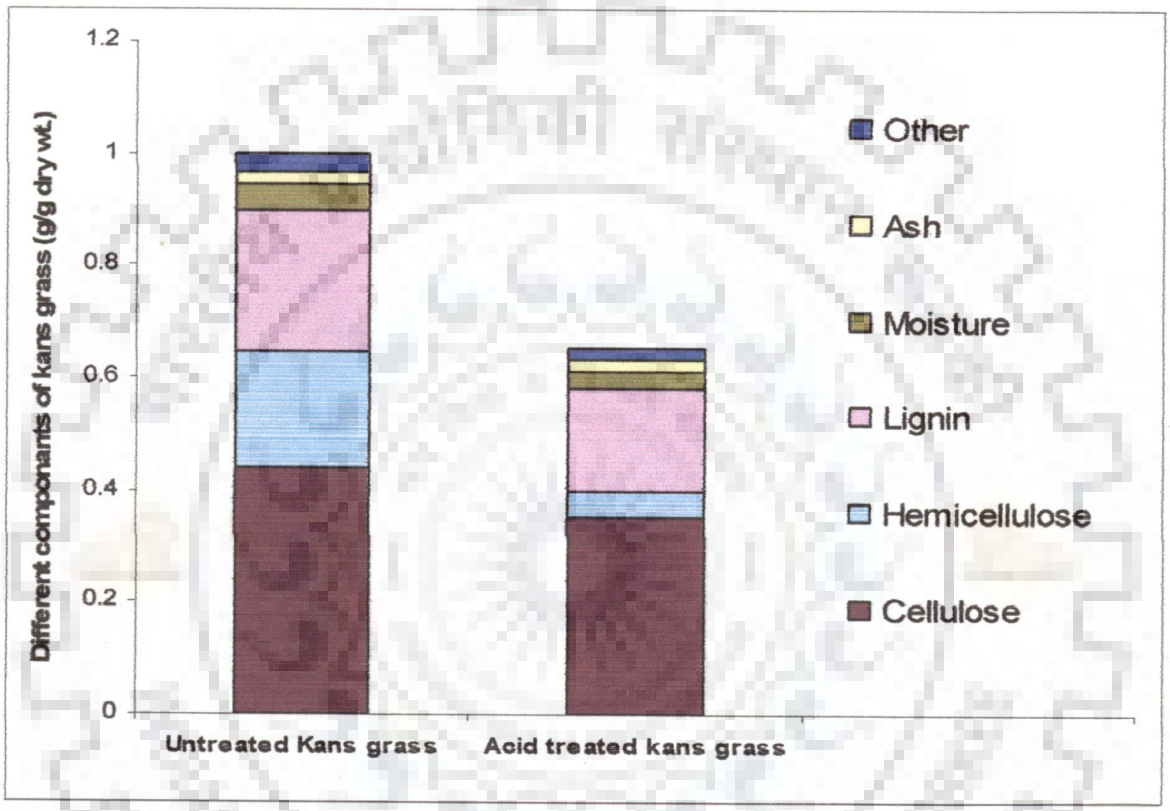
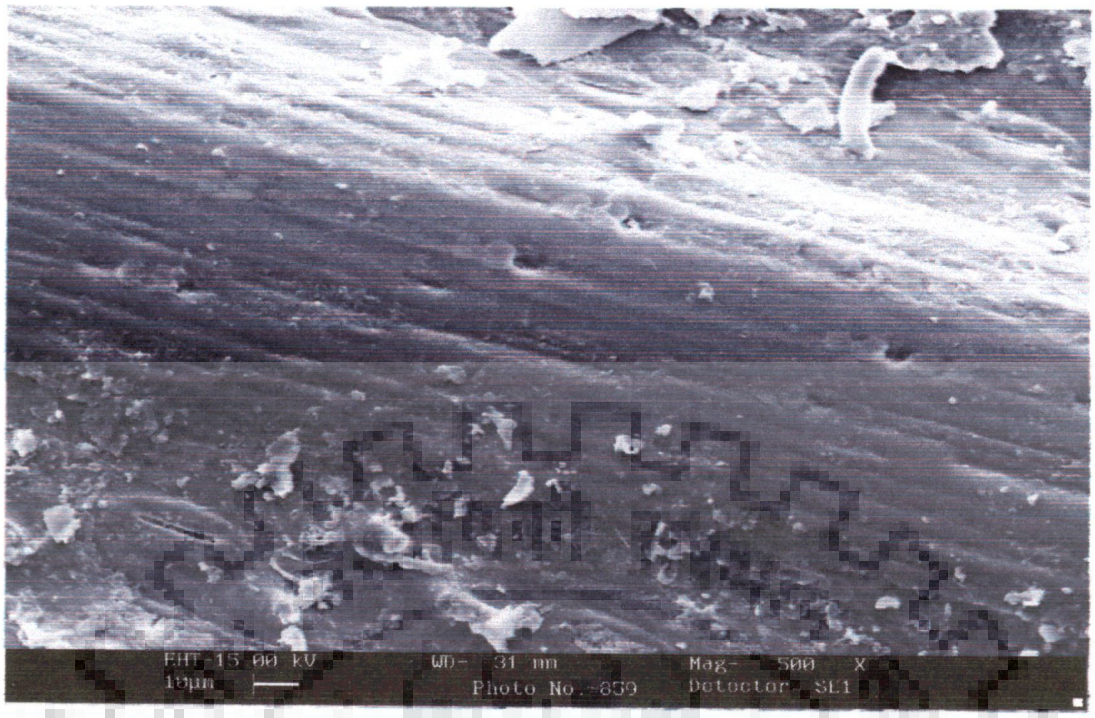


Fig. 4.5 Comparison in composition of Kans grass biomass before and after dilute acid pretreatment (2% H_2SO_4 at 120°C for 90 minutes)

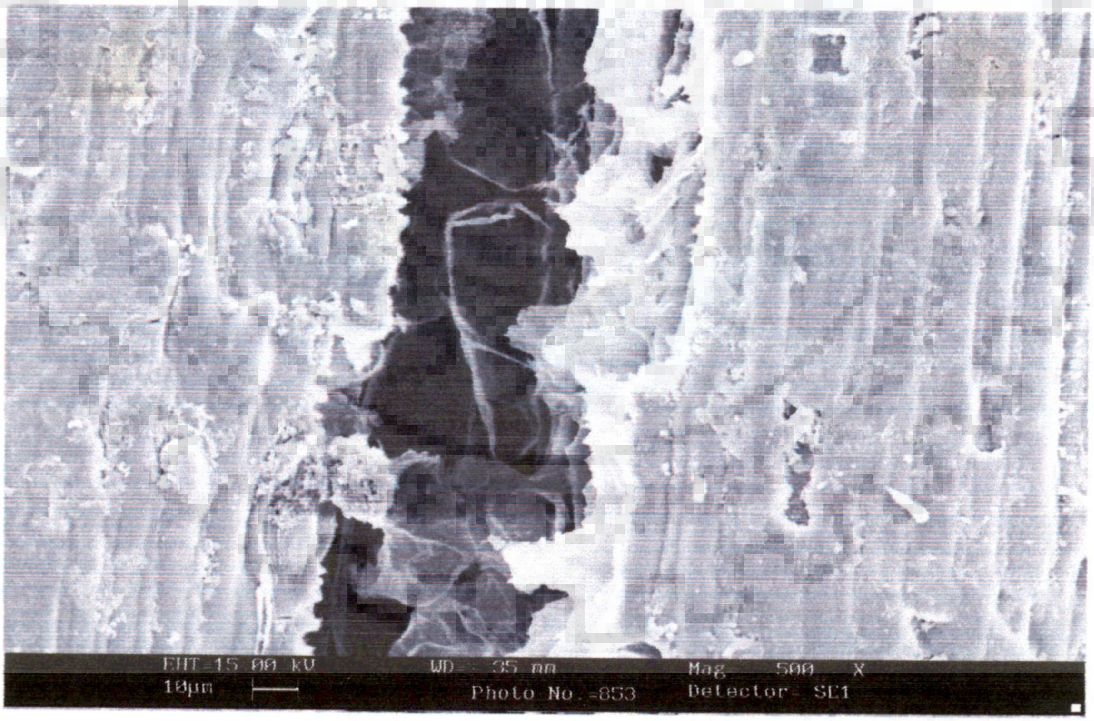
(a)

(b)

Fig. 4.6 Physical appearance of Kans grass biomass **(a)** before pretreatment **(b)** after dilute acid pretreatment (2% H_2SO_4 at 120°C for 90 minutes)



(a)



(b)

Fig. 4.7 Configuration of Kans grass biomass observed under Scanning electron microscope (SEM) **(a)** Before pretreatment **(b)** After pretreatment with 2% H_2SO_4 with 90 minutes of residence time at 120°C

4.3 Study of NaOH pretreatment

NaOH pretreatment of Kans grass (5%w/v) was carried out with different concentrations of NaOH (0.5, 1, 1.5 and 2%) for different duration of time (30, 60, 90, 120 minutes) and at variable temperatures (100, 110 and 120°C). After NaOH pretreatment the solid residue was separated from the liquid fraction, washed with distilled water and stored at room temperature after drying at 60°C overnight in an incubator and was used for further estimations of remaining solid residue, lignin content and holocellulose (Table 4.8-4.10). The liquid fraction was stored at -80°C for the estimations of liberated total reducing sugars (TRS) and xylose (Fig. 4.7-4.9).

4.3.1 Lignin and sugars liberation

NaOH mediated pretreatment is more susceptible than other chemical pretreatment due to no or low toxic compounds production during pretreatment reaction. NaOH pretreatment is estimated to be one of the most effective chemical for pretreatment of different lignocellulosic feedstocks including sunflower hull, corn stover, cotton stalk and sunflower stalk [296, 297, 298, 299, 300]. The total solid recovery, lignin removal as well as holocellulose content are shown in Tables 4.7-4.9. The total solid residue recovery after pretreatment decreased as the reaction time increased which may be due to solubilization of lignin and sugars (that mainly composed of xylose). During pretreatment at 100°C nearly 12.77-24.6% (Table 4.7) of solid residue loss was observed while with increase in temperature to 110 and 120°C (Table 4.8 & 4.9) the range of solid residue lost was found to be in the range of 14.47-39.79 and 15.67-47.53 % respectively. So it was evident from the Tables 4.7-4.9 that as the temperature was increased there was more loss in solid residue.

Higher concentration of NaOH is favourable to achieve the maximum removal of lignin but solubilization of the carbohydrates was also carried out which cause the less yield of holocellulose (carbohydrates). At 100°C the lignin solubilization (%) was found to be in the range of 4.01-31.19 while at 110°C this range was enhanced to 6.24-50.13 with all NaOH concentration and duration of pretreatment. The maximum lignin removal of 79.3% was observed in 90 minutes of reaction time with 2% NaOH concentrations at 120°C, on the other hand under the same conditions a loss of 6.6% of xylan with respect to holocellulose was also observed. The higher concentrations of NaOH (1.5% and 2%) were significant during pretreatment reaction at 120°C with no major difference in lignin removal. and the maximum delignification was found to be in the range of 72.8-79.3%.

However at 120°C with reaction time of 120 minutes and for all concentrations (0.5, 1, 1.5 and 2%), more than 70% (70.13-73.23%) delignification was observed, but at the same time loss of holocellulose content was also observed. In a similar investigation 65.63% of delignification was found with 2% NaOH concentration in 90 minutes of duration [301].

The liquid fraction obtained after NaOH pretreatment was investigated for the total reducing sugars and xylose estimation. Xylose is one of the major pentose sugars of hemicellulose that released during alkali pretreatment with all other sugars. At 100°C the total reducing sugars and xylose were found to be in range of 0.83-1.47 and 0.32-1.23 g/l (Fig. 4.7) respectively at all pretreatment conditions. At 110°C these ranges enhanced to 1.24- 2.37g/l for total reducing sugars and 0.68-1.45 g/l for xylose (Fig. 4.8). However the maximum removal of total reducing sugars was estimated to be in range of 2.20-3.26 g/l with all conditions (Different NaOH concentration and residence time) at 120°C (Fig.4.9), here NaOH concentrations of 1.5 and 2% are important as both of these concentrations resulted approximately the same amount of total reducing sugars and xylose for all reaction time.

As NaOH pretreatment is more favoured towards lignin removal and this was favoured that high temperature (120°C) and high reaction time in which lignin removal was found to be in the range of 70.43-72.86% of total lignin. The maximum lignin removal and xylose liberation was observed to be 79.31% and 4.2% respectively at 90 minutes of duration at 120°C with 2% NaOH. The TRS were liberated to be 6% which may be due to low amount of glucose and other sugars liberation. However, in another study Silverstein et al. [299] under the same pretreatment conditions (2% NaOH, 90 min, 120°C) reported 65% lignin removal in cotton stalk. Lignin removal in present study is comparable to other reported data where the lignin removal was observed to be 77% in straw [123]. As lignin inhibits the function of cellulase enzymes so the results from sugarcane bagasses and soft woods by various authors [301, 302] suggest that lignin removal is needed to maximize the yield of fermentable sugars during the enzymatic hydrolysis. Hence removal of the lignin depends upon the lignocellulosic material and pretreatment conditions including temperature as well as residence time.

Table 4.7 Total lignin removal as well as total residual solid and holocellulose remained after dilute NaOH pretreatment with different NaOH concentration (0.5, 1, 1.5 and 2%) and time (30, 60, 90 and 120 minutes) at 100°C

Pretreatment conditions		Total solid (g/ 100g dry wt.)	Lignin removal (%)	Holocellulose remained (g/100g dry wt.)
Time (min)	NaOH conc. (%)			
Untreated		100	25.14±0.55	64.73±0.55
30	0.5	87.23 ±0.71	4.019±0.59	62.19 ±0.27
	1.0	85.23 ±0.89	12.97 ±1.24	61.18 ±0.36
	1.5	82.12 ±1.06	18.18 ±2.65	60.58 ±0.19
	2.0	79.71 ±0.62	23.55 ±2.34	59.29 ±0.21
60	0.5	85.45 ±0.63	11.92 ±1.97	62.15 ±0.12
	1.0	81.34 ±1.07	17.39 ±2.18	61.27 ±0.24
	1.5	78.34 ±0.88	24.91 ±2.98	60.11 ±0.17
	2.0	77.10 ±1.24	27.65 ±1.62	59.87 ±0.13
90	0.5	81.34 ±1.87	13.17 ±1.76	59.78 ±0.12
	1.0	79.34 ±1.65	21.28 ±2.10	58.28 ±0.17
	1.5	77.4 ±2.13	28.25 ±2.12	57.78 ±0.21
	2.0	77.12 ±1.43	28.81 ±1.98	56.14 ±0.26
120	0.5	79.2 ±1.19	17.31 ±1.34	58.21 ±0.19
	1.0	78.34 ±1.37	26.14 ±1.22	57.23 ±0.12
	1.5	76.9 ±0.94	30.56 ±1.63	56.32 ±0.13
	2.0	75.4 ±1.45	31.19 ±1.92	56.27 ±0.15

Table 4.8 Total lignin removal as well as total residual solid and holocellulose remained after dilute NaOH pretreatment with different NaOH concentration (0.5, 1, 1.5 and 2%) and time (30, 60, 90 and 120 minutes) at 110°C

Pretreatment conditions		Total solid (g/ 100g dry wt.)	Lignin removal (%)	Holocellulose remained (g/100g dry wt.)
Time (min)	NaOH conc. (%)			
Untreated		100	25.14±0.55	64.73±0.55
30	0.5	85.53 ±0.65	6.24 ±1.51	62.13 ±0.78
	1.0	76.86 ±1.35	30.13 ±0.43	61.76 ±0.83
	1.5	71.06 ±0.87	39.39 ±1.67	60.56 ±0.59
	2.0	68.12 ±1.44	43.81 ±0.31	59.21 ±0.67
60	0.5	80.97 ±1.39	15.08 ±1.43	61.56 ±0.56
	1.0	75.84 ±0.76	22.16 ±0.54	59.81 ±0.74
	1.5	69.54 ±0.87	39.39 ±1.29	58.23 ±0.83
	2.0	65.32 ±1.32	46.99 ±0.63	57.23 ±0.57
90	0.5	76.32 ±0.47	26.58 ±0.54	57.12 ±0.53
	1.0	73.57 ±1.39	42.57 ±0.87	56.39 ±0.83
	1.5	65.89 ±0.65	48.38 ±1.73	56.13 ±0.63
	2.0	64.32 ±1.54	45.92 ±0.39	54.19 ±0.52
120	0.5	73.42 ±0.62	38.95 ±0.87	56.13 ±0.45
	1.0	71.23 ±1.32	46.20 ±0.87	54.13 ±1.08
	1.5	63.42 ±0.27	45.60 ±0.76	53.67 ±0.74
	2.0	60.21 ±0.37	50.13 ±0.66	53.13 ±0.82

Table 4.9 Total lignin removal as well as total residual solid and holocellulose remained after dilute NaOH pretreatment with different NaOH concentration (0.5, 1, 1.5 and 2%) and time (30, 60, 90 and 120 minutes) at 120°C

Pretreatment conditions		Total solid (g/ 100g dry wt.)	Lignin removal (%)	Holocellulose remained (g/100g dry wt.)
Time (min)	NaOH conc. (%)			
Untreated		100	25.14±0.55	64.73±0.55
30	0.5	84.33 ±1.21	14.25 ±0.56	61.59 ±0.78
	1.0	77.57 ±0.76	46.84 ±0.63	60.79 ±0.34
	1.5	69.25 ±1.67	52.01 ±0.84	55.85 ±0.45
	2.0	67.77 ±1.43	75.57 ±0.69	56.44 ±0.63
60	0.5	74.93 ±0.51	19.14 ±1.45	57.79 ±0.12
	1.0	67.77 ±0.82	59.29 ±0.75	55.80 ±0.76
	1.5	65.7 ±1.54	71.83 ±0.48	54.76 ±0.96
	2.0	67.53 ±0.63	76.80 ±0.52	51.35 ±0.43
90	0.5	74.01 ±1.48	34.10 ±1.49	55.97 ±0.43
	1.0	65.40 ±1.39	61.64 ±0.98	54.22 ±0.85
	1.5	63.22 ±1.45	74.33 ±1.21	49.84 ±0.35
	2.0	60.66 ±0.73	79.30 ±0.78	51.90 ±0.42
120	0.5	69.95 ±1.45	70.43 ±1.87	55.69 ±0.52
	1.0	58.56 ±1.54	72.94 ±0.61	47.84 ±0.82
	1.5	52.47 ±1.42	71.91 ±0.58	43.66 ±0.32
	2.0	57.36 ±0.73	72.86 ±0.50	44.93 ±0.44

4.3.2 Compositional and Configuration change in Kans grass biomass after NaOH pretreatment

After optimization of pretreatment conditions, NaOH concentration of 2% and residence time of 90 minutes was found to be best in terms of lignin removal (79.31%). The estimation of composition of Kans grass biomass before and after pretreatment was done (Fig.4.11). The holocellulose content of NaOH treated biomass was deceased from 64.73 g to 51.90 g per 100 g of biomass. This is due to the solubilization of the carbohydrate content during pretreatment. However the lignin content in original biomass was found to be 25.15 g/100g biomass which was decreased to 5.28 g/100 g biomass after pretreatment. The graphical representation of the composition change is shown in Fig. 4.11. The corresponding physical as well as structural changes are shown in Fig. 4.12 and 4.13 respectively. SEM was used to study the morphological features and surface characteristics of materials after the pretreatment and compared with untreated biomass (Kans grass). The pretreatment resulted in significant structural changes (Fig. 4.13). Sodium hydroxide pretreatment disrupted the structure of the fibers mainly by removing lignin. Furthermore, the structure of the lignocellulosic biomass was exposed up and provides higher surface area for subsequent enzymatic reactions. The similar observations of SEM analysed were also observes on pine wood by Hui Wang [303].

4.4 Comparative analysis of dilute H_2SO_4 and NaOH pretreated Kans grass biomass

Kans grass biomass is composed of 64.73% of holocellulose or total carbohydrate. The pretreatment strategy is an important first step for bioethanol production. During dilute H_2SO_4 pretreatment 77.05% of hemicellulosic portion liberated with removal of 28.03% lignin (Table 4.10). While with NaOH pretreatment only 20.6% of the hemicellulosic portion was solubalize with 79% of delignification (Table 4.10). Lignin is one of the obstacle for hydrolysis of crystalline sugars, so this NaOH pretreated biomass may be further enzymatically hydrolyzed to obtained the soluble sugars, as removal of lignin provide more surface area for the enzyme action to the carbohydrate part [304, 305]. Hence dilute H_2SO_4 pretreatment is more susceptible for solubilization of hemicellulosic sugars with little removal of lignin while major effect of alkaline pretreatment is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides. In addition, alkali pretreatments remove acetyl and the various uronic acid substitutions on hemicellulose and facilitate access of enzyme to the hemicellulose and cellulose surface [306].

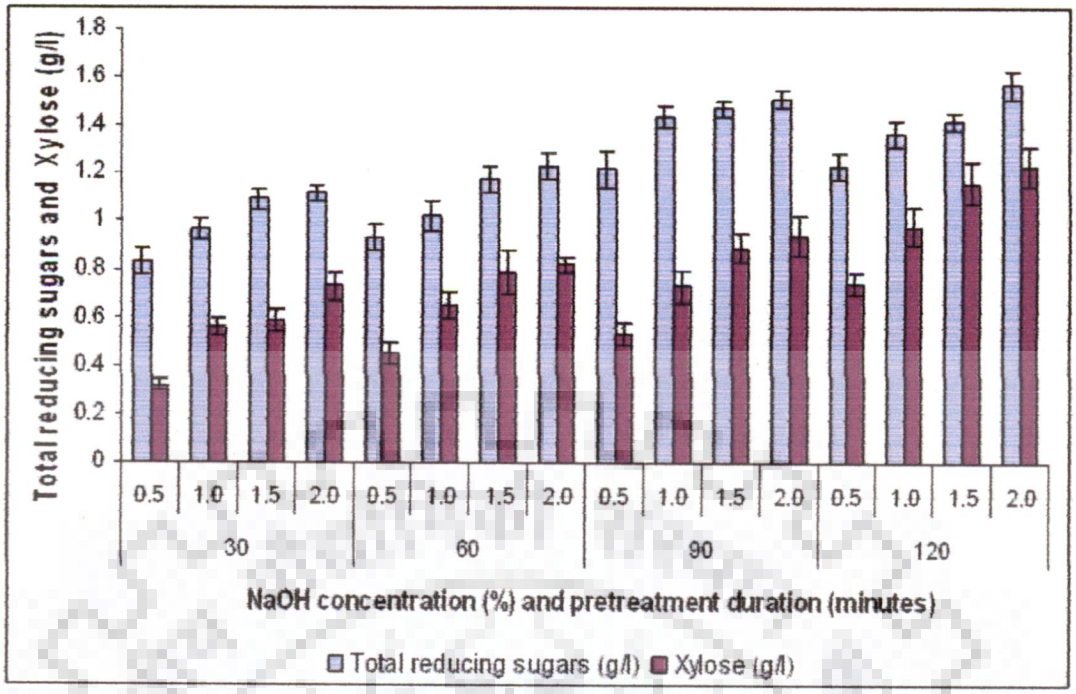


Fig. 4.8 Concentration of total reducing sugars and xylose liberated during NaOH pretreatment with different NaOH concentrations (0.5, 1, 1.5 and 2%) and reaction time durations (30, 60, 90 and 120 minutes) at 100°C

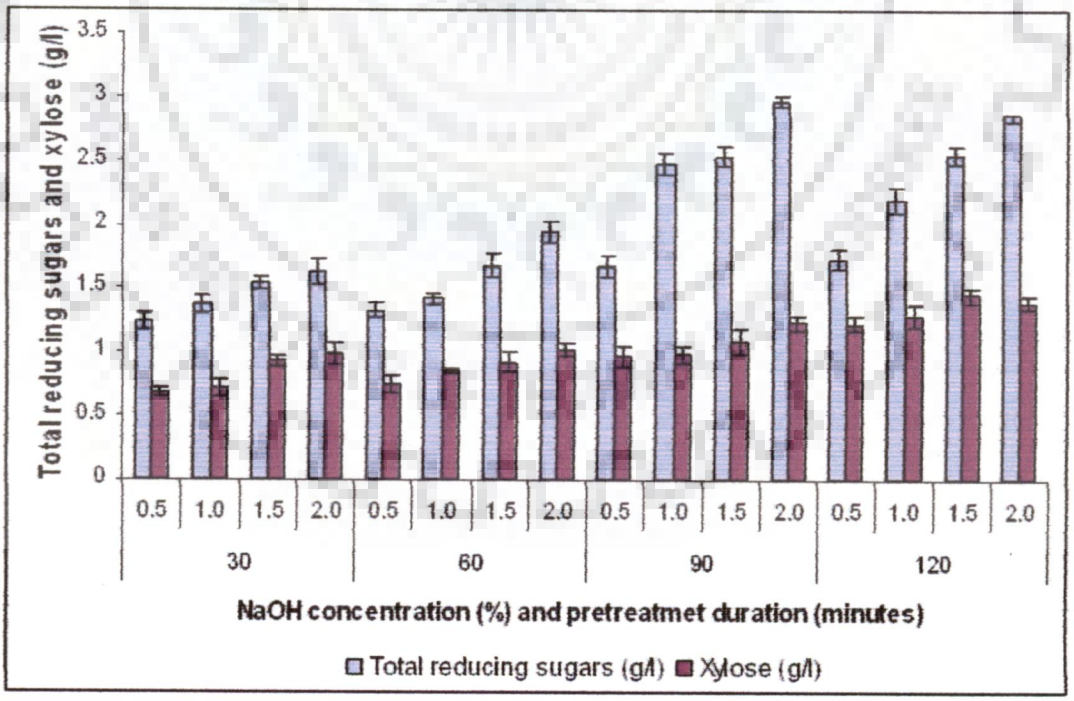


Fig. 4.9 Concentration of total reducing sugars and xylose liberated during NaOH pretreatment with different NaOH concentrations (0.5, 1, 1.5 and 2%) and reation time durations (30, 60, 90 and 120 minutes) at 110°C

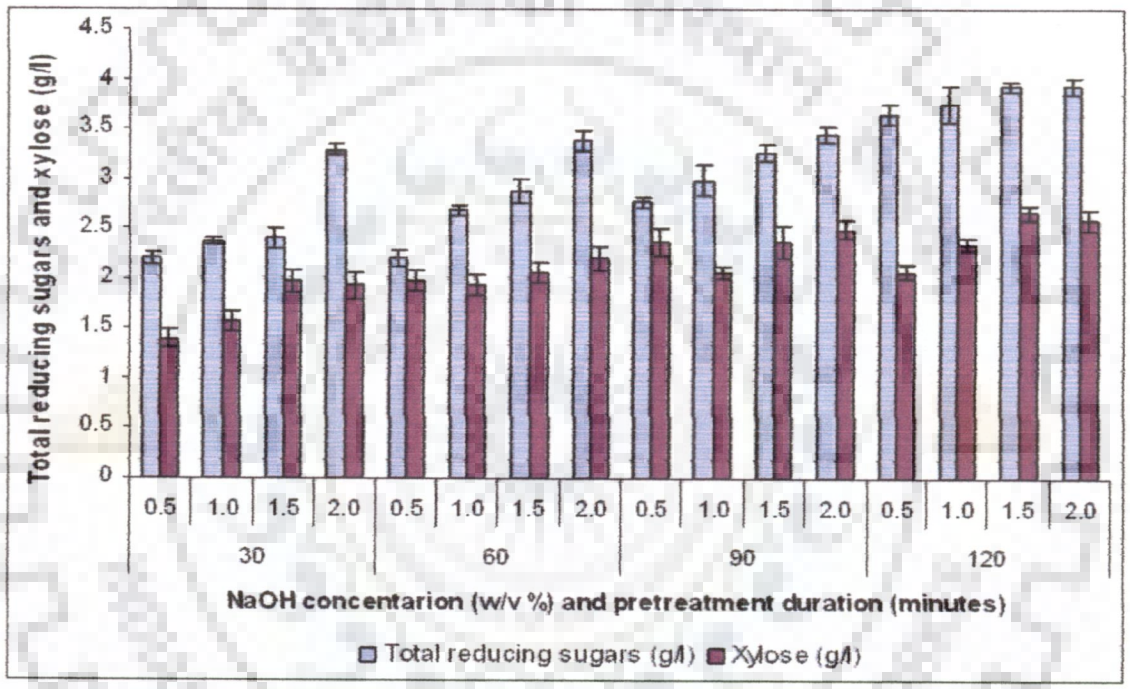


Fig. 4.10 Concentration of total reducing sugars and xylose liberated during NaOH pretreatment with different NaOH concentrations (0.5, 1, 1.5 and 2%) and reation time durations (30, 60, 90 and 120 minutes) at 120°C

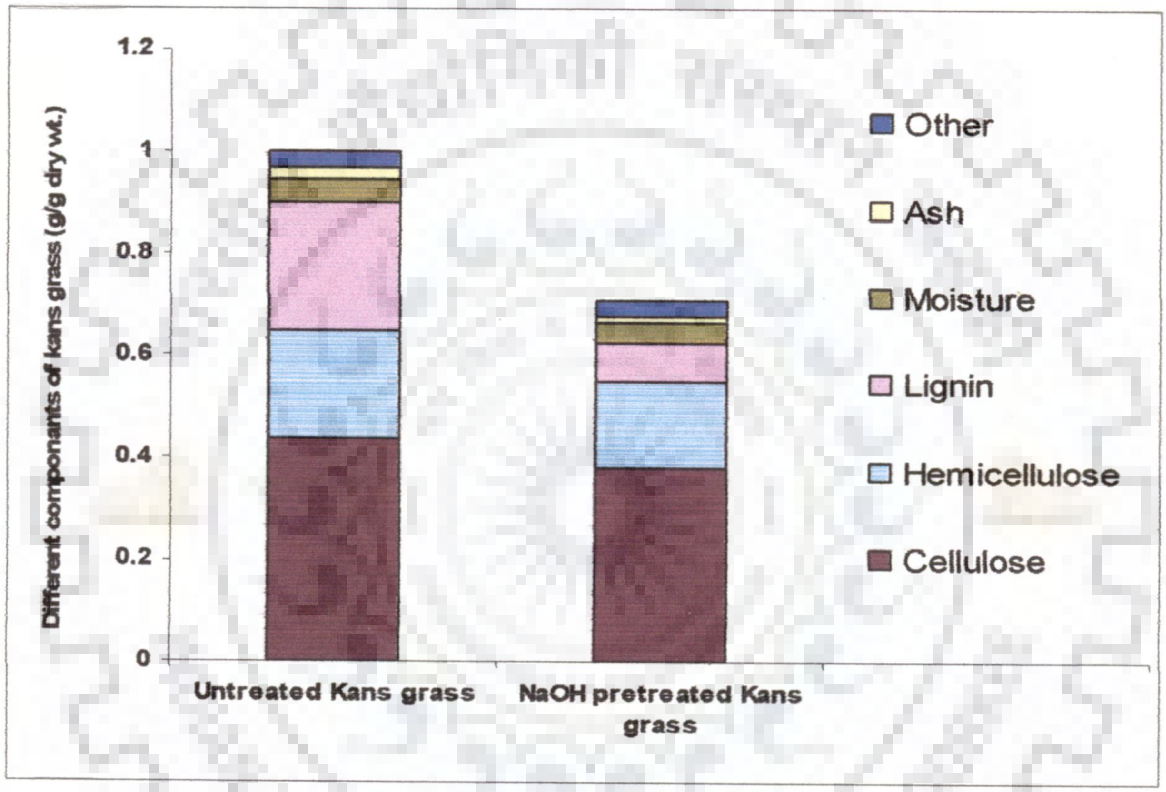
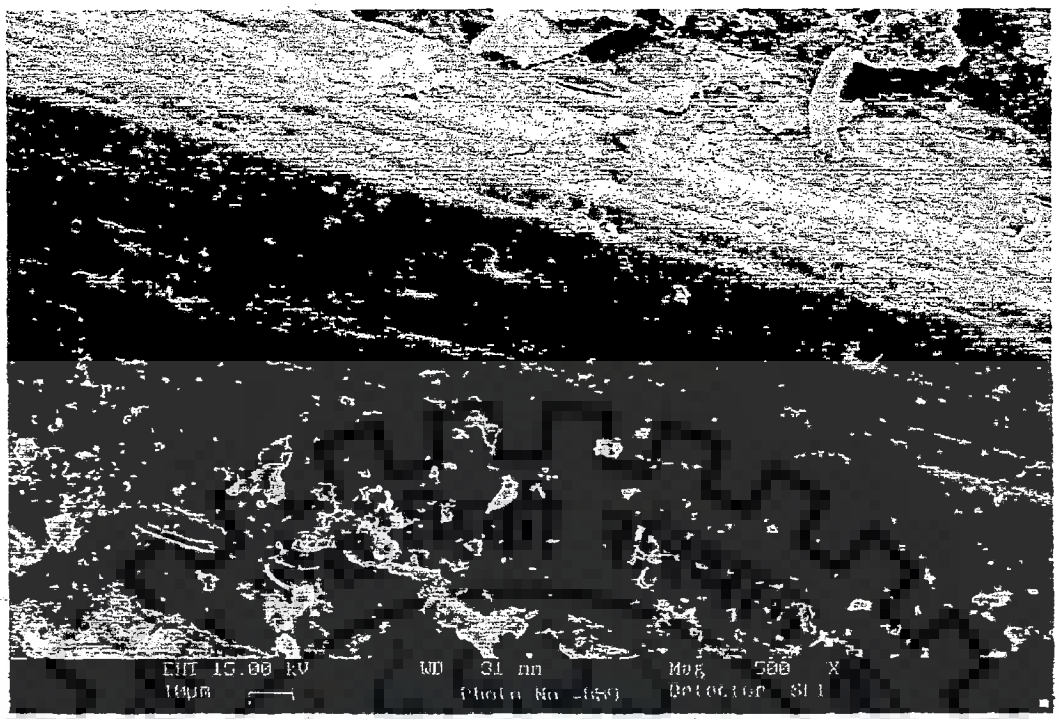


Fig. 4.11 Comparison in composition of Kans grass biomass before and after dilute NaOH pretreatment (2% NaOH for 90 min and at 120°C)

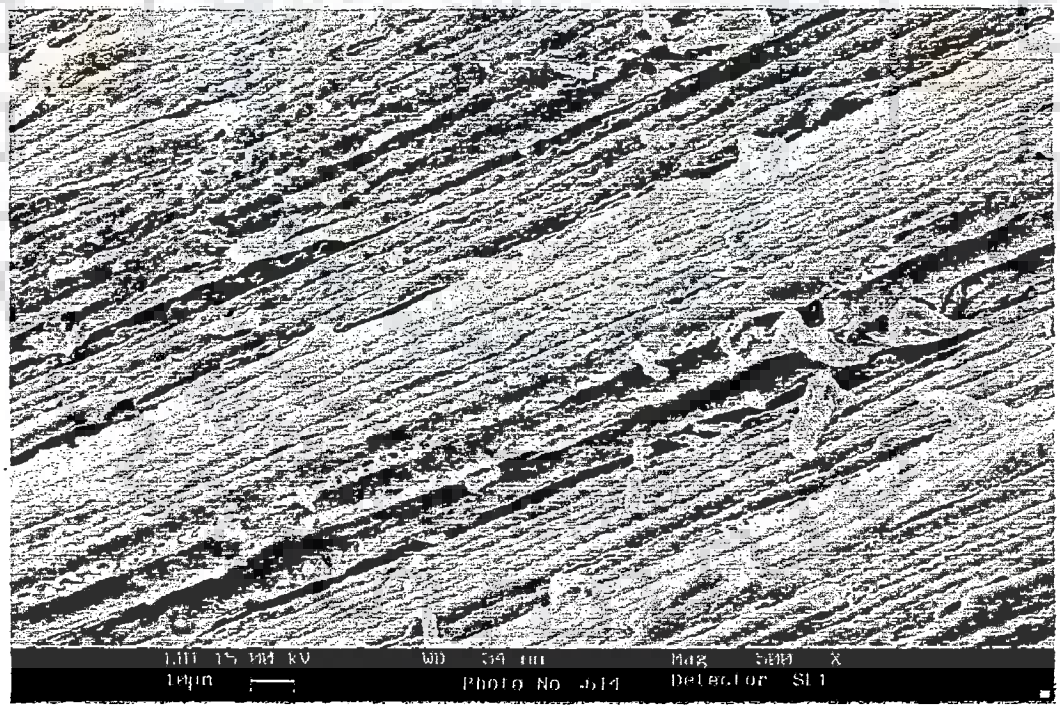
(a)

(b)

Fig. 4.12 Physical appearance of Kans grass biomass **(a)** before pretreatment **(b)** after pretreatment with 2% NaOH with 90 minutes of reaction time



(a)



(b)

Fig. 4.13 Configuration of Kans grass biomass observed under Scanning electron microscope (SEM) **(a)** Before treatment **(b)** After pretreatment with 2% NaOH with 90 minutes of reaction time

Table 4.10 A comparison of composition in original, dilute H_2SO_4 pretreated and NaOH pretreated Kans grass biomass

Components	**Untreated Biomass (g/100 gdb)**	**Dil.H_2SO_4 pretreatment (g/100 gdb)**	**Dil NaOH pretreatment (g/100 gdb)**
Holocellulose	64.73±0.55	40.0±0.58	51.90±0.37
Cellulose	43.68±0.54	35.07±0.53	35.20±51
Hemicellulose	21.05±0.46	04.83±0.50	16.7±32
Lignin	25.15±0.55	18.10±0.31	5.28±0.13
Moisture	4.70±0.055	3.0±0.21	3.2±0.11
Ash	2.10±0.21	2.0±0.12	2.0±0.08

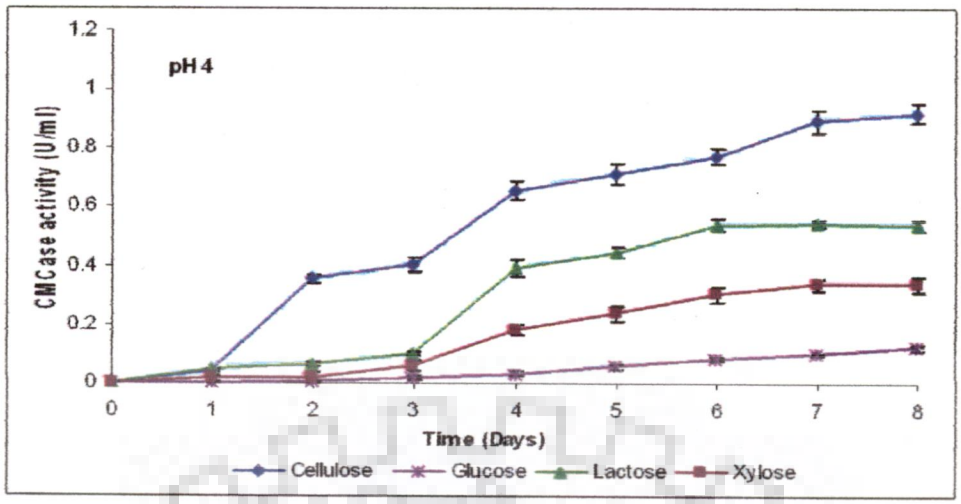
4.5 Endoglucanase or CMCase enzyme Production

T. reesei (NCIM 1052) was used for CMCase production with different carbon sources (cellulose, glucose, lactose and xylose), pH (4, 5 and 6), temperatures (25, 28 and 30°C) and for variable duration of time (Fig. 4.14-4.16). The Samples were withdrawn periodically and were analyzed for CMCase activity, cell biomass and total extra cellular protein. The results are given in Table 4.11.

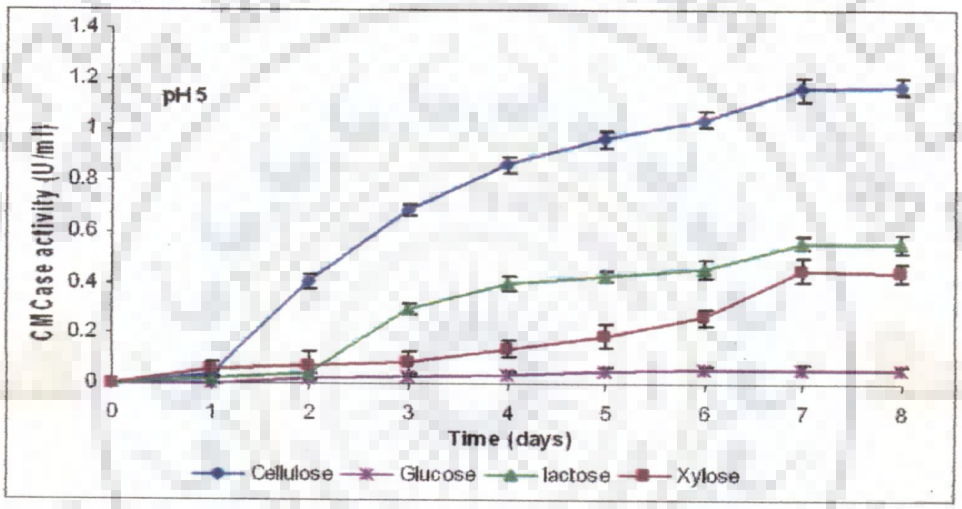
Among the four carbon sources used in this study, cellulose was found to be most effective one for CMCase production (Fig. 4.14-1.16) which goes in the range of 0.523 U/ml (pH 6 and 30°C) to 1.46 U/ml (pH 5 and 28°C) whereas that for lactose was found to be in the range of 0.247 U/ml (pH 4 and 30°C) to 0.559 U/ml (pH 5 and 25°C) and the same for xylose and glucose were 0.192-0.450 U/ml and 0.053-0.268 U/ml respectively. Hence, lactose was found to be second most important sugar for CMCase production. As reported by other researchers [307, 308, 309] lactose relives the fungus from catabolic repression and acts as an inducer however exact mechanism is not known yet. Glucose which was found to be the least producer of CMCase was the most favoured substrate for cell biomass formation.

Cellulose and lactose were found to be best substrate for CMCase production and showed maximum activity at pH 5 (Table 4.11) across all temperatures. Xylose also showed maximum activity at pH 5 with temperature 25°C (0.450 U/ml) and 28°C (0.302 U/ml). However, it showed higher CMCase activity at pH 6 (0.0321 $U.ml^{-1}$) when temperature was maintained at 30°C. For glucose though it showed maximum activity at pH 4 across all temperature, not considered as a favoured substrate for CMCase production. It was observed that when cellulose was used as a substrate for CMCase production at 25°C temperature, the CMCase activity was found to be in the range from 0.806 to 1.17 U/ml. When temperature was enhanced to 28°C, the corresponding values were also enhanced and found in the range of 0.953 (pH 6) to 1.46 (pH 5) U/ml. However, when temperature was further increased to 30°C the corresponding range reduced to 0.523 from 0.949 U/ml. Almost similar results for CMCase activity were also observed when lactose (it shows almost same maximum activity at 25 and 28°C) and glucose were used as a substrate.

Hence it is evident that 28°C is the optimum temperature for CMCase production with cellulose as substrate.



(a)



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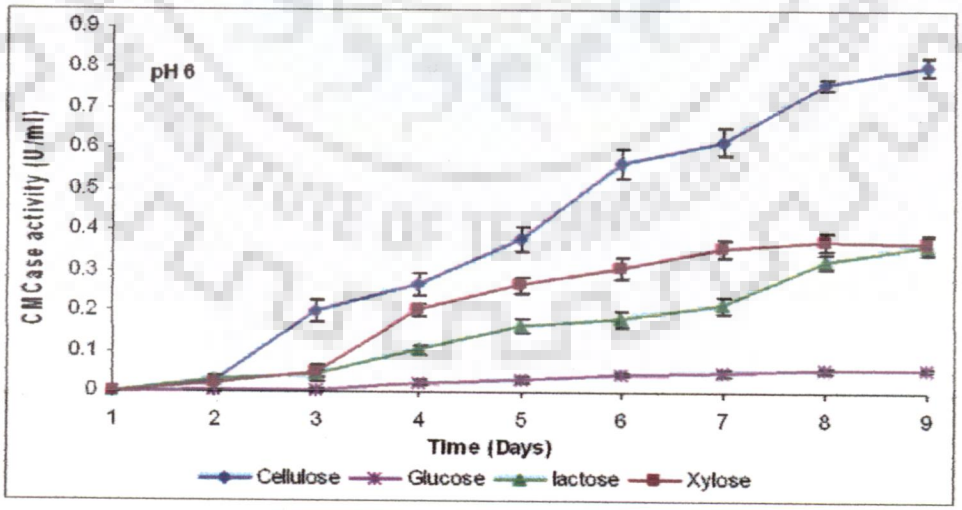
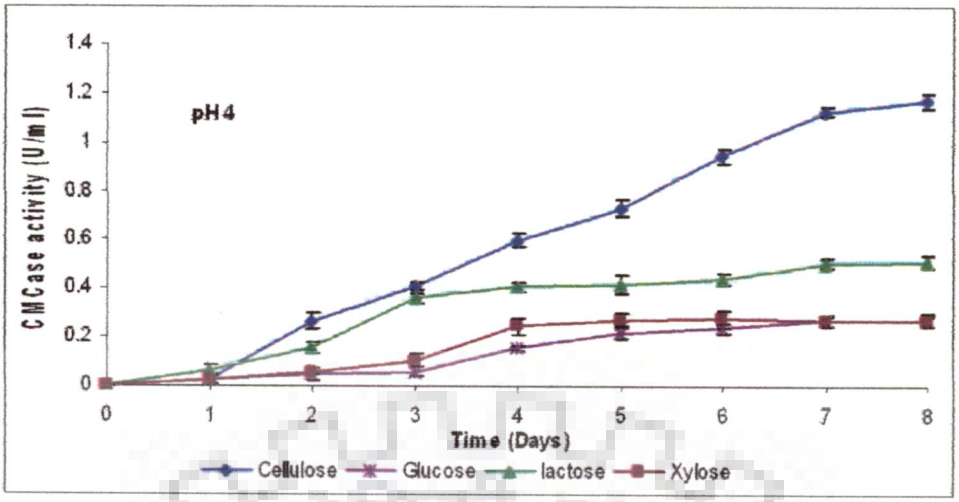
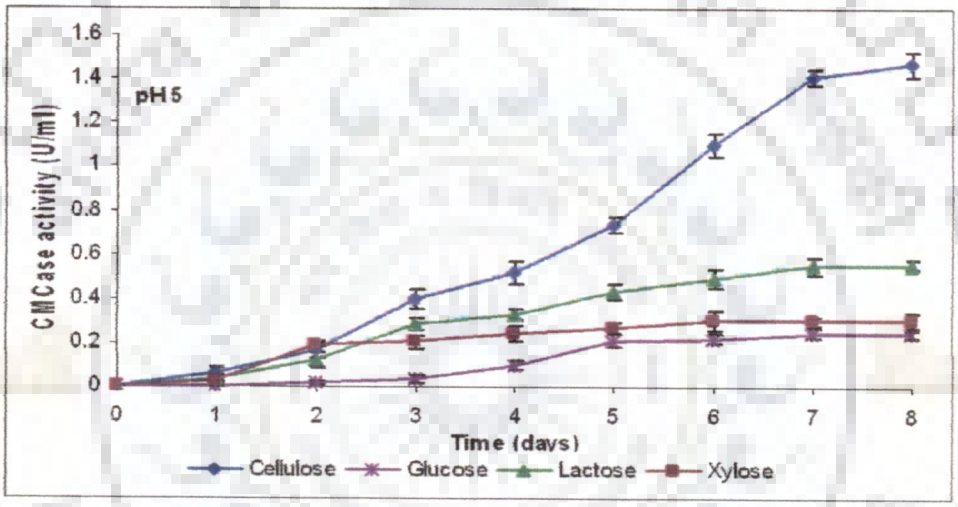


(c)

Fig. 4.14 CMCase activity at 25°C with different carbon sources (cellulose, glucose, lactose and xylose) at different pH **(a)** 4 **(b)** 5 and **(c)** 6



(a)



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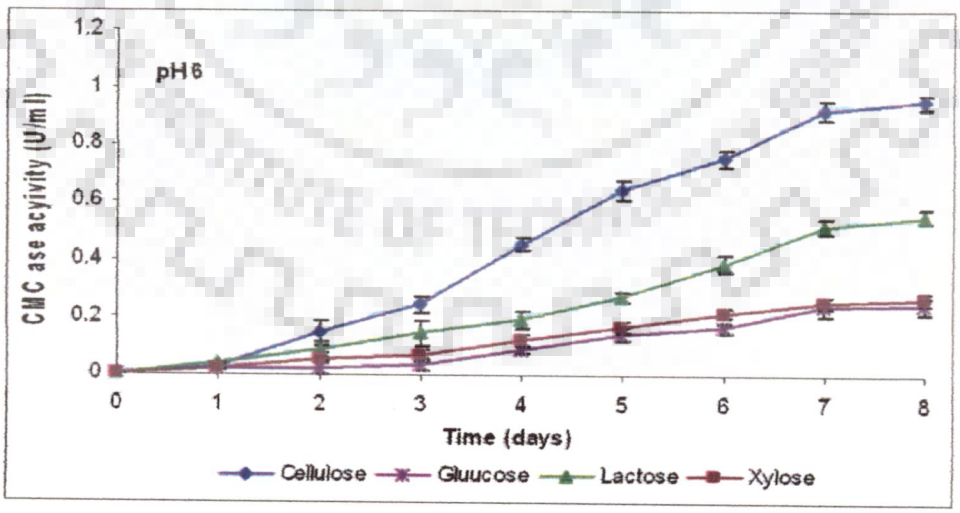
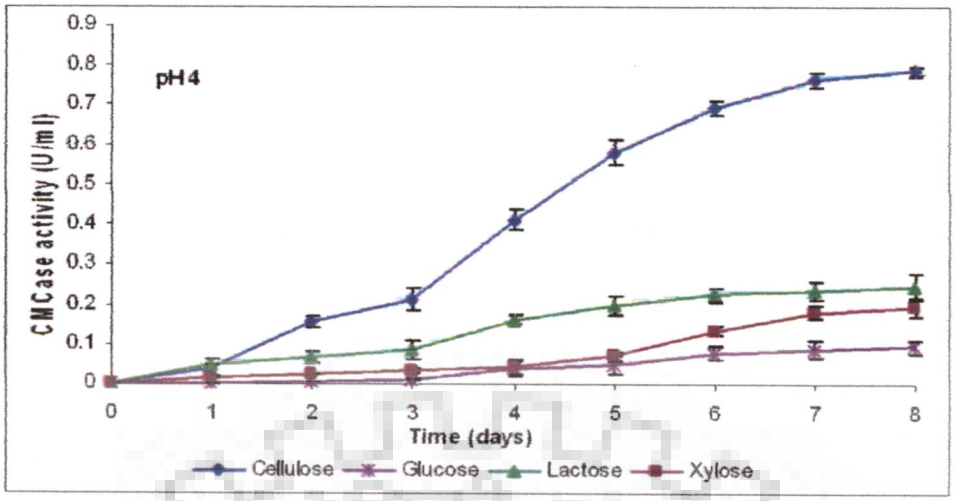
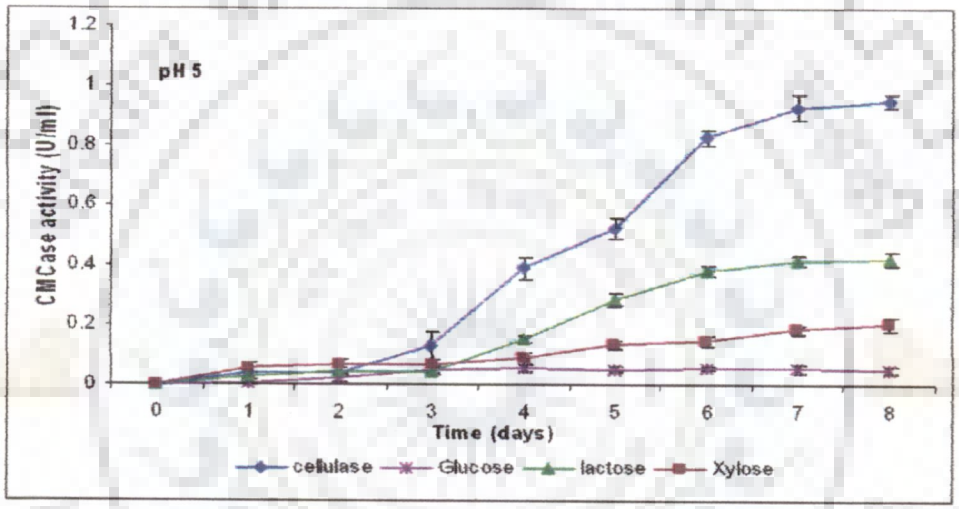


(c)

Fig. 4.15 CMCase activity at 28°C with different carbon sources (cellulose, glucose, lactose and xylose) at different pH **(a)** 4 **(b)** 5 and **(c)** 6



(a)



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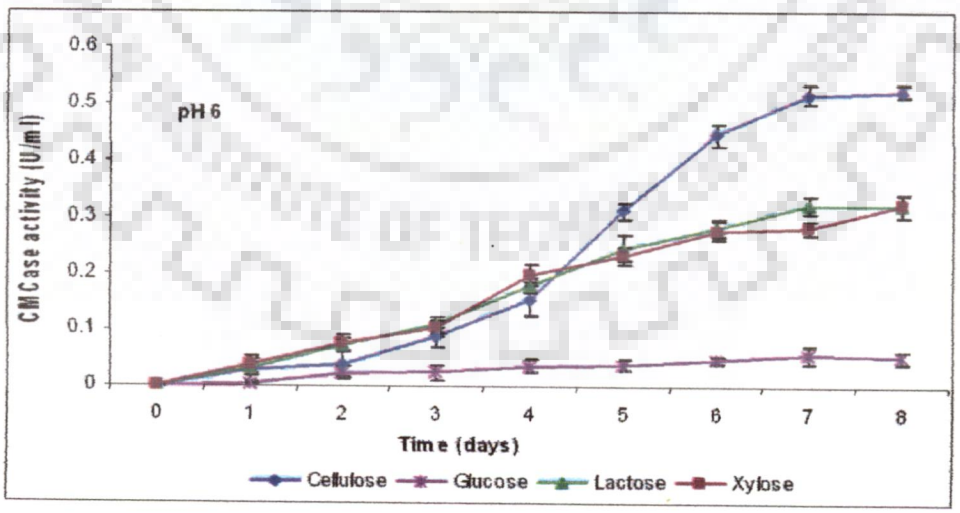


(c)

Fig. 4.16 CMCase activity at 30°C with different carbon sources (cellulose, glucose, lactose and xylose) at different pH **(a)** 4 **(b)** 5 and **(c)** 6

Table 4.11 CMCase production at different temperatures, pH and carbon source/substrates

Substrate	Temperature	pH	CMCase (U/ml)	Biomass (mg/ml)	Total protein (mg/ml)	Time (days)
Cellulose	25	4	0.921±0.042	2.45±0.17	0.159±0.062	8
		5	1.171±0.021	2.32±0.21	0.149±0.031	8
		6	0.806±0.017	2.27±0.10	0.132±0.010	8
	28	4	1.16±0.018	2.41±0.16	0.166±0.023	8
		5	1.46±0.031	3.22±0.24	0.165±0.014	8
		6	0.953±0.051	3.01±0.20	0.155±0.057	8
	30	4	0.786±0.013	3.14±0.25	0.129±0.036	8
		5	0.949±0.037	2.36±0.17	0.107±0.015	7
		6	0.523±0.016	2.53±0.21	0.112±0.058	8
Lactose	25	4	0.543±0.068	1.80±0.18	0.041±0.007	7
		5	0.559±0.039	1.64±0.14	0.040±0.003	7
		6	0.359±0.018	1.38±0.13	0.045±0.006	8
	28	4	0.512±0.034	3.01±0.19	0.051±0.006	8
		5	0.557±0.017	3.71±0.12	0.066±0.003	8
		6	0.553±0.060	3.63±0.14	0.061±0.008	8
	30	4	0.247±0.059	3.62±0.19	0.049±0.006	8
		5	0.421±0.075	3.09±0.21	0.053±0.004	8
		6	0.322±0.030	3.08±0.23	0.037±0.008	7

Substrate	Temperature	pH	CMCase (U/ml)	Biomass (mg/ml)	Total protein (mg/ml)	Time (days)
Xylose	25	4	0.338±0.061	1.64±0.13	0.030±0.007	7
		5	0.450±0.085	1.52±0.49	0.094±0.004	7
		6	0.376±0.030	1.38±0.22	0.046±0.008	7
	28	4	0.276±0.073	2.72±0.37	0.029±0.002	6
		5	0.302±0.062	2.92±0.31	0.021±0.009	6
		6	0.213±0.012	2.85±0.16	0.025±0.005	8
	30	4	0.192±0.013	2.96±0.21	0.043±0.008	8
		5	0.204±0.054	2.97±0.27	0.044±0.002	8
		6	0.321±0.031	3.04±0.19	0.043±0.006	8
Glucose	25	4	0.121±0.044	1.72±0.21	0.044±0.008	8
		5	0.061±0.016	2.20±0.78	0.040±0.002	7
		6	0.058±0.061	2.02±0.17	0.041±0.006	8
	28	4	0.268±0.022	4.08±0.31	0.054±0.007	7
		5	0.245±0.059	3.28±0.21	0.045±0.004	7
		6	0.242±0.068	3.17±0.56	0.046±0.06	8
	30	4	0.093±0.052	3.51±0.27	0.025±0.008	8
		5	0.053±0.029	3.42±0.31	0.037±0.006	8
		6	0.057±0.072	3.31±0.36	0.034±0.007	7

The crude enzyme preparation obtained after optimized condition (cellulose as carbon source, pH 5 and 25°C) with 1.46 U/ml CMCase activity may contain several other enzymes as well. The activity of this preparation was also evaluated for total cellulase activity and xylanase activity. The total cellulase activity was expressed in termes of filter paper units (FPU) and was found to be 1.14 FP U/ml whereas xylanase activity was estimated to be 6.60 U/ml. This crude preparation was used for saccharification of raw material, as described in material and method section.

4. 6 Enzymatic saccharification of dilute H_2SO_4 pretreated Kans grass

The crude enzyme mixture obtained at optimized conditions had CMCase as well as xylanase activity (6.60U/ml) was further used for enzymatic hydrolysis of the acid pretreated Kans grass biomass. The 20 FPU/g of dry biomass of crude enzyme was used for the enzymatic reaction and resulted sugars were analyzed as total reducing sugars and xylose. To determine the effectiveness of the crude enzyme, commercial enzyme (20 FPU/g dry biomass) was also studied in parallel experiments.

4.6.1 Saccharification of acid (H_2SO_4) pretreated Kans grass with crude and commercial enzyme

Hydrolysis of acid treated biomass with 20 FPU/gdb crude enzymes was studied with biomass loading of 1, 2, 2.5, 5 and 6% (w/v) and shown in Fig. 4.17 (a). With biomass loading of 1% the total reducing sugars (TRS) released was found to be 36.15 mg/gdb in 84 hours, but with increasing the biomass loading to 2%, TRS release was as high as 69.08 mg/gdb for the same duration of time. However when this biomass loading was further enhanced to 2.5, 5 and 6%, the TRS released was found to be less (47.79, 40.15 and 29.27 mg/gdb respectively). When release of xylose sugars was observed at 2% biomass loading it was found to be the maximum (14.13 mg/gdb) and for other biomass loading line (1, 2.5, 5 and 6%) the values were found to be less (13.22, 12.19, 12.18, 11.26 g/gdb respectively). When same experiment was conducted with commercial cellulase preparation on similar biomass as above (20 FPU/gdb), The TRS and xylose released was found to be maximum at 2% biomass loading (63.21 mg/gdb and 7.4 mg/gdb respectively) (Fig. 4.17 (b)). Hence with increasing the time from zero hour concentration of TRS increasing and it is getting stationary after 72 hours. The same pattern was observed in both treated and untreated biomass with enzymatic hydrolysis (Fig 4.17 and 4.18).

Hydrolysis with crude enzyme preparation released higher amount of TRS as compared to pure enzyme preparation (Fig.4.17 (a) and (b)). This may be attributed on the

fact that presence of certain amount of xylanase along with cellulases in the crude preparation which is absent in commercial enzyme preparation.

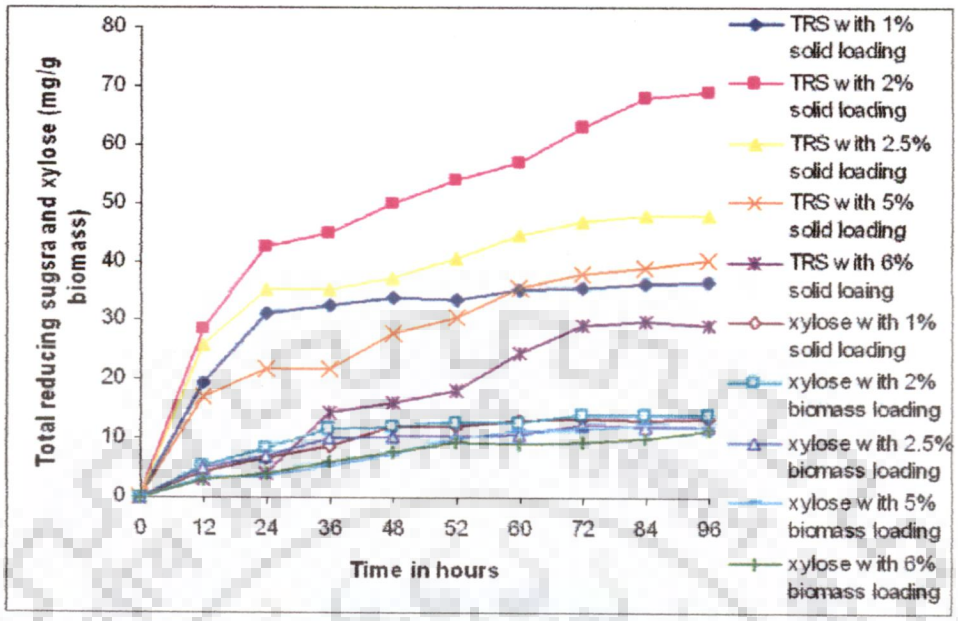
4.6.2 Saccharification of untreated Kans grass with crude and commercial enzyme

When untreated biomass (prior to acid treatment) was directly treated with crude (Fig. 4.18 (a)) and commercial (Fig. 4.18(b)) enzyme preparation, the corresponding maximum TRS released was found to be 34.67 and 31.82 mg/gdb for 2% biomass loading respectively in 72 hours with negligible amount of xylose released. It was also analysed that TRS released for untreated biomass was found to be much less as compared to acid pretreated biomass. This may be due to the fact that lignin association with cellulosic and hemicellulosic structure of the biomass was more rigid in case of untreated biomass hence access of enzyme was not available as much as compared to acid pretreated biomass. Acid pretreatment not only partially removed but also made the association of lignin with the cellulosic fibres less rigid that gives better access for the enzymes to the cellulosic and hemicellulosic structure of the biomass.

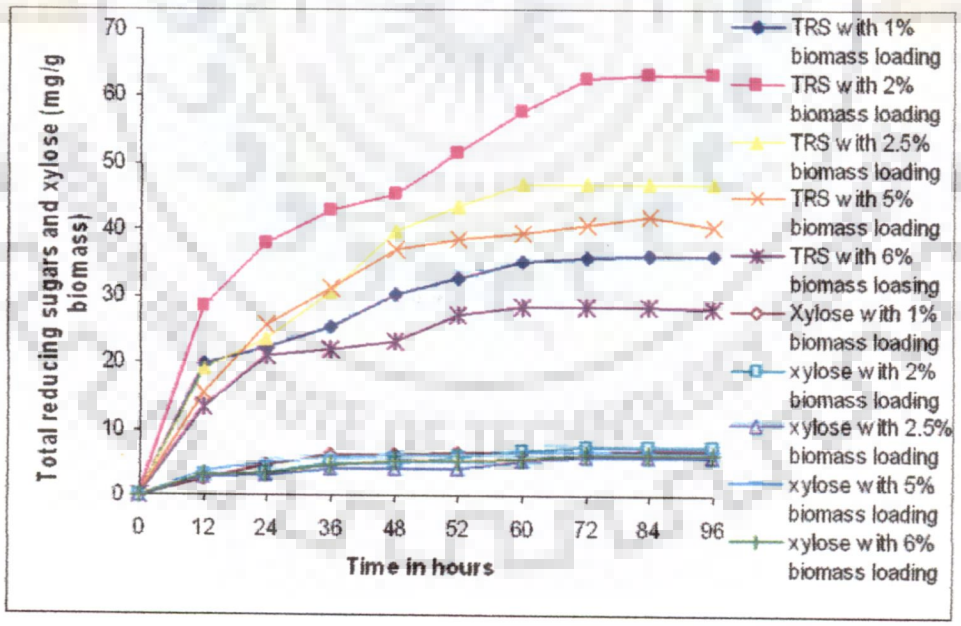
When untreated biomass that was composed of 64.73% of holocellulose, was first pretreated with dilute acid (2% v/v H_2SO_4, 90 minutes and 120°C), there was removal of lignin (28.03% of total lignin) along with hemicellulose (77.05% of total hemicellulose) and cellulose (19.71 % total cellulose content). When this acid pretreated biomass was further treated with crude enzyme 34.8% of total holocellulose removal was observed (Fig. 4.19). The corresponding changes are evident in the photographs (Fig. 4.20). Fig. 4.20 (a) showed the intact Kans grass structure and compared with Fig. 4.20 (b) (structure of acid treated biomass) where hemicellulosic portion was the most affected by acid treatment along with associated cellulosic structure. When this biomass was further treated with commercial cellulase (2% w/v biomass loading, 20 FPU/g gdb), mainly cellulose was removed (Fig. 4.20 (c)) along with residual fraction of hemicellulose. However lignin and other components remained intact.

4.6.3 Best condition for optimization

Biomass loading to 2%, TRS released was as high as 69.08 mg/gdb released (14.13 mg/gdb) for the same duration of time 84 hours. So 17.25% of holocellulose sugars were converted to soluble sugars. Hence, less released of TRS with commercial cellulase (63.21 mg/gdb) as compared to crude enzyme (69.08 mg/gdb) was observed. This may be due to the fact that crude enzyme mixture is composed of xylanase enzyme (6.6 U/ml) with the cellulase activity and during saccharification it resulted in xylose formation with glucose sugar.

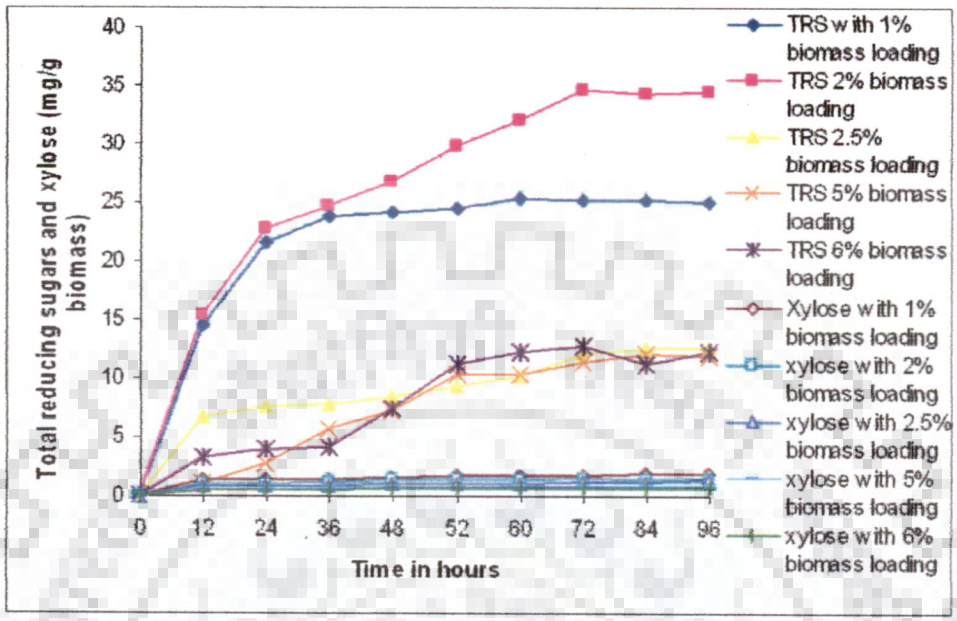


(a)

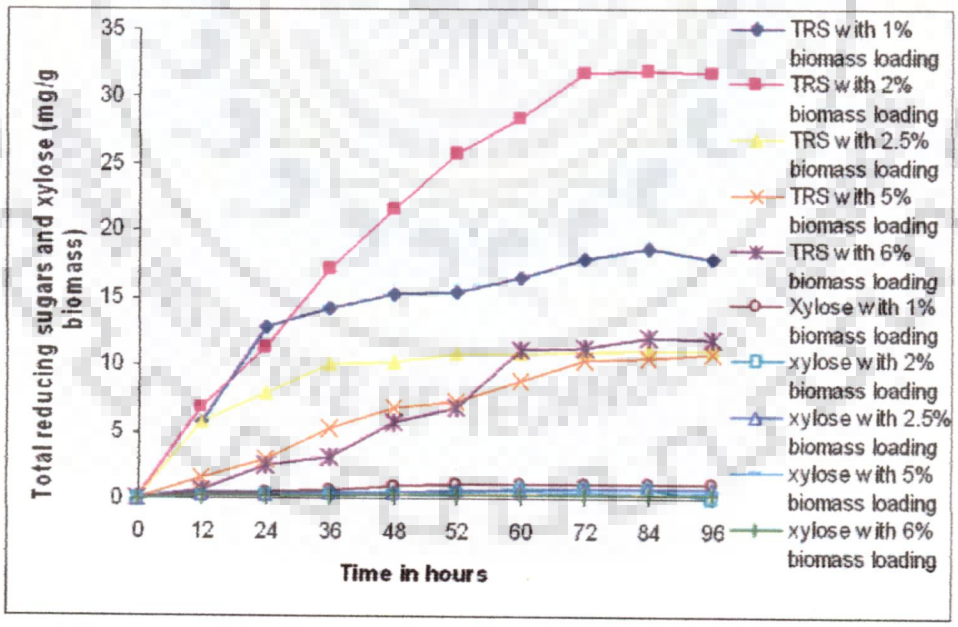


(b)

Fig. 4.17 Enzymatic saccharification of H_2SO_4 pretreated Kans grass with **(a)** crude enzyme **(b)** Commmercial enzyme and formation of Total reducing sugars (TRS) and xylose



(a)



(b)

Fig. 4.18 Enzymatic saccharification untreated (original) Kans grass with **(a)** crude enzyme **(b)** Commercial enzyme and formation of Total reducing sugars (TRS) and xylose

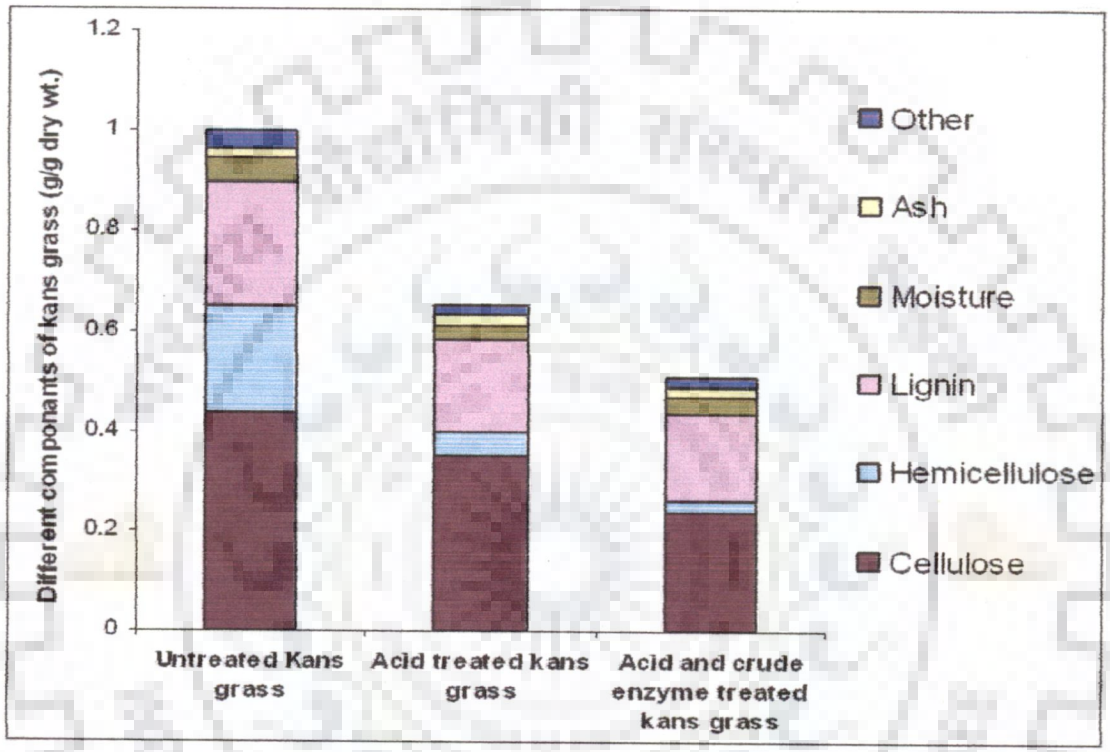


Fig. 4.19 Compositional change in Kans grass biomass before pretreatment, after dilute H_2SO_4 pretreatment and enzymatic saccharification

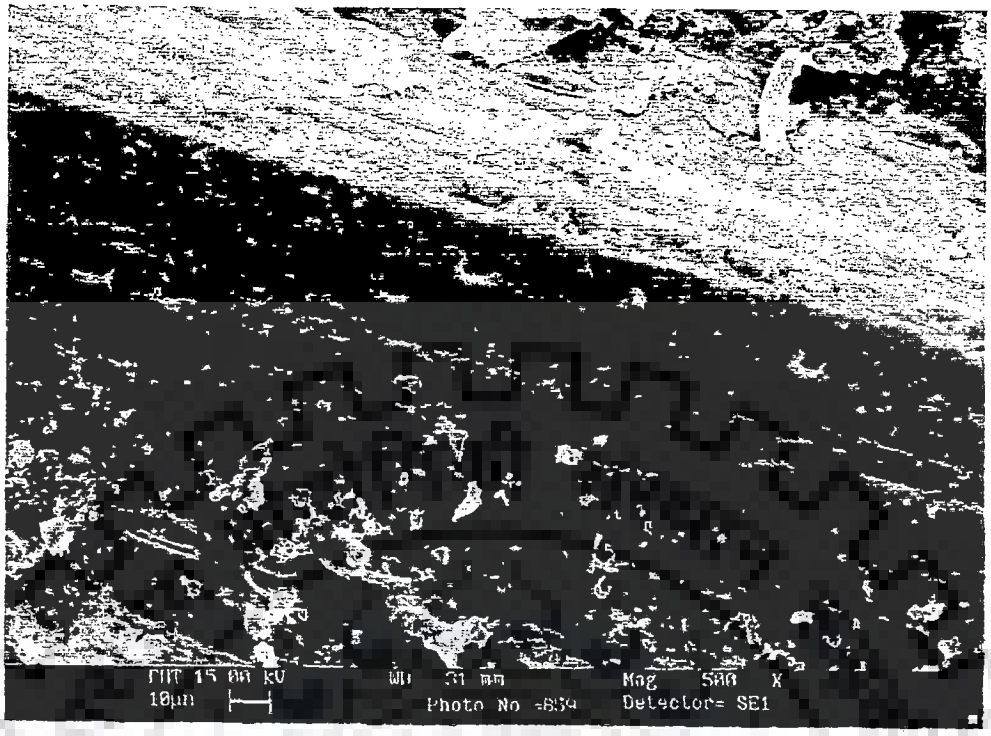


Fig. 4.20 (a) Configuration of Kans grass biomass (original) before pretreatment

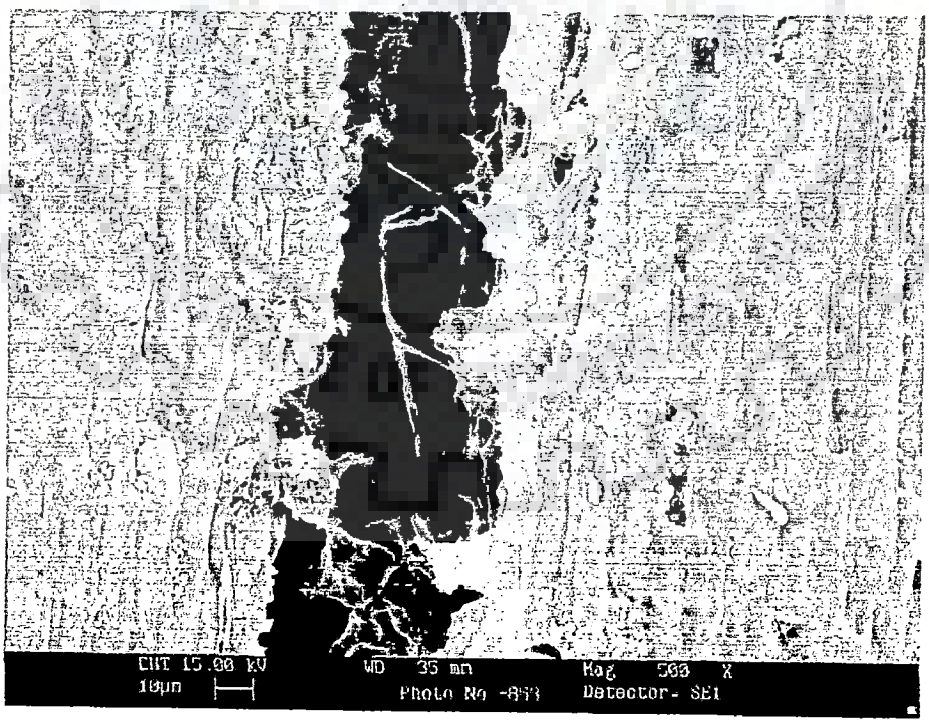


Fig. 4.20 (b) Configuration change in Kans grass biomass after dilute acid (H_2SO_4) pretreatment (with 2% H_2SO_4 with 90 minutes of reaction time at 120°C)

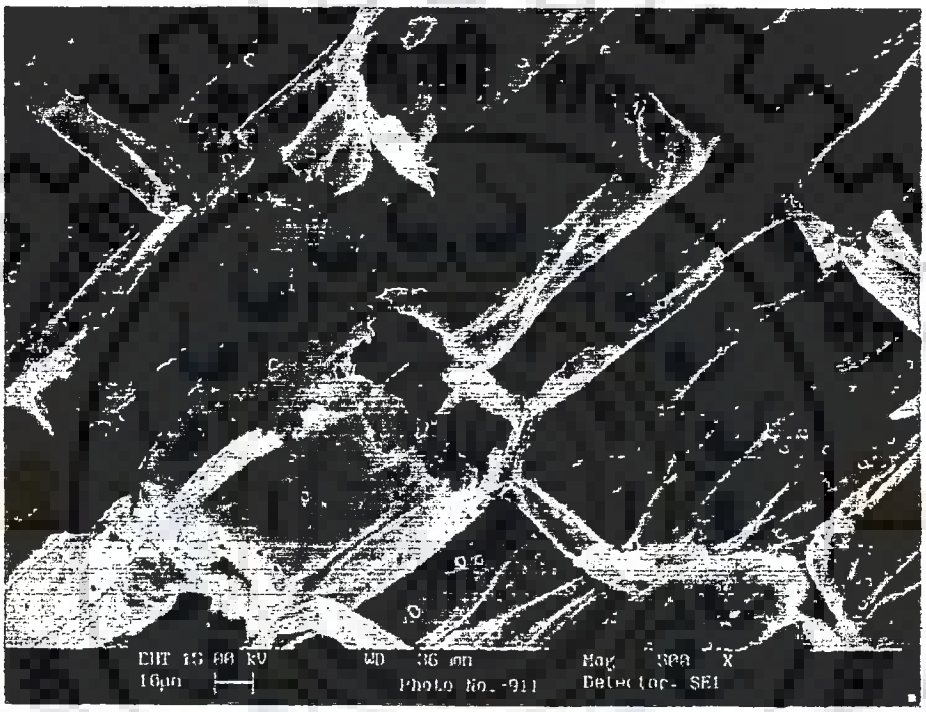


Fig. 4.20 (c) Configuration change in Kans grass biomass after dilute acid (H_2SO_4) pretreatment (with 2% H_2SO_4 with 90 minutes of reaction time at 120°C) and enzymatic saccharification (2% biomass loading, with 20 FPU/gdb of crude enzymes at 50°C for 96 minutes)

4.6.4 Structural and compositional changes

When Kans grass biomass was first pretreated with dilute H_2SO_4, it resulted in solubilization of hemicellulosic portion (77.05% of total hemicellulose) with little removal of lignin content (28.03% of total lignin) (Fig 4.19).Again, when this pretreated biomass was treated with crude enzyme mixture solubilization of 35% of holocellulose portion as total reducing sugars was observed.

The structural changes in Kans grass biomass before any pretreatment, after acid pretreatment and after acid as well as enzyme treatment were also studied. This is evident in the photographs (Fig. 4.20) As Fig. 4.20 (a) showed the intact Kans grass structure with out any pretreatment and was compared with Fig. 4.20 (b) (structure of acid treated biomass) where hemicellulosic portion was the most affected by acid treatment along with associated cellulosic structure. When this biomass was further treated with crude cellulase (2% w/v biomass loading, 20 FPU/g gdb), mainly cellulose was removed (Fig. 4.20 (c)) along with residual fraction of hemicellulose. However lignin and other components remained intact and the removal of cellulosic content by crude cellulase was observed.

4.7 Saccharification of NaOH pretreated Kans grass biomass

NaOH pretreatment was found to be more effective for the lignin removal; more than 50% of lignin was removed at pretreatment temperature of 120°C. Hence the solid residue remained after NaOH pretreatment at 120°C treated with different concentration of NaOH (0.5, 1, 1.5 and 2%) and duration (30, 60, 90 and 120 minutes) was further used for enzymatic saccharification with crude cellulase enzyme (20 FPU/gdb) with different biomass loading (2, 4, 5 and 6% w/v).

To investigate the optimum conditions for the enzymatic hydrolysis of pretreated Kans grass biomass (pretreated with NaOH at 120°C), was supplied with 20 FPU/gdb of crude mixture of enzyme (derived from *T.reesei* with 1.14 FPU total cellulase activity, 1.46 U/ml of CMCase and 6.6 U/ml of xylanase activity) in citrate buffer for 96 hours for different biomass loading (2, 4, 5 and 6% w/v) and then sugar liberated was estimated.

The overall reducing sugars production pattern after crude enzymatic saccharification can be clearly observed in Fig. 4.21 (a, b, c and d). A decreased in TRS after hydrolysis was observed with the increased concentrations of NaOH during pretreatment. As with maximum NaOH concentration (2%) the TRS was found to be in the range of 0.48-101 mg/gdb, the same was found to be in the range of 20.81-110.70 and 49.32-182.27 mg/gdb for 1.5 and 1% NaOH respectively while with 0.5% NaOH pretreated biomass yielded 80-350 mg/gdbTRS by enzymatic saccharification with crude enzyme.

The biomass obtained after pretreatment condition of 0.5% NaOH for 120 minutes was found to be the most suitable for the maximum recovery of TRS after enzymatic hydrolysis with different biomass loadings (2, 4, 5and 6%). With 2% Biomass loading 169mg/gdb TRS was obtained after saccharification with crude cellulase. When biomass loading was increased to 4% and 5% the TRS concentration was also increased to 298 and 350 mg/gdb respectively. However no further enhancement was observed with 6% biomass loading, rather a drop in TRS yield was observed (328 mg/gdb).It was a general observation in all experiments that with increase in biomass loading there was decrease in TRS. This may be due to stirring difficulties and low aqueous movable phase. A similar observations also recorded by Szczodrak and Lee [310, 311]. As higher concentration of NaOH and long residence time found to be more suitable for maximum removal of the lignin and low solid residue recovery may cause low total reducing sugars formation. The 120 minutes of pretreatment duration lead to maximum recovery of sugars during enzymatic saccharification (Fig. 4.22). At 0.5% NaOH concentration with 120 minutes of

pretreatment duration maximum sugars recovery of 62.85% was observed on the other hand a higher concentrations (2%) and longer duration (90 minutes) (which is the best condition of maximum lignin removal of 79.3%) resulted in lesser sugar release (15.58%) The reason may be due to presence of low holocellulose content after removal of 79.3% lignin. The NaOH concentration of 0.5% w/v also showed significant results with supply of different biomass loading and residence time by different researchers where 60-72% of lignin removal was observed [312, 313] which is an important step for hydrolysis.

4.7.1 Best condition for optimization

As per the experiments, best pretreatment condition was selected as 0.5% NaOH pretreatment for 120 minutes duration with 70.75% of lignin removal and 63% of the holocellulose was released as total reducing sugars. However the maximum removal of lignin was obtained (79.21%) at 2% NaOH and 90 minutes residence time, but at this condition only 15.41% of holocellulose release as soluble sugar was reported (Fig. 4.22). Since, lignin removal should not be the only criteria for an effective pretreatment as large amount of loss of carbohydrate at more sever conditions was found that may affect the enzymatic hydrolysis step. Hence, utilisation of low concentrations of chemical as well as loading of crude enzyme mixture was observed to be cost effective processes during this study.

4.7.2 Structural and compositional changes

The configuration changes were observed after SEM analysis of Kans grass biomass. A smooth structure of Kans grass biomass was observed before pretreatment (4.23 (a)). However due to NaOH pretreatment the patches formed that may be due to disruption of lignin (Fig. 4.23 (b)). Again, when this NaOH pretreated biomass was further treated with 20 FPU of crude cellulase, the disruption of the biomass structure was observed (Fig. 4.23 (c)) due to solubilization of holocellulosic portion due to enzyme action. The changes in composition also confirmed that lignin component was maximum removed by NaOH pretreatment, whereas the holocellulosic portion was solubalized in further treatment with crude cellulase enzyme (Fig. 4.24).

4.8 Comparison of enzymatic saccharification/hydrolysis with dilute H_2SO_4 and NaOH pretreated biomass

As both acid as well as NaOH pretreatments are important in majorly solubilization of hemicellulosic and lignin portion of lignocellulosic biomass respectively.

The over all comparison in both pretreatments (NaOH and H_2SO_4) and then further enzymatic saccharification (with crude enzyme) is shown in Fig. 4.25. When original biomass was supplied for H_2SO_4 pretreatment, it resulted in solubilization of hemicellulosic sugars (77.05%), cellulosic (19.17%) as well as lignin (28.03%). Hence, 71.97% of lignin was still present in acid treated biomass and when this biomass was supplied with crude cellulase enzyme (from *T.reesei*), Lignin acted as the obstacle between the enzyme and cellulosic portion (Fig. 4.25). This biomass was further applied for enzymatic saccharification and 34.9% of total holocellulose (H_2SO_4 pretreated biomass) was solubalize in to total reducing sugars.

On the other hand when the original biomass was pretreated with NaOH, it resulted in the removal of lignin (70.75%) with little liberation of total reducing sugars from holocellulose. Now in the next step when this biomass (NaOH pretreated) was further used for enzymatic saccharification, it resulted in the removal of 350mg/g of biomass of sugar which is 63% to total NaOH pretreated holocellulose. This is due to better exposure of cellulosic fiber to enzyme as a result of lignin removal; where dilute H_2SO_4 pretreatment could not do this job which only solubalize hemicellulosic portion better. Thus it can be concluded from this study that as far as cellulose solubilization is concerned lignin is a obstacle which needs to be targeted first (in term of lignin removal) Then the non crystalline inter linking hemicellulose should be solubalize.

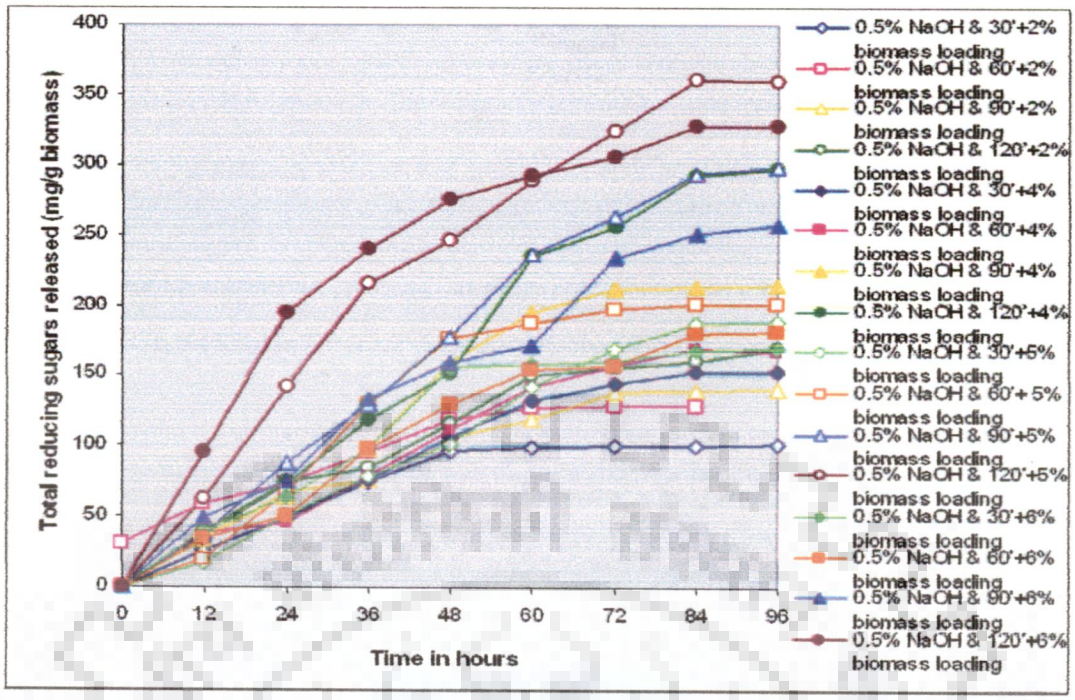


Fig. 4.21 (a) Enzymatic saccharification of 0.5% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120°C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v)

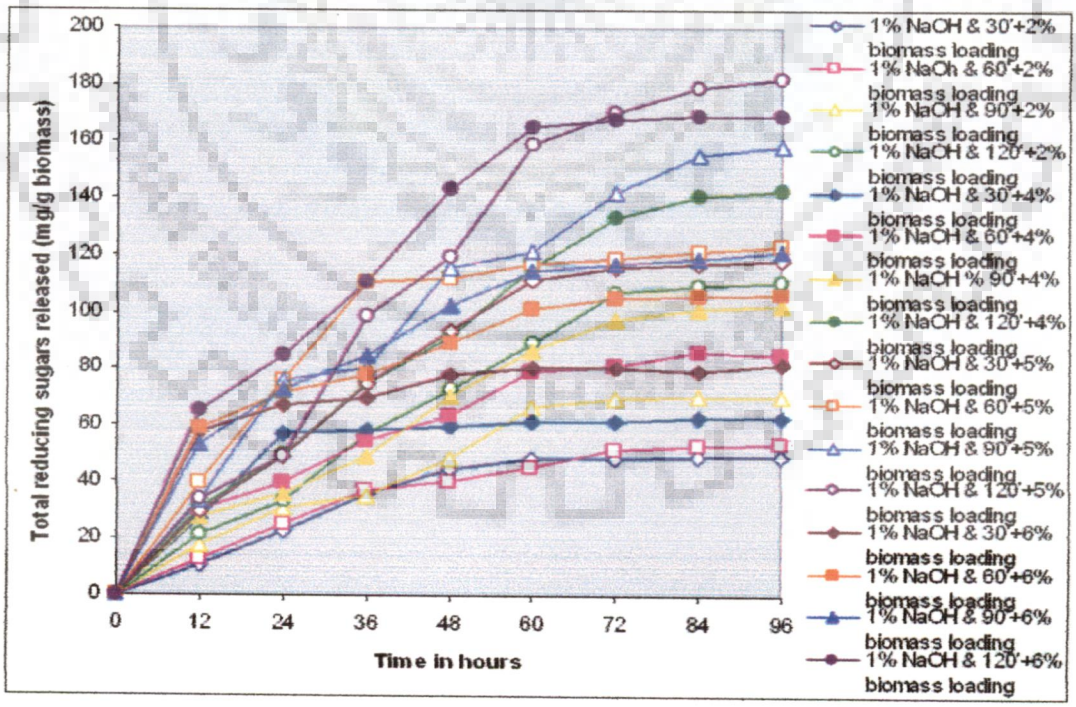


Fig. 4.21 (b) Enzymatic saccharification of 1 % (w/v) NaOH pretreated Kans grass biomass (pretreated at 120°C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v)

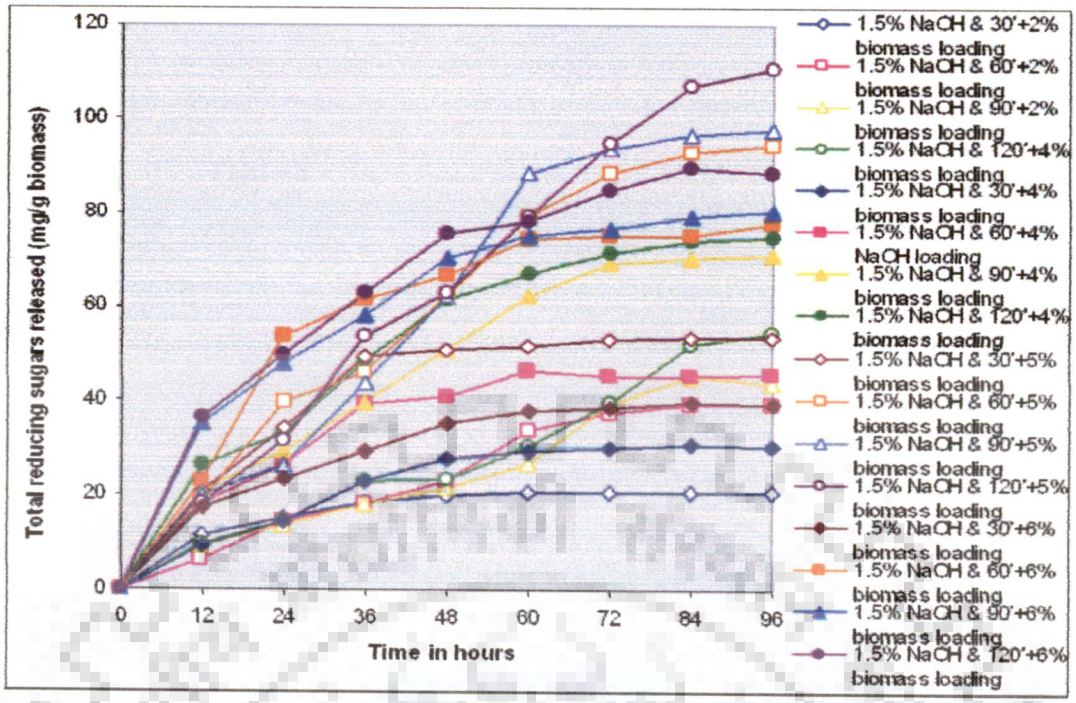


Fig. 4.21 (c) Enzymatic saccharification of 1.5% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120°C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v)

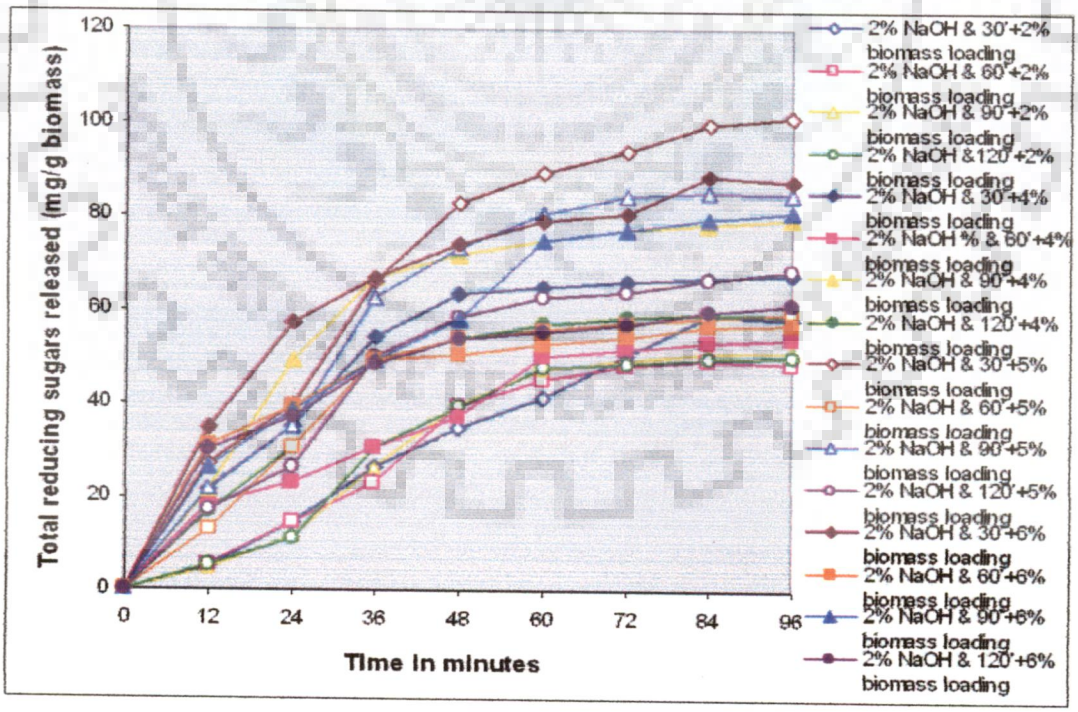


Fig. 4.21 (d) Enzymatic saccharification of 2% (w/v) NaOH pretreated Kans grass biomass (pretreated at 120°C for different duration) with crude cellulase (20 FPU/gdb) for different biomass loadings (2, 4, 5 and 6% w/v)

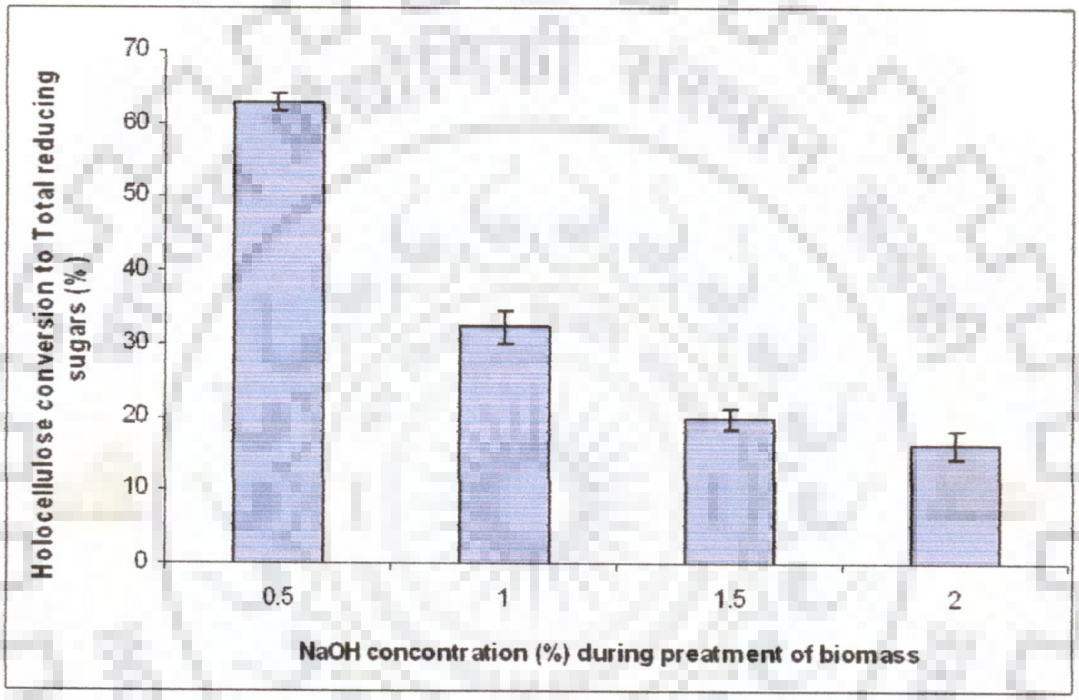


Fig. 4.22 Conversion of holocellulose (%) to total reducing sugars after crude enzymatic saccharification of NaOH pretreated Kans grass with different concentration of NaOH (0.5, 1, 1.5 and 2% w/v) at pretreatment reaction time of 120 minutes

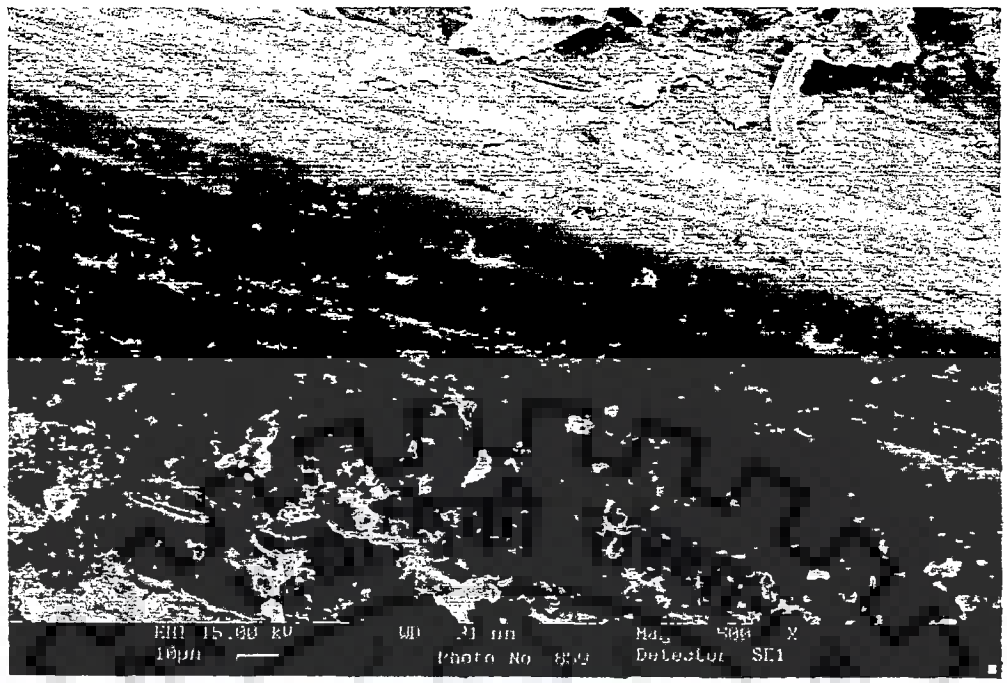


Fig. 4.23 (a) Configuration of Kans grass biomass before NaOH pretreatment (Original biomass)

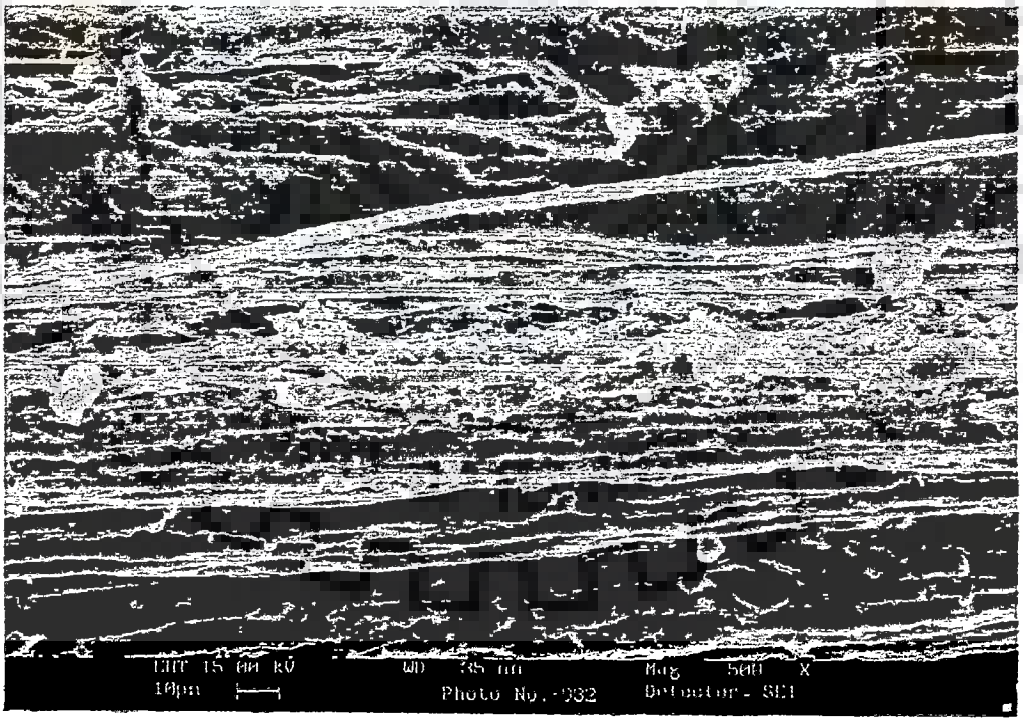


Fig. 4.23 (b) Configuration change in Kans grass biomass after NaOH pretreatment (with 0.5 % NaOH with 120 minutes of residence time at 120°C)

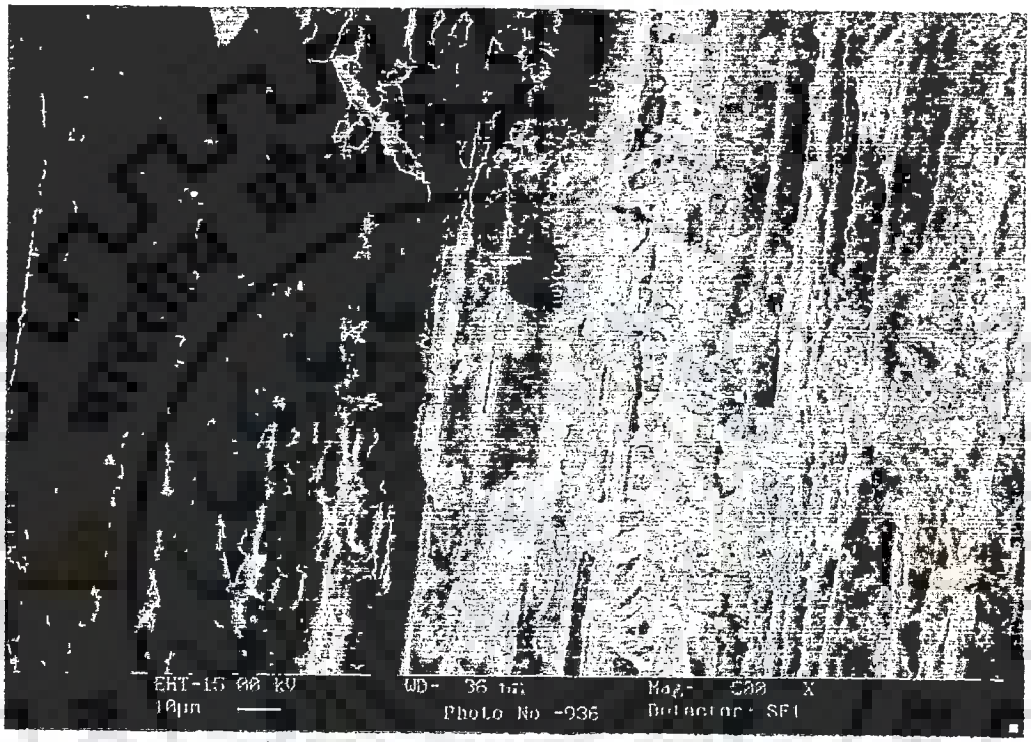


Fig. 4.23 (c) Configuration change in Kans grass biomass after alkali pretreatment (with 2% NaOH at 120°C for 90 minutes) and enzymatic saccharification (2% biomass loading, with 20 FPU/gdb of crude enzymes at 50°C for 96 minutes)

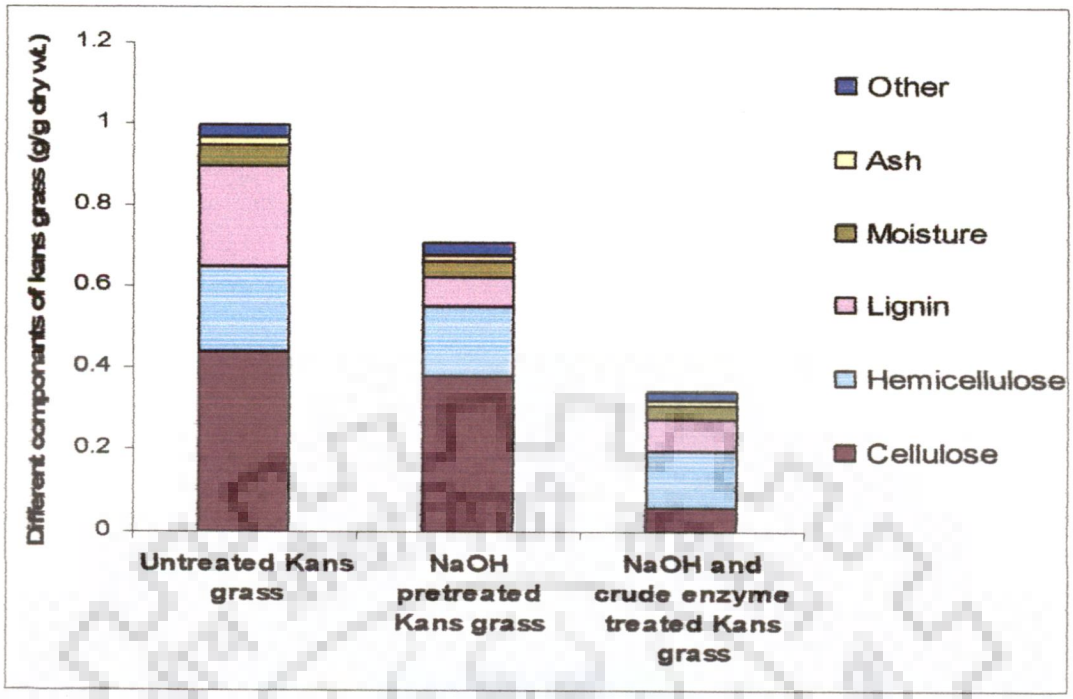


Fig. 4.24 Compositional change in Kans grass biomass before pretreatment, after dilute NaOH pretreatment and enzymatic saccharification with crude cellulase

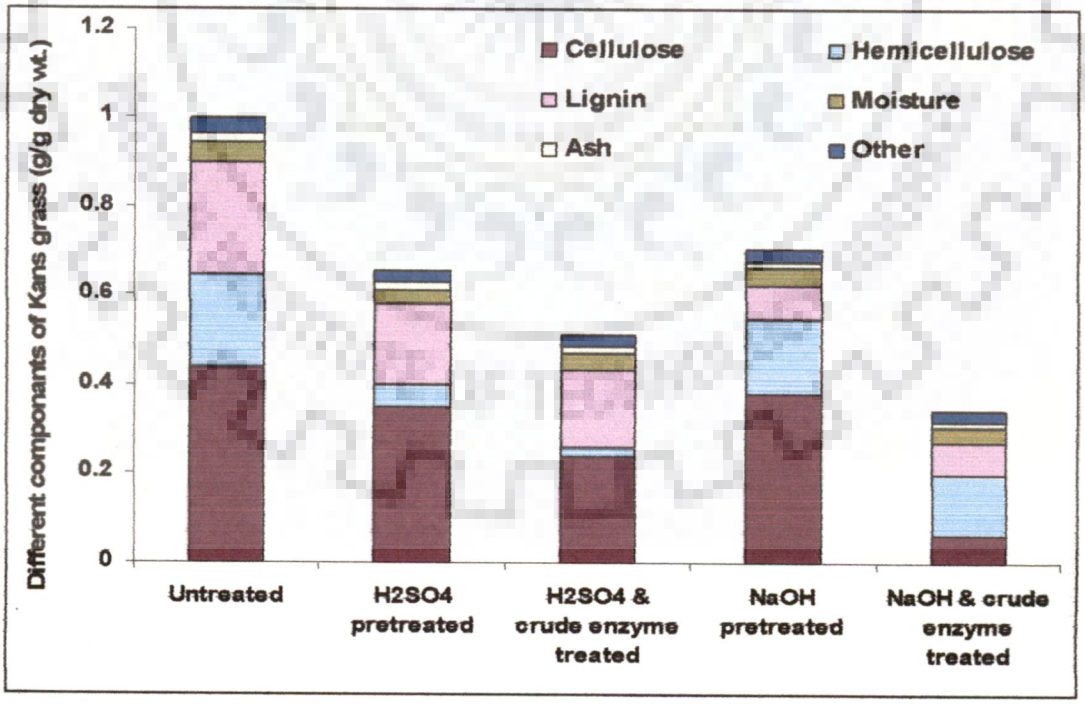


Fig. 4.25 A comparison in untreated, dilute H_2SO_4 and NaOH pretreated biomass treated with crude enzyme

4.9 Ethanol production

TRS obtained from different routes including dilute acid pretreatment as well as sugar hydrolysate obtained after enzymatic saccharification of H_2SO_4 and NaOH pretreated biomass was utilized for ethanol production by using *P. stipitis* and *S. cerevisiae*. As acid hydrolysate was composed of mixture of sugars (xylose, arabinose, mannose, galactose and glucose), hence *P. stipitis* was utilized for ethanol production. An adaptive strain of *P.stipitis* was also developed and compared with the wild strain. This strain was further used in entire studies. *S. cerevisiae* is a glucose utilizing yeast and was used for ethanol production from enzymatically saccharified hydrolysate.

4.9.1 Development of acid adaptive strain

In order to utilize low-cost lignocellulosic feedstocks for bioethanol production, the lignocelluloses need to be degraded to simple sugars for microbial utilization in fermentation. However, commonly used dilute acid hydrolysis generates many inhibitory compounds which suppress yeast growth. Consequently, after detoxification process partial removal of toxic compounds is observed, these remained inhibitors in the hydrolysate may inhibit the fermentation step.

Acid hydrolysate obtained after dilute H_2SO_4 pretreatment is composed of 11.66 g/l TRS sugars (Table 4.12) with 2.26 g/l of furfural and 1.19 g/l acetic acid concentration (Fig. 4.26). As concentration of furfural above 2g/l may inhibit the microbial growth as well as metabolism [232], hence a detoxification process was performed to minimize the inhibitory compounds concentration so that microbes can tolerate and grow in this DAH media. However, after detoxification step a trace of toxic compounds including acetic acid (0.33 g/l) and furfural (1.11 g/l) remained in detoxified acid hydrolysate (DAH) (Fig. 4.26) which causes the delay in growth and low ethanol production.

So, adaptation against inhibitors in yeast was performed by sequentially transferring and growing the cell in the media containing different concentration of non detoxified acid hydrolysate (20, 40, 60 and 80%) with supplementation of other media components (given in section material and methods). The growth pattern of the *P. stipitis* is given in Fig. 4.27 which shows a very long lag phase when hydrolysate media composed of 20% untreated acid hydrolysate. The prolong lag phase and slow growth is due to presence of inhibitory compounds (furfural and acetic acid). When the concentration of hydrolysate increased to 40 and 60% a slower growth pattern was observed, however with 80% acid hydrolysate no growth was observed. Hence a strain that can tolerate up to 60% non detoxified acid hydrolysate was used for the further experiments. Several studies also

have reported for development of adaptive strains for improved bioethanol yield [314, 315]. A control experiment without acid hydrolysate (0% hydrolysate) was also performed with all other media components and comparative early growth pattern was observed as no toxic compounds were present.

4.9.2 Comparison with wild strain by ethanol production in detoxified acid hydrolysate (DAH)

DAH was further used for the fermentation experiments with adaptive as well as with wild strains of *P. stipitis*. In wild strain 2.32 g/l of ethanol was determined at the end of 30 hours (Fig. 4.28 (a)) whereas in adaptive strain maximum ethanol production was estimated to be 2.67 g/l in 22 hours (Fig. 4.29 (a)) The ethanol yield (Yp/s) and biomass yield (Yx/s) was found to be 0.28 g/g and 0.56 g/g respectively in adaptive strain which are higher than the wild strain (Yp/s, 0.23g/g; Yx/s, 0.48) (Table 4.13). A parallel control experiment for wild as well as adaptive strain in synthetic sugars media was also performed (Fig. 4.28 (b) and 4.29 (b)). A shorter lag phase was observed with adapted strain in comparison to wild strain during the fermentation of DAH (Fig. 4.28 (a) and 4.29 (a)). This may be due to the fact that the adapted strain, during the prolonged cultivation on none detoxified acid hydrolysate (20, 40 and 60%) had developed resistance to different stresses (inhibitory compounds) present in the hydrolysate and thus strain was found to be more tolerance against inhibitory compounds. It is also suggested that under these unfavourable conditions microorganism might have geared to synthesize new enzymes and/or metabolites which lead to efficient metabolism of sugars and tolerance to the inhibitors [153].

This study described the use of an adaptive strain of *P. stipitis* tolerant to toxic compounds (furfural and acetic acid) which might have remained even after detoxification of Kans grass acid hydrolysate. The adaptive strain was found to be more efficient than wild strain in terms of bioethanol (0.28 g/g) as well as biomass yield (0.56 g/g).

The neutralized acid hydrolysate media that is composed of neutralized acid hydrolysate (no detoxification step only pH was adjusted) was also tested for the fermentation with wild as well as 'adaptive' strain ((Fig. 4.28 (c) and 4.29 (c)). As neutralized media is composed of inhibitory and phenolic compounds that may inhibit the microbial growth, hence a slow growth with extended lag phase of 38 and 22 hours for wild and adaptive strain was observed respectively. However the maximum ethanol production was observed after 72 hours using wild strain and 40 hours using adaptive strain with ethanol yield of 0.12 g/g and 0.22 g/g respectively (Table 4.13). Adaptive strain able to

tolerate inhibitory compounds generated during acid pretreatment was better due to genetic changes or activation/deactivation of certain genes thereby change in metabolite level and their secretion. So the ethanol production was better in case of adaptive strain. A detail comparative analysis in fermentation parameters of wild as well as adaptive strain in DAH, synthetic and neutralized media is also given in Table 4.13

Hence, based on the observations of increasing non detoxified hydrolysate yeast response to toxic compounds, an adaptation method is developed. This adapted *P. stipitis* strain was also evaluated for producing high ethanol yield and demonstrated it's significantly higher levels of tolerance to toxic compared with the parental strains under same conditions. This adapted and more tolerant strain showed significant improved cell growth and produced normal yield of ethanol in the presence of furfural or acetic acid

Table 4.12 Composition of dilute acid (H_2SO_4) hydrolysate of Kans grass biomass after optimization for maximum sugars formation

Component	**Values in g/l**
Total reducing sugars	11.66±0.32
Xylose	5.13±0.25
Arabinose	3.34±0.21
Glucose	0.94±0.089
Other fermentable sugars (mannose, cellobiose , galactose)	2.19±0.17

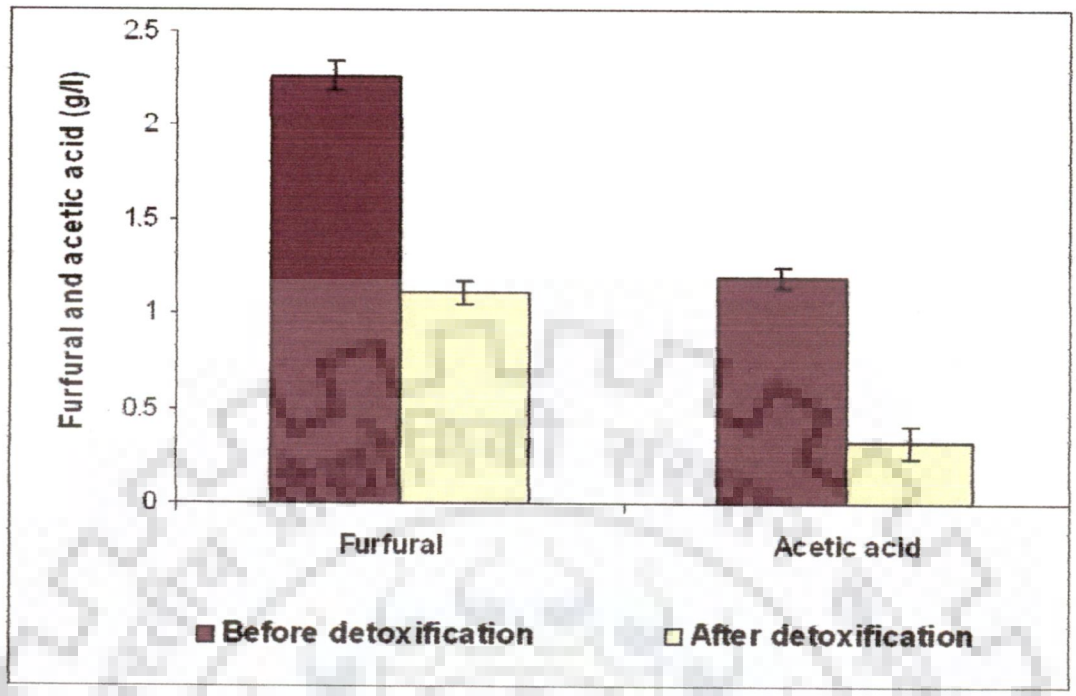


Fig. 4.26 Toxic compounds present before and after detoxification of acid hydrolysate (11.66g/l). The detoxification resulted in the reduction of the furfural and acetic acid concentration to 1.21 and 0.32 respectively

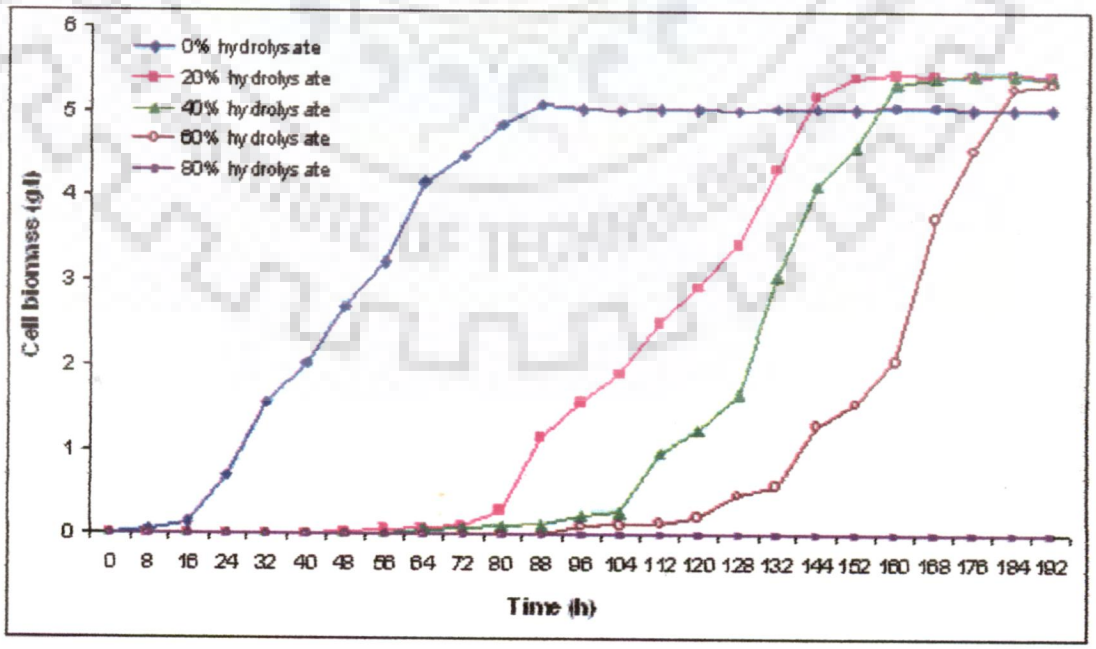


Fig. 4.27 Growth pattern of *P. stipitis* in hydrolysate media different concentration of untreated acid hydrolysate (0, 20, 40, 60 and 80%)

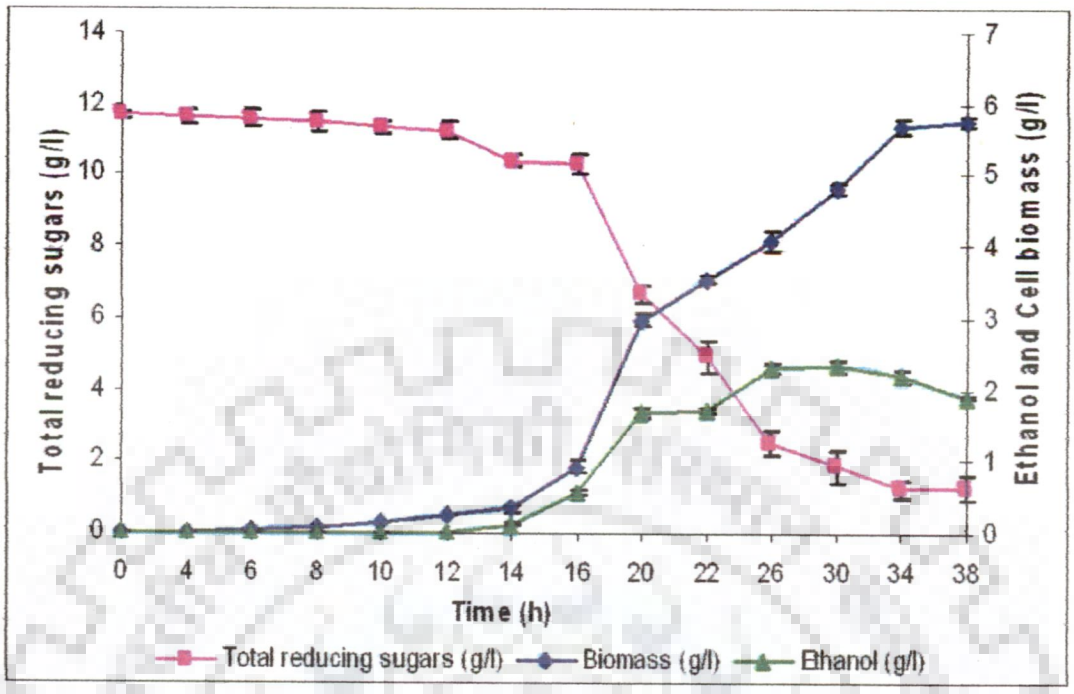


Fig. 4.28(a) Fermentation profile of wild strain of *P. stipitis* in detoxified acid hydrolysate (DAH) media

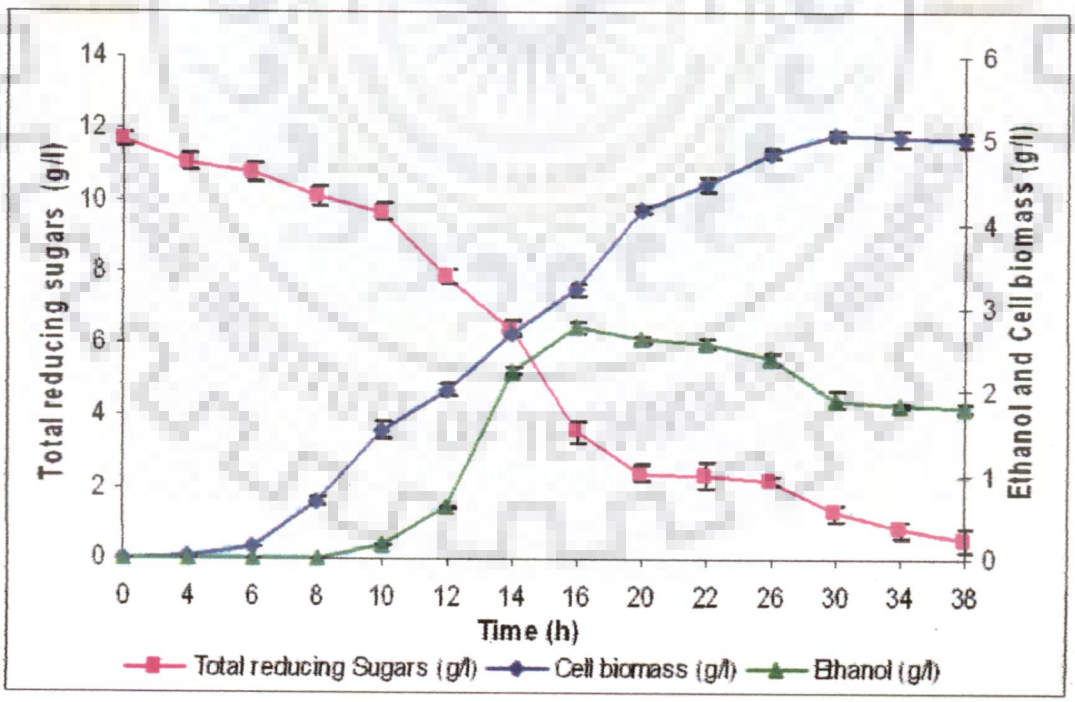


Fig. 4.28(b) Fermentation profile of wild strain of *P. stipitis* in synthetic media

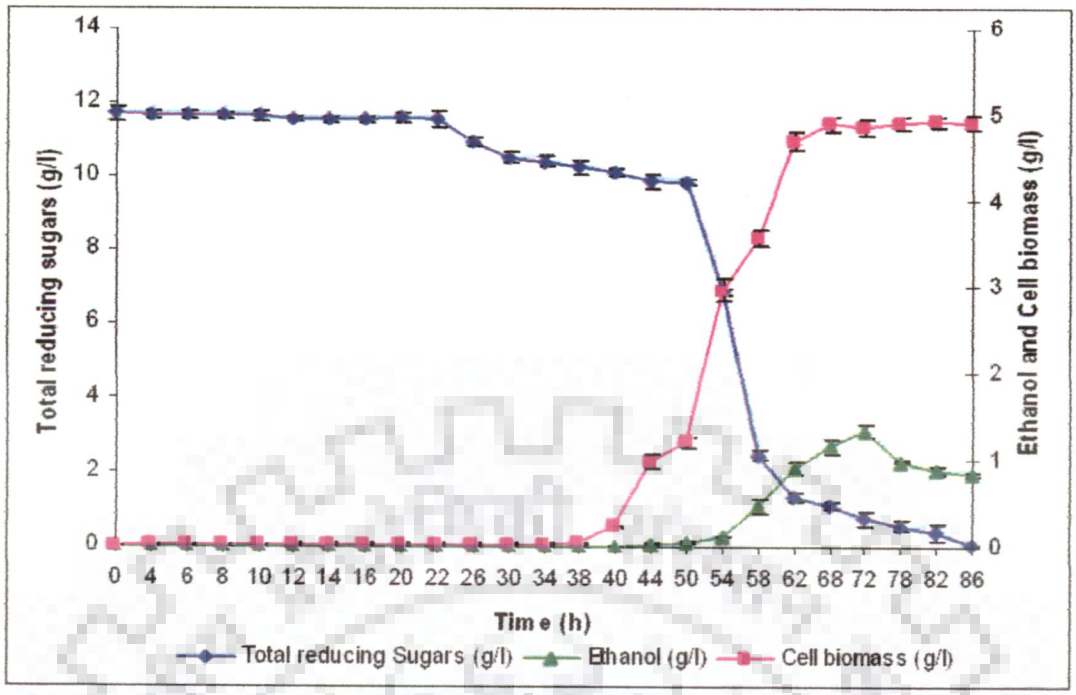


Fig. 4.28(c) Fermentation profile of wild strain of *P. stipitis* in neutralised media

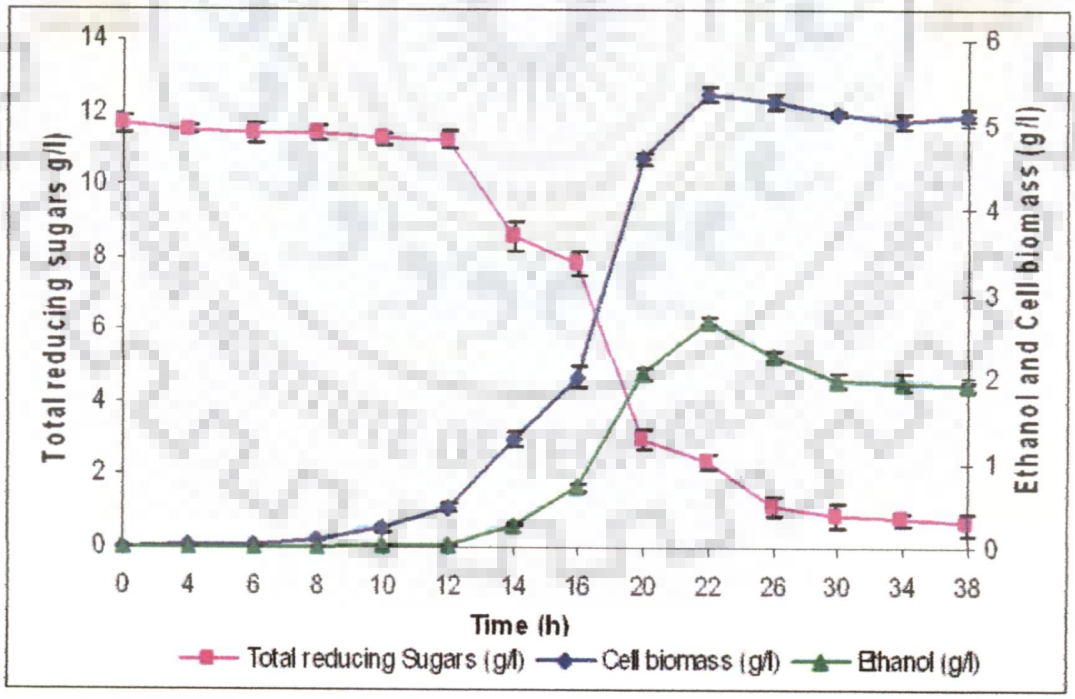


Fig. 4.29(a) Fermentation profile of 'adaptive strain' of *P. stipitis* in detoxified acid hydrolysate (DAH) media

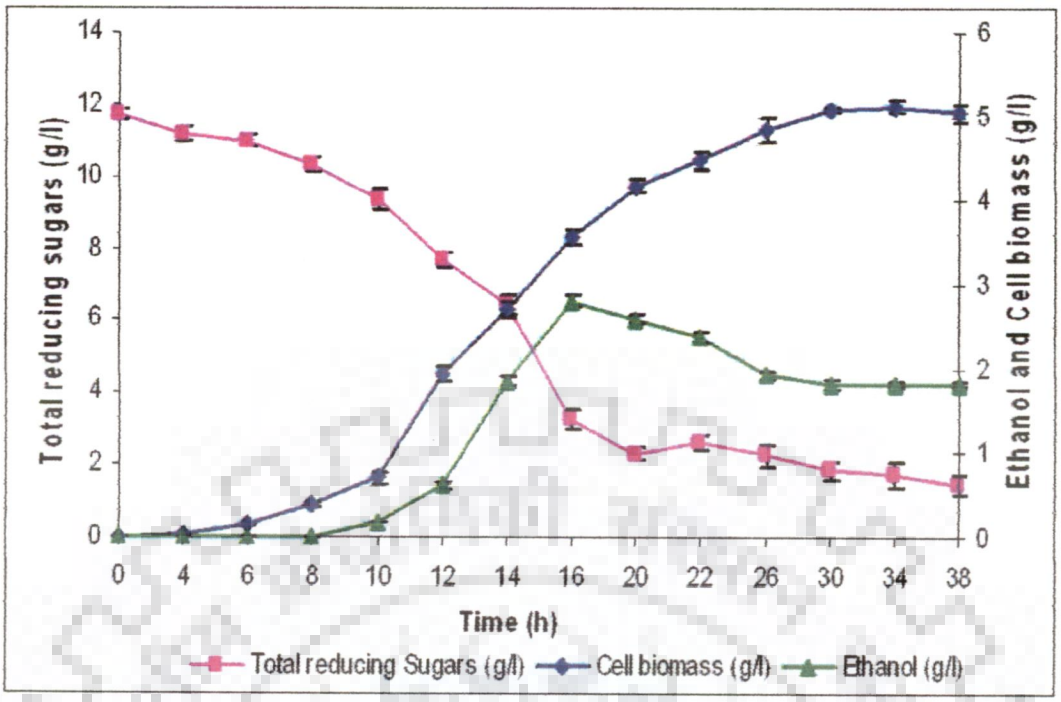
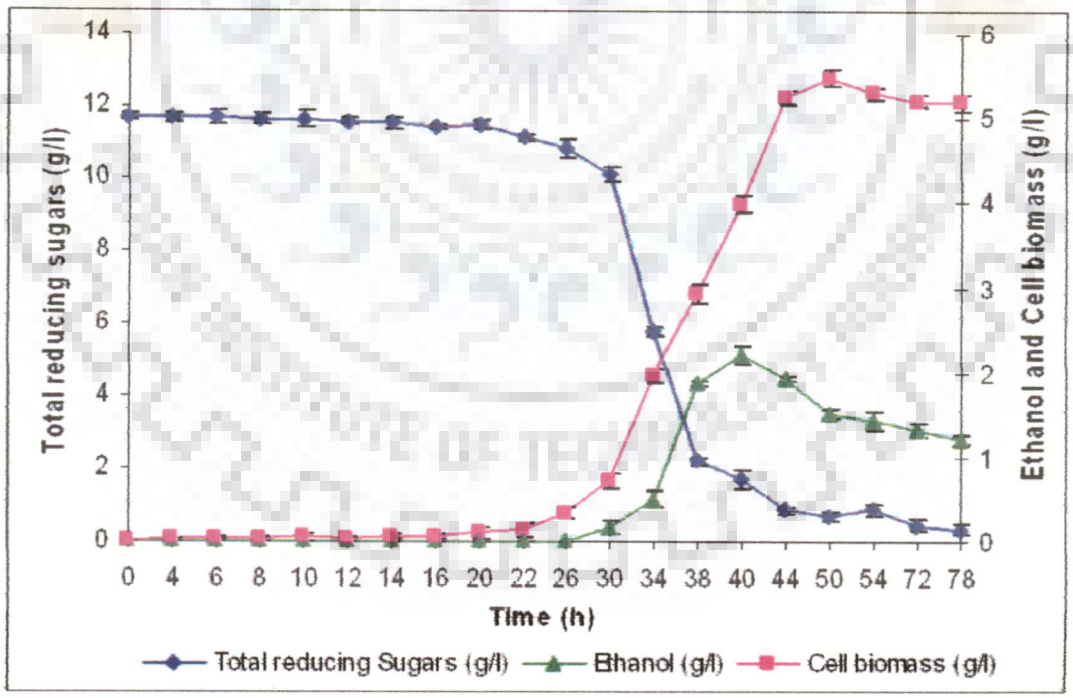


Fig. 4.29(b) Fermentation profile of 'adaptive strain' of *P. stipitis* in synthetic media



(c)

Fig. 4.29(c) Fermentation profile of 'adaptive strain' of *P. stipitis* in neutralised media

Table 4.13 comparative table of different fermentation parameter for wild (W) and adaptive (A) strain in detoxified acid hydrolysate (DAH), synthetic and neutralised media

Fermentation parameters	*P.stipitis* (W) in DAH media	*P.stipitis* (W) in synthetic media	*P.stipitis* (W) in neutralised media	*P.stipitis* (A) in DAH media	*P.stipitis* (A) in Synthetic media	*P.stipitis* (A) in neutralised media
Initial total reducing sugars, (g/l)	11.66±0.23	11.66±0.35	11.66±0.26	11.66±0.14	11.66±0.35	11.66±0.32
Maximum ethanol concentration (g/l)	2.32±0.032	2.76±0.054	1.33±0.041	2.67±0.022	2.80±0.021	2.21±0.32
Time (hours)	30	16	72	22	16	40
Sugar consumed (%)	84.91±1.32	69.05±2.21	93.96±0.156	80.05±1.72	72.11±2.21	85.77±1.26
Specific growth rate, μ (h^{-1})	0.29±0.021	0.409±0.022	0.22±0.015	0.29±0.016	0.41±0.021	0.18±0.12
Ethanol yield coefficient, Yp/s (g/g)	0.23±0.015	0.33±0.019	0.12±0.010	0.28±0.012	0.33±0.014	0.22±0.011
Biomass yield coefficient ,Yx/s (g/g)	0.48±0.032	0.39±0.029	0.44±0.15	0.56±0.019	0.42±0.023	0.40±0.12
Ethanol productivity, Qp (g/l/h)	0.077±0.003	0.20±.008	0.018±0.0025	0.122±0.011	0.17±0.021	0.05±0.0021
Growth rate, Qx (g cells/l/h)	0.16±0.021	0.20±0.018	0.067±0.0021	0.239±0.032	0.22±0.026	0.099±0.0021
Max. sugars consumption rate, Qs (g/l/h)	0.32±0.011	0.72±0.013	0.16±0.012	0.422±0.019	0.52±0.021	0.29±0.0010
Theoretical yield, □ (%)	46.19±1.32	56.53±1.52	23.92±1.21	56.04±1.76	65.±1.36	43.55±0.97

4.9.3 Ethanol production from concentrated DAH (60 and 90.50 g/l) released from acid pretreated biomass

Dilute H_2SO_4 pretreatment resulted in the liberation of 11.66 g/l of total reducing sugars. The total reducing sugars concentration of acid hydrolysate was enhanced from 11.66 g/l to 60 and finally to 90.50 g/l using vacuum evaporation techniques. As the total reducing sugars concentration was increased the content of the constituent sugars was also found to be enhanced. Xylose, a major component of the hydrolysate, was also enhanced to 20.54 and 27.60 g/l in both concentrated hydrolysate (60 and 90.50 g/l). The concentrations of all other sugars components are listed in Table 4.14. With the increase of concentration of sugars in the acid hydrolysate the concentrations of toxic compounds were also enhanced. The furfural and acetic acid concentrations was increased to 2.47 and 8.46 g/l respectively in 60 g/l concentrated acid hydrolysate (Fig.4.30 (a)) however the same were enhanced to 2.65 and 10.02 g/l respectively in 90.50 g/l acid hydrolysate (Fig. 4.30 (b)). After detoxification step the concentrations of these toxic compounds were minimized (approximately 2 times in furfural and 4 times in acetic acid concentrations) to prevent the inhibition of yeast.

Fermentation of the hydrolysate media was carried out by adapted *P.stipitis* for ethanol production. In 60 g/l sugars concentration, a prolong lag phase was observed and after 24 hours, the cell biomass concentration was found to be 0.30 g/l and the maximum ethanol (20.13 g/l) was found after 72 hours (Fig. 4.31 (a)), however cell biomass in hydrolysate media with TRS 90.50 g/l was of 0.35 g/l for same duration of time (24 hours) (Fig. 4.32 (a)). Also, the maximum concentration of ethanol was found to be 27.18 g/l after 72 hours. A parallel set of experiments were also performed with synthetic media as a control for both concentrations of TRS (Fig. 4.31 (b) and 4.32 (b).

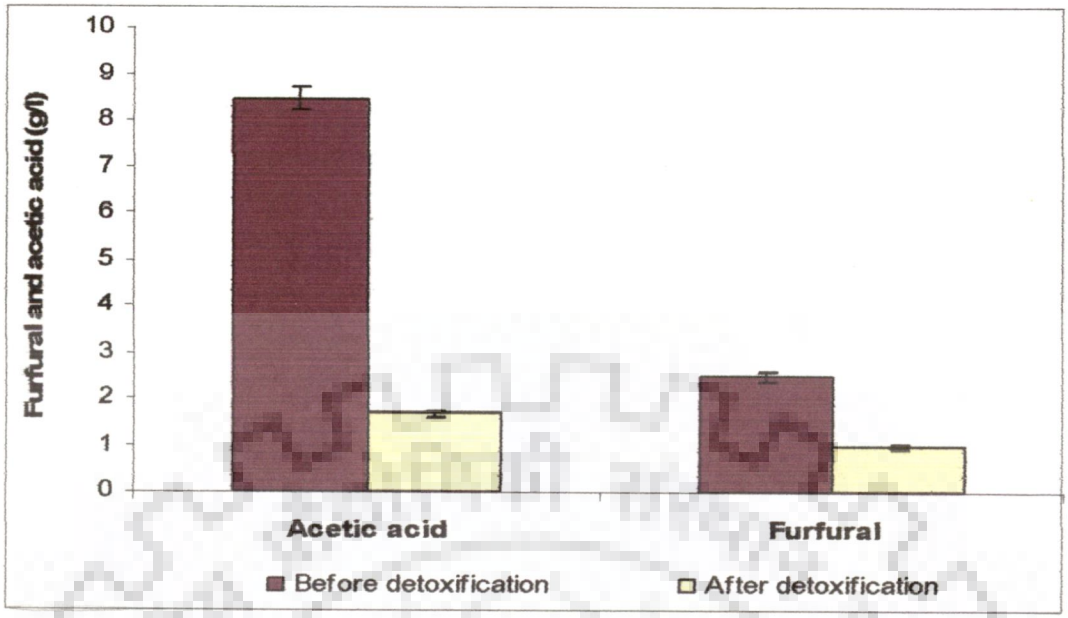
The ethanol yield was found to be 0.41 g/g in 60 g/l TRS hydrolysate whereas in synthetic media it was observed to be 0.46 g/g (Table 4.15). In hydrolysate media with TRS concentration of 90.50 g/l the Yp was 0.38 g/g. The low ethanol yield in 90.50 g/l hydrolysate may be due to presence of little more toxic compounds (2.65 acetic acid and 1.5 g/l furfural) in comparison to 60 g/l (2.47 g/l acetic acid and 1.17 g/l furfural) or substrate inhibition. Hence low concentration of total reducing sugars (60 g/l) resulted in a better ethanol yield in comparison to the high concentration after detoxification.

Several other studies have been done with olive tree [216]; wheat straw [217]; Water-hyacinth [218] and sugarcane bagasses [219] for ethanol synthesis in acid hydrolysate. The ethanol yield obtained from present study is comparable to the several other studies carried out by different authors (Table 4.16). Hence, the ethanol yield by

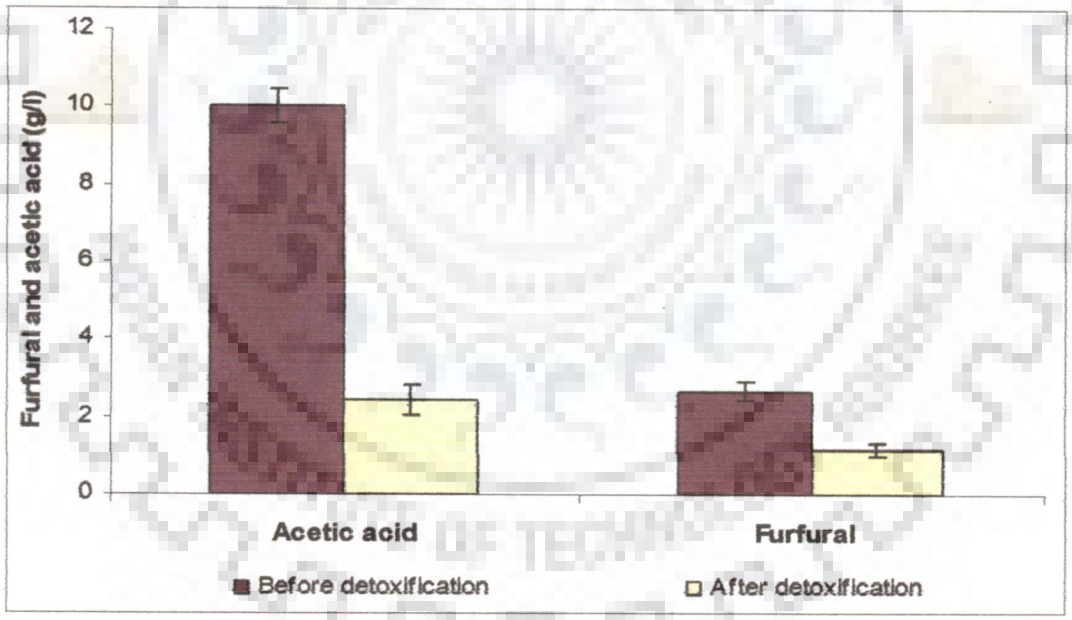
adaptive *P. stipitis* at different concentration of TRS in detoxified acid hydrolysate were found to be 0.28, 0.41 and 0.38 g/g for 11.66, 60 and 90.50g/l of TRS respectively (Fig. 4.33). However the hydrolysate with TRS concentration of 60g/l was found to be optimum concentration for a better yield.

Table 4.14 Sugars composition in acid hydrolysate with of 60 and 90.50 g/l total reducing sugars (TRS)

Component	60 g/l TRS	90.50 g/l TRS
Total reducing sugars	60.50±0.52	90.50±0.45
Xylose	20.54±1.12	27.60±0.98
Arabinose	18.26±1.52	28.43±0.62
Glucose	6.25±0.89	8.29±0.87
Other fermentable sugars (Mannose, cellobiose, galactose etc.)	14.95±0.94	27.32±0.53

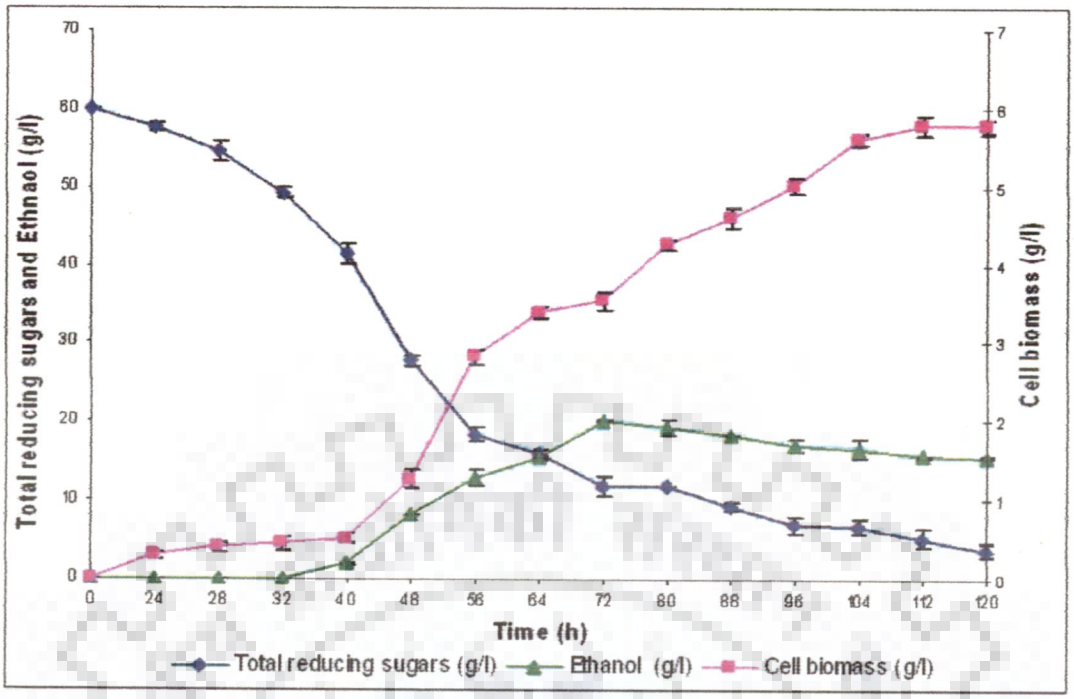


(a)

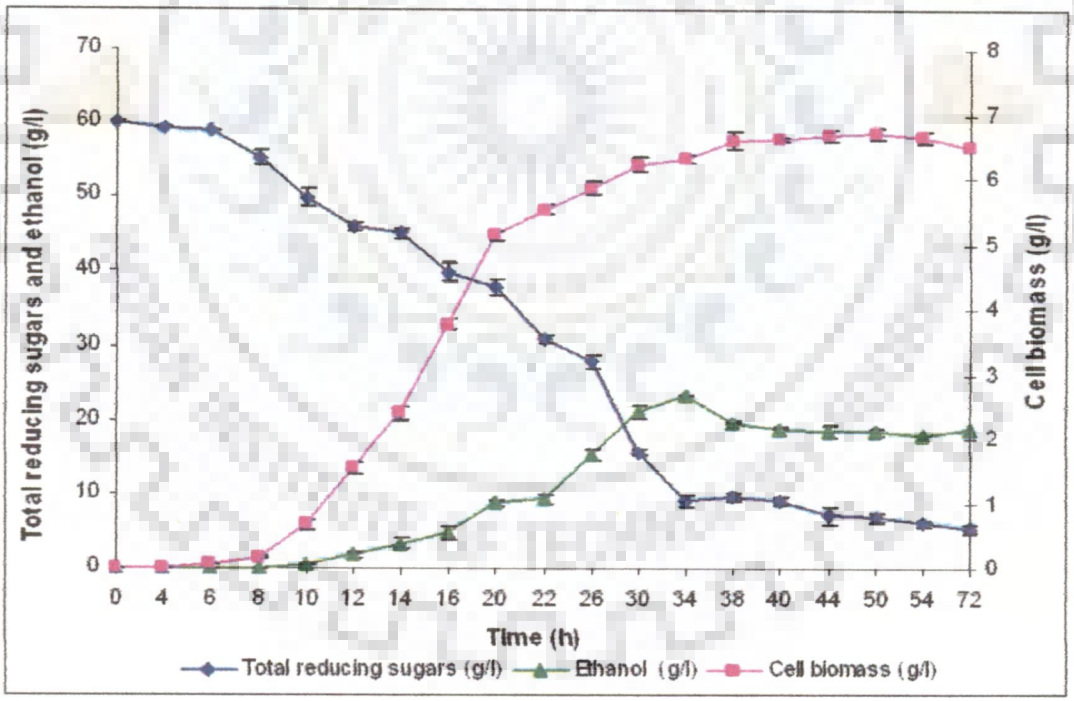


(b)

Fig. 4.30 Toxic compounds, Acetic acid and furfural concentration before and after detoxification in **(a)** 60 g/l of acid hydrolysate **(b)** 90 g/l acid hydrolysate

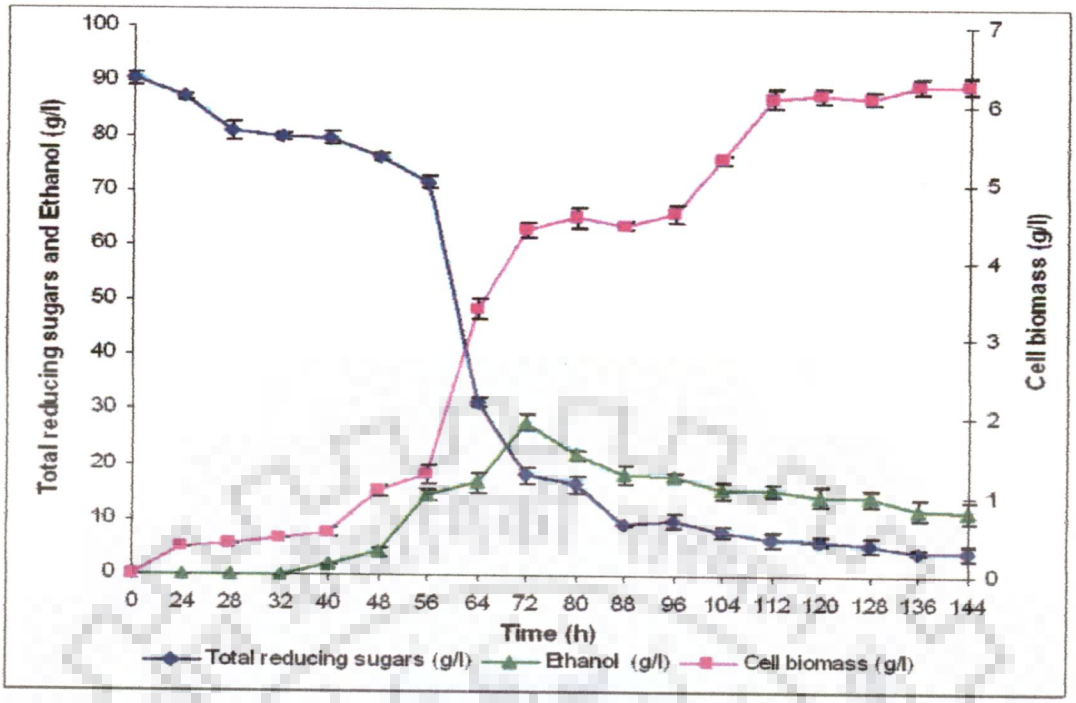


(a)

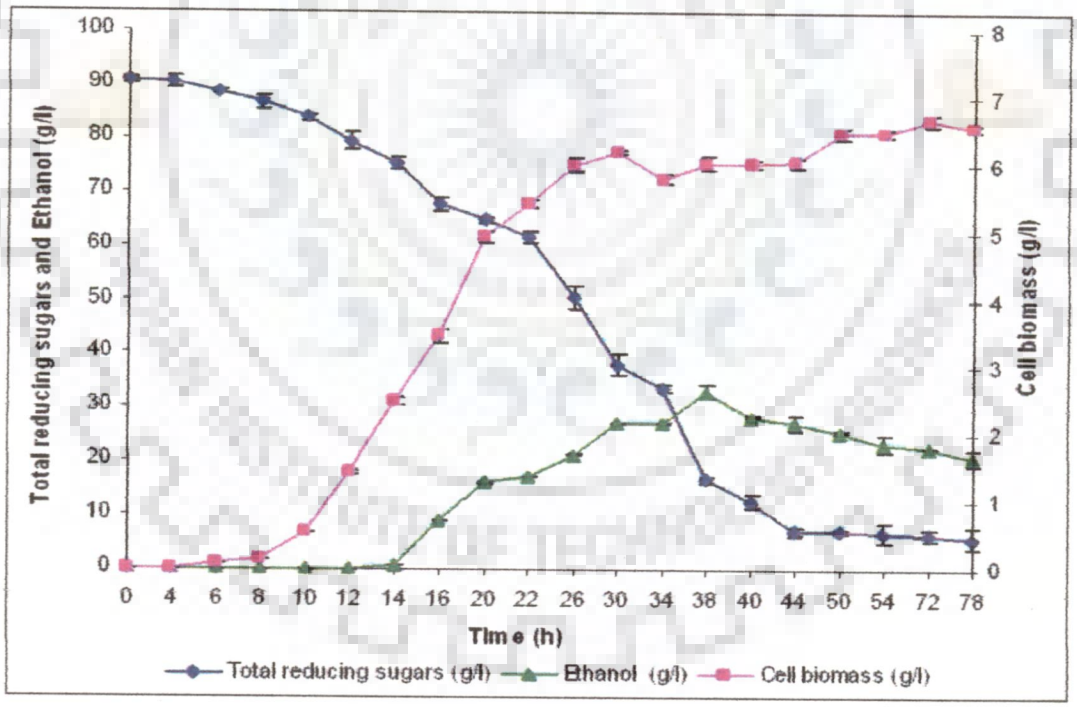


(b)

Fig. 4.31 Fermentation profile of adaptive strain of *P. stipitis* **(a)** hydrolysate (60 g/l) **(b)** synthetic media (60 g/l)



(a)



(b)

Fig. 4.32 Fermentation profile of adaptive *P. stipitis* (a) hydrolysate (90 g/l) (b) synthetic media (90 g/l)

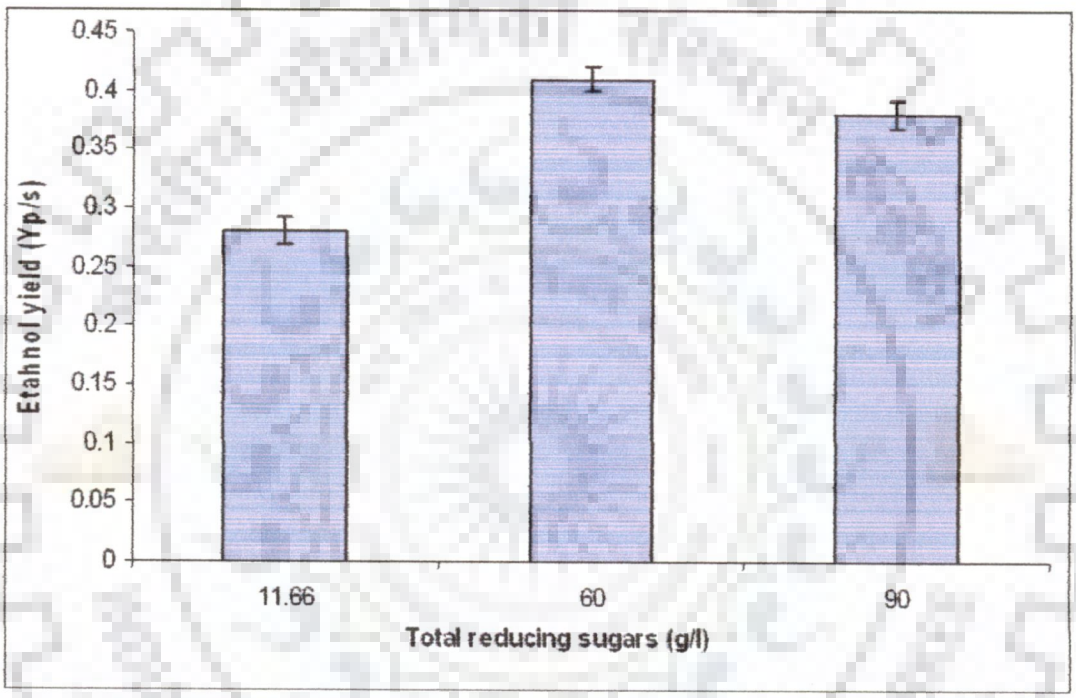


Fig. 4.33 A comparison in ethanol yield (Yp) in different concentration of total reducing sugars (11.66, 60 and 90.50g/l)

Table 4.15 Comparative table of different fermentation parameter for *P.stipitis* in detoxified acid hydrolysate (DAH) and synthetic media with concentration of 60 and 90 g/l

Fermentation parameters	60 g/l		90 g/l	
	Hydrolysate media	**Synthetic media**	**Hydrolysate media**	**Synthetic media**
Initial total reducing sugars, (g/l)	60±0.15	60±0.20	90.50±0.21	90.50±0.35
Maximum ethanol concentration (g/l)	20.13±0.51	23.45±0.62	27.81±0.78	33.12±0.93
Time (hours)	72	34	72	38
Sugar consumed (%)	80.21±1.25	84.70±2.32	72.23±1.68	73.78±1.93
Specific growth rate, μ (h^{-1})	0.117±0.032	0.211±0.010	0.104±0.016	0.237±0.003
Ethanol yield coefficient, Yp/s (g/g)	0.41±0.011	0.46±0.028	0.38±0.012	0.44±0.042
Biomass yield coefficient ,Yx/s (g/g)	0.07±0.005	0.12±0.035	0.06±0.019	0.08±0.005
Ethanol productivity, Qp (g/l/h)	0.28±0.027	0.69±.075	0.39±0.011	0.87±0.01
Growth rate, Qx (g cells/l/h)	0.05±0.002	0.19±0.032	0.06±0.032	0.16±0.031
Max. sugars consumption rate, Qs (g/l/h)	0.67±0.031	1.49±0.012	1.0±0.019	1.94±0.041
Theoretical yield, □ (%)	82.00±1.32	90.05±1.21	75.51±1.81	88.02±1.52

Table 4.16 Ethanol productions from acid hydrolysate of various lignocellulosic materials by different researchers

Substrate	Microrganism	Yp (g/g)	Qp (g/l/h)	Reference
Wheat straw NREL Y-7124	*P. stipitis*	0.35	0.30	[255]
Wheat straw NREL Y-7124 (Adaptive strain)	*P. stipitis*	0.41	0.54	[255]
Sugarcane baggages	*P.tannophilis*	0.34	0.57	[259]
Water-hyacinth NCIM-3497	*P. stipitis*	0.42	0.17	[260]
Olive tree CECT No.1922	*P. stipitis*	0.35-0.42	0.17-0.36	[257]
Kans grass NCIM-3497	***Pichia stipitis***	***0.41***	***0.28***	***Present work***

4.9.4 Ethanol production by dilute acid (H_2SO_4) pretreated Kans grass hydrolysate obtained after enzymatic hydrolysis

The acid pretreated Kans grass biomass was enzymatically hydrolysed with crude enzyme mixture (obtained after optimization) and the resulted sugar obtained was concentrated to 10 g/l for fermentation. The fermentation was carried out by using *P. stipitis* and the highest ethanol concentration of 3.15 g/l was achieved at 28 hours (Fig. 4.34 (a)) with the ethanol yield (Yp/s) of 0.43 g/g (Table 4.17) However, a control experiment was also performed with synthetic sugars (glucose 10g/l) in place of hydrolysate with the same other media components, the highest ethanol concentration was found to be 3.64 g/l after 24 hours, whereas the ethanol yield (Yp/s) was found to be 0.46 g/g (Fig. 4.34 (b)). The specific growth rates were found to be 0.22 and 0.26 h^{-1} in hydrolysate and synthetic media respectively. A higher biomass yield (Yx/s) was also observed in synthetic media in comparison to the hydrolysate media which may be due to presence of some inhibitory compounds that might interfere in the process of fermentation. The maximum ethanol productivity and maximum sugars consumption rates were found to be 0.11 and 1.0 g/l/h respectively.

The hydrolysate obtained after enzymatic hydrolysis was also utilised for the ethanol production by using *S. cerevisiae*. The fermentation experiments were carried out with hydrolysate as well synthetic media (Fig. 4.35 (a) and (b)). The maximum ethanol concentration was found to be 3.09 g/l after 28 hours with ethanol yield (Yp/s) of 0.46 g/g. when the similar experiment was carried out with synthetic sugars media the ethanol yield of 0.47g/g was observed after 20 hours. However, the ethanol productivity was 0.11 g/l/h (Table 4.17).

Hence *S. cerevisiae* was found to be better yeast for ethanol yield in comparison to the *P. stipitis*. The ethanol yield obtained in sugar hydrolysate (0.46 g/g) (after enzymatic hydrolysis) with *S. cerevisiae* was found to be approximately to that obtained in synthetic media (0.47 g/g) (Fig. 4.36). Several studies have been reported in the literature for separate hydrolysis (acid pretreated biomass) and fermentation by different authors (Table 4.18). And when their results were compared with that of present study it was found that the results in term of ethanol yield were comparable

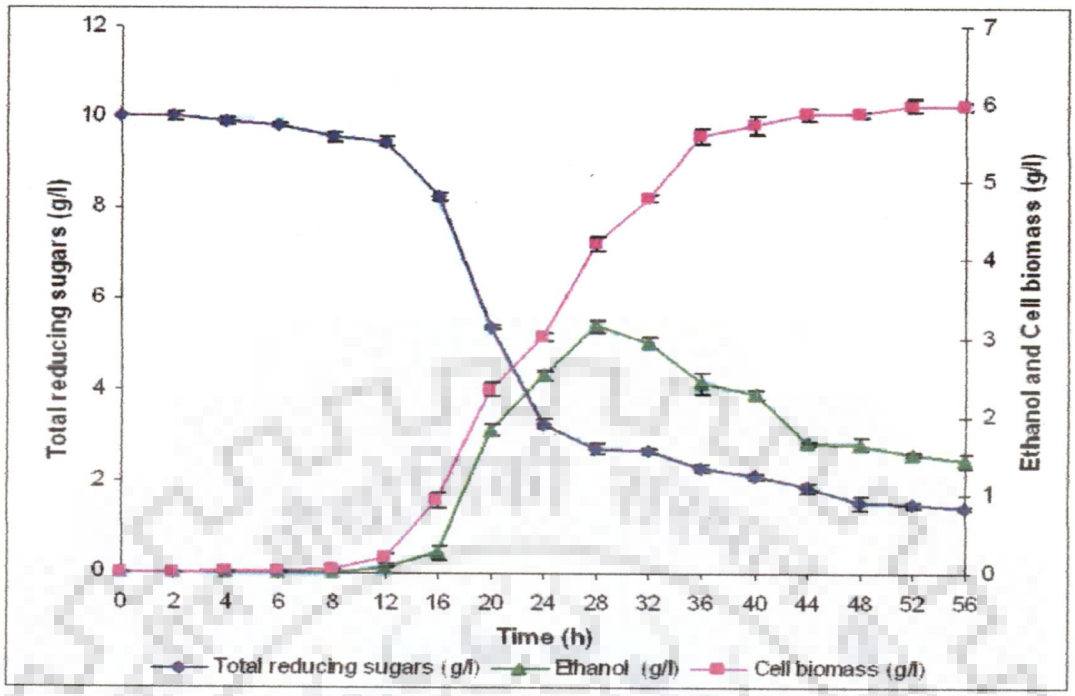
4.9.5 Ethanol production using NaOH pretreated Kans grass hydrolysate obtained after enzymatic hydrolysis

The sugar hydrolysate obtained after enzymatic hydrolysis (using crude enzyme mixture obtained from *T. reesei*) of NaOH pretreated Kans grass biomass was utilized for

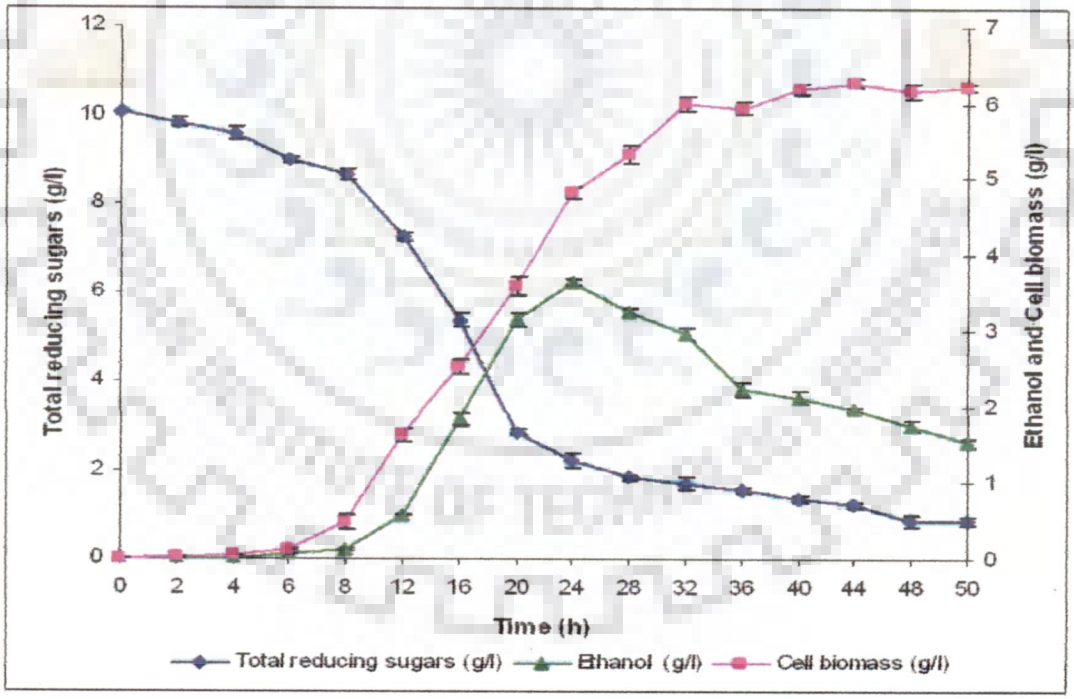
bioethanol production by using yeast strains of *S. cerevisiae* and *P. stipitis* shown in Fig. 4.37 (a) and Fig. 4.37 (b) respectively. When the fermentation was carried out by using *S. cerevisiae* adjusting initial TRS concentration at 10g/l, the ethanol concentration was found to be 2.89g/l with ethanol yield (Yp) 0.38 g/g after 32 hours (4.37 (a)). The specific growth rate was estimated to be 0.22 h^{-1} (Table 4.19).

Similarly when, fermentation was carried out using *P. stipitis* in hydrolysate media with 10g/l of initial TRS concentration. The ethanol concentration was estimated to be 3.2 g/l after 24 hours. Complete sugar consumption (g/l), ethanol production (g/l) as well as cell biomass formation (g/l) profile is shown in Fig. 3.37 (b). The bioethanol yield was found to be 0.44 g/g with specific growth rate of 0.22 h^{-1} (Table 4.17).

Hence a better ethanol yield (0.44 g/g) was observed when *P. stipitis* was used for fermentation. As *P. stipitis* is one of the yeast strains that can utilize wide range of hexose and pentose sugar including glucose, mannose, galactose, xylose and cellobiose. As the NaOH pretreated biomass is mainly composed of cellulose and hemicellulosic portion after removal of lignin content during pretreatment. The enzyme mixture that was used for enzymatic hydrolysis was composed of xylanase and cellulase activity. Hence during hydrolysis both type of sugars (pentose and hexose, mainly glucose) were solubalize and were present in this sugar mixture. The *P. stipitis* which can utilize both types of sugars for ethanol fermentation was found to be more efficient in term of ethanol yield (0.44 g/g) to *S. cerevisiae* (0.38 g/g) which mainly can utilize glucose sugar for ethanol fermentation.

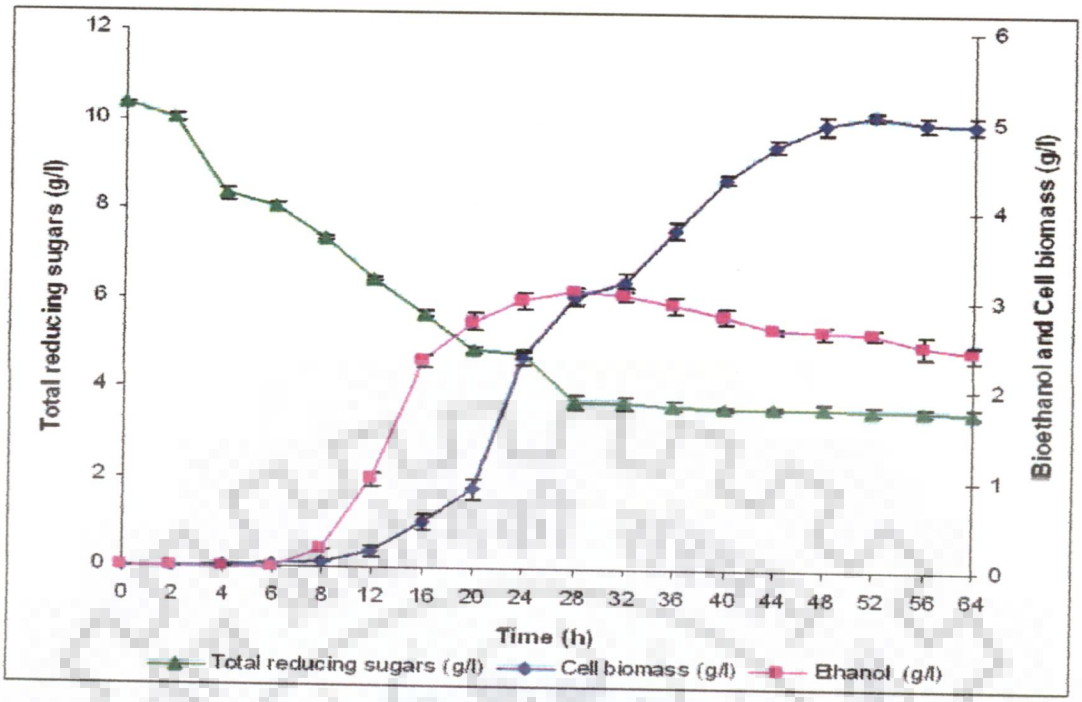


(a)

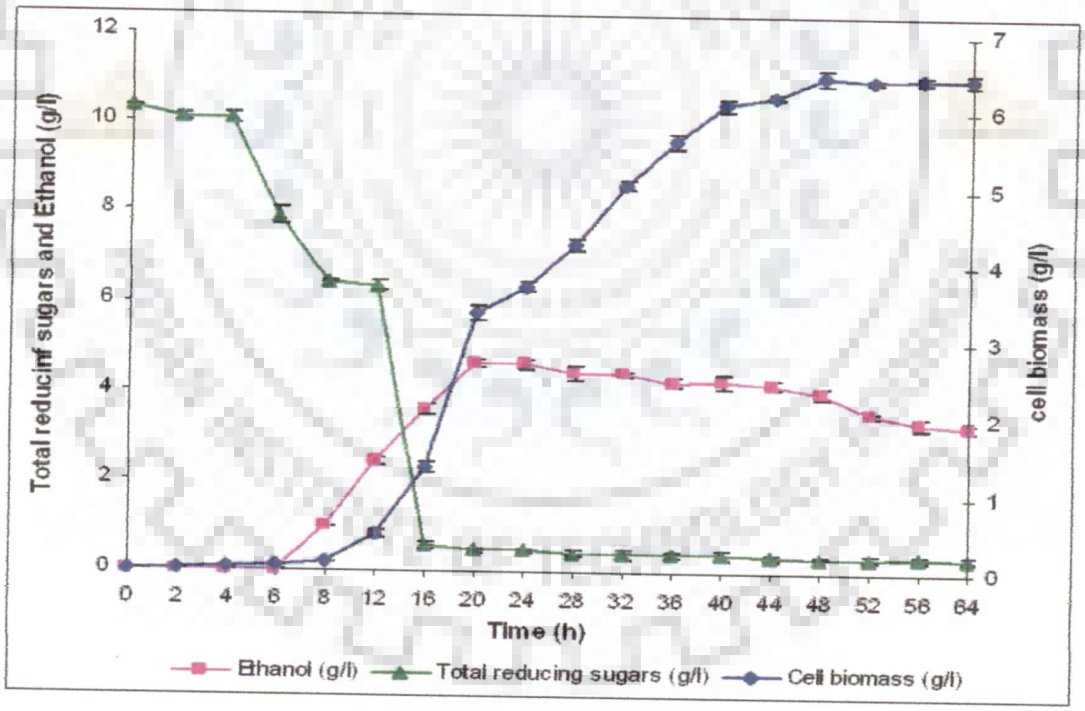


(b)

Fig. 4.34 Fermentation profiles of adaptive *P. stipitis* in **(a)** hydrolysate obtained after enzymatic hydrolysis of acid pretreated Kans grass biomass **(b)** synthetic media



(a)



(b)

Fig. 4.35 Fermentation profiles of S. *cerevisiae* in (a) hydrolysate obtained after enzymatic hydrolysis of acid pretreated Kans grass biomass) synthetic media

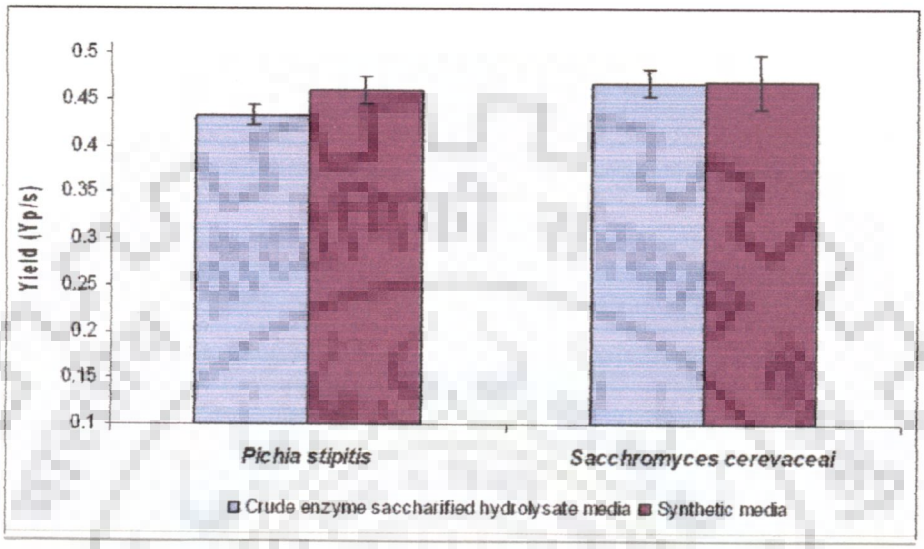


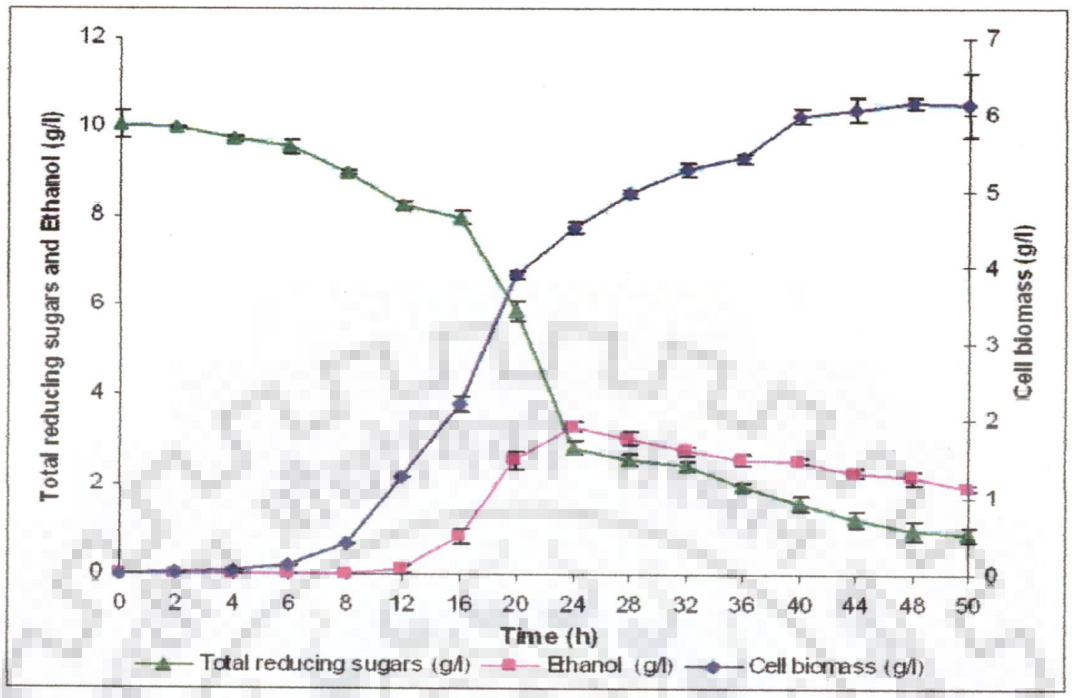
Fig. 4.36 A comparison in maximum ethanol yield (Yp/s) during ethanol fermentation by *P. stipitis* and *S. cerevisiae* in Crude enzymatically saccharified Kans grass biomass (dilute H_2SO_4 pretreated) hydrolysate and synthetic media

Table 4.17 Fermentation by *S. cerevisiae* and *P. stipitis* in sugar hydrolysate obtained after H_2SO_4 and crude enzyme treated biomass

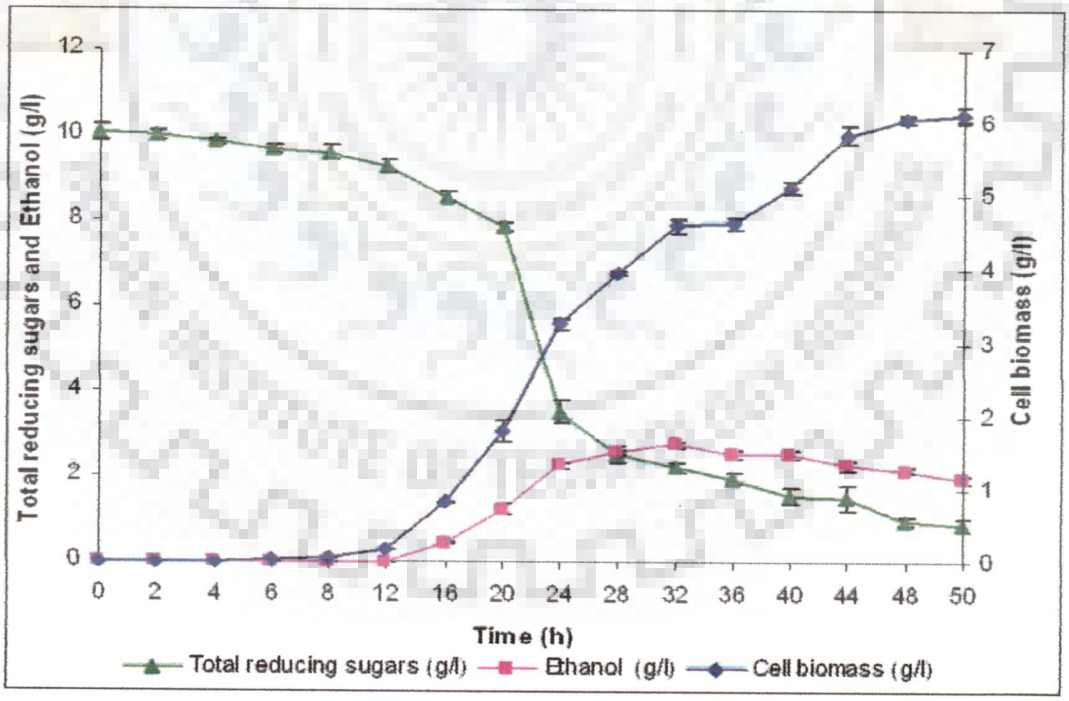
Fermentation parameters	*S. cerevisiae*		*P. stipitis*	
	Hydrolysate media	**Synthetic media**	**Hydrolysate media**	**Synthetic media**
Initial total reducing sugars, (g/l)	10.33±0.32	10.33±0.45	10.33±0.21	10.33±0.36
Maximum ethanol concentration (g/l)	3.09±0.13	4.64±0.17	3.15±0.08	3.64±0.03
Time (hours)	28	20	28	24
Sugar consumed (%)	64.28±2.43	95.16±1.31	72.80±1.11	77.81±0.91
Specific growth rate, μ (h^{-1})	0.12±0.021	0.17±0.032	0.22±0.012	0.26±0.004
Ethanol yield coefficient, Yp/s (g/g)	0.46±0.015	0.47±0.029	0.43±0.011	0.46±0.014
Biomass yield coefficient ,Yx/s (g/g)	0.45±0.029	0.34±0.032	0.58±0.011	0.68±0.004
Max. ethanol productivity, Qp (g/l/h)	0.11±0.052	0.23±0.062	0.11±0.021	0.15±0.023
Max.growth rate, Qx (g cells/l/h)	0.18±0.032	0.23±0.062	0.15±0.024	0.20±0.027
Max. sugars consumption rate, Qs (g/l/h)	0.23±0.019	0.16±0.041	1.0±0.017	1.94±0.038
Theoretical yield, □ (%)	91.12±1.32	92.55±1.21	84.84±1.52	91.17±1.21

Table 4.18 Comparison of Separate hydrolysis and fermentation results from various lignocellulosic materials pretreated by dilute H_2SO_4

Substrates	**Enzyme source for Saccharification**	**Microorganisms for ethanol production**	**Ethanol Yield ($g.g^{-1}$)**	**References**
wheat starch pre fermentation effluent	Commercial enzyme	Recombinant *Sacchromyces cerevaceai* strain REF	0.47	[320]
wheat starch post fermentation effluent	Commercial enzyme	Recombinant *Sacchromyces cerevaceai* strain REF	0.46	[320]
Prosopis juliflora	Commercial enzyme	*Sacchromyces cerevaceai*	0.49	[321]
Saccharum spontaneum	*A. oryzae*	*P.stipitis*	0.38	[322]
Barley straw	Commercial enzyme	Recombinant *E.coli*	0.48	[232]
Kans grass	***Crude from T.reesei***	***Sacchromyces cerevaceai***	***0.46***	***Present study***



(a)



(b)

Fig. 4.37 Fermentation profiles of **(a)** *P. stipitis* and **(b)** *S. cerevisiae* in hydrolysate obtained after enzymatic hydrolysis of dilute NaOH pretreated Kans grass biomass

Table 4.19 Fermentation of hydrolysate (obtained after NaOH and crude enzyme pretreatment of Kans grass biomass) by using *S. cerevisiae* and *P. stipitis*

Fermentation parameters	Hydrolysate media	
	S. cerevisiae	***P. stipitis***
Initial total reducing sugars, (g/l)	10.21±0.32	10.13±0.27
Maximum ethanol concentration (g/l)	2.89±0.23	3.29±0.31
Time (hours)	32	24
Sugar consumed (%)	78.19±1.87	74.60±2.31
Specific growth rate, μ (h^{-1})	0.22±0.04	0.220.03
Ethanol yield coefficient, Yp/s (g/g)	0.38±0.05	0.44±0.07
Biomass yield coefficient ,Yx/s (g/g)	0.58±0.08	0.64±0.08
Ethanol productivity, Qp (g/l/h)	0.090±0.01	0.13±0.06
Growth rate, Qx (g cells/l/h)	0.14±0.02	0.20±0.03
Max. sugars consumption rate, Qs (g/l/h)	0.24±0.02	0.31±0.02
% Theoretical yield, □ (%)	75.16±1.22	86.47±2.31

4.9.6 Comparison in ethanol yield in hydrolyzates obtained after enzymatic hydrolysis of acid (H_2SO_4) and alkali (NaOH) pretreated biomass

The hydrolysate obtained (10g/l) after enzymatic hydrolysis of dilute H_2SO_4 pretreated biomass was utilized for the further fermentation process by using *S. cerevisiae* as well as *P. stipitis*. It was observed that *S. cerevisiae* resulted better yield (0.46 g/g) than the *P. stipitis* (0.43 g/g) on same time period of 28 hours.

Hydrolysate (10 g/l of TRS) obtained after hydrolysis of alkali pretreated biomass was used for fermentation again by using yeasts, *S. cerevisiae* and *P.stipitis*. Ethanol yield of 0.38 g/g was obtained after fermentation by using *S. cerevisiae* while 0.44 g/g was found by *P. stipitis*

Hence a highest yield of 0.46 g/g was found by using *S. cerevisiae* in H_2SO_4 pretreated hydrolysate (after enzymatic hydrolysis). As dilute H_2SO_4 pretreatment caused the exclusion of mainly hemicellulosic portion with removal of lignin, thus resulted

biomass mainly composed of cellulose (glucose polymer). When this biomass was treated with crude enzyme mixture, the librated sugars mainly composed of glucose with little xylose. As *S. cerevisiae* is known to be the organism for glucose utilizing yeast for ethanol fermentation so, it resulted in the better yield than the *P. stipitis*. In a study, SO_2 pretreated spruce by using recombinant strain of *S. cerevisiae* gave 0.38 g/g of ethanol yield [324].

On the other hand NaOH pretreated biomass composed of both hemicellulose and cellulose with removal of major portion of lignin. When crude enzyme was supplied for hydrolysis it resulted in the solubilization of hemicellulosic part (mainly xylose, from xylanase action present in crude enzyme mixture) with cellulosic part. As *S. cerevisiae* is not able to utilize the xylose hence low ethanol yield was obtained (0.38 g/g) in comparison to *P. stipitis*. As *P. stipitis* able to utilized xylose efficiently and also other sugars hence a better yield of ethanol was obtained (0.44 g/g).

Chapter 5
Conclusions

As without taking any share of agricultural lands used for food and feed production, forest and for other use, there are waste and marginal lands where Kans grass (*Saccharum spontaneum*) which is a weed can be cultivated and can be used for the production of bioethanol.

- This study investigated the potential of a novel biomass (Kans grass) that can be cultivated on waste land throughout the year and can be utilized for bioethanol production. This biomass do not required special cultivation care as it can grow on margin/waste land and no extra pressure is there on crops land. Addition of this, biomass is available through out of year.
- One of the objectives was characterisation of this novel biomass (Kans grass), Holocellulose was found to be 64.73% on dry weight basis. The total lignin was observed to be 25.15% with ash and moisture contents of 2.1 and 4.7% respectively. Hence a high amount of carbohydrate is available that can be further solubalize into fermentable sugars for bioethanol production.
- The most substantial effect of sulphuric acid pretreatment on Kans grass biomass is the solubilization of hemicellulose portion. For dilute sulphuric acid pretreatment different concentrations of H_2SO_4 as well as four reaction time duration with three different temperatures (4x4x3) were used to obtain the optimum condition for maximum sugars solubilization. The condition with 2% H_2SO_4, 90 minutes of reaction time and 120°C of pretreatment temperature the total reducing sugars concentration was found to be 11.66 g/l. Compositional results of optimised condition (2% H_2SO_4, 90 minutes and 120°C) showed removal of 77.06% of hemicellulosic and 19.72% cellulosic portion with 28.04% of lignin removal. Low concentration of H_2SO_4 and low temperatures make this process more economically feasible. Fewer amounts of toxic compounds generated which removed by detoxification process and minimized the toxic compounds concentration by 3 to 4 times.
- Dilute NaOH pretreatment was performed using four dilute NaOH concentrations with four reaction time and three temperatures (4x4x3). The maximum removal of lignin (79.30%) was observed at 2% NaOH concentration with reaction time of 90 minutes at 120°C. NaOH pretreatment was found to be more significant for the

maximum removal of lignin & made biomass less rigid for better action of enzyme on holocellulose in further step of saccharification.

- As for bioethanol production saccharification for lignocellulosic material, cellulase enzyme production accounts of 40% of total cost in bioethanol synthesis. Therefore, in the present study effort were done for on site production of crude enzyme from the *T. reesei* and further hydrolysis of pretreated biomass was performed using the crude enzyme preparation and was compared with commercial enzyme preparation. Interestingly, soluble sugars released from lignocellulosic biomass were approximately similar with both crude enzyme preparation and commercial enzyme. Therefore, this crude enzyme mixture was equally efficient to that of the commercial one. When comparison was done for enzymatic hydrolysis of acid and alkali pretreated biomass, a five times higher total reducing sugars released in case of alkali pretreated biomass. Hence, on site produced crude enzyme (from *T. reesei*) to obtain total reducing sugars may be more cost effective process during this study.
- Pretreatment is important for hydrolysis step as it delignify the biomass with liberation of sugars and result the better enzyme action. Hence in the present study NaOH pretreatment was found to be better strategy in comparison of dilute acid pretreatment which resulted about 5-times higher TRS formation after enzymatic hydrolysis.
- An acid adaptive strain of *P. stipitis* was developed that showed improved bioethanol yield as well as less fermentation time in acid hydrolysate in comparison to the wild strain. Dilute acid pretreatment can solubalize the hemicellulosic crystalline sugars into different monomeric sugars including Xylose, arabinose, glucose and other sugars. After concentrating this hydrolysate it was fermented using this acid adaptive *P. stipitis* which provided better yield.
- The hydrolysate obtained after enzymatic hydrolysis of acid and alkali pretreated biomass was further utilized for bioethanol production by using yeasts *S. cerevceai* and *P. stipitis* and a high ethanol yield comparable to the other authors was obtained. The ethanol yield obtained was found to be close/comparable to the other studies for ethanol production.

Future prospects

- To study acid pretreatment at higher biomass loading (10-15%) as well as high temperatures (up to 160°C) to obtained higher hemicellulosic sugar liberation.
- Development of SSF (simultaneous saccharification and fermentation) strategy, in which both hydrolysis and fermentation steps can be combined.
- Scale-up of fermentation process in bioreactor by using batch, fed batch as well continuous strategies
- To develop co-fermentation technology by using two different microorganism, one for pentose and other for hexose utilizing organism

Chapter 6
References

[1] Howard, R.L., 2003. Lignocellulose biotechnology: issues of bioconversion and enzyme production. African Journal of Biotechnology. 2 (10), 602-619.

[2] Somerville, C., 2006. Editorial: The Billion-Ton Biofuels Vision. Science 312:1277.

[3] United StatesDepartment of Energy. Energy Information Administration. U.S. Dept. of Energy, Washington, DC, 2007.

[4] Planning Commission, GOI. Report of committee on development of biofuel. Planning Commission, Government of India, 2003.

[5] Campbell, C.J., Laherrere, H., 1998. The end of cheap oil. Sci Am. 3, 78–83.

[6] Zhang, Y., Dube, M.A., McLean, D.D., Kates, M., 2003. Biodiesel production from waste cooking oil: Process design and technological assessment. Bioresource Technology, 89, 1-16.

[7] Islam,A.A.K.M., Yaakob, Z., Anuar, N., Osman M., 2010. Propagation Potentials of Genotypes and Different Physiological Ages of Stem Cuttings in *Jatropha curcas* L. Journal of Agricultural Science. 2(4), 75-82.

[8] Akbar, E., Yaakob, Z., Kartom, S.K., Ismail, M., Salimon, J., 2009. Characteristic and Composition of Jatropha Curcas Oil Seed from Malaysia and its potential as Biodiesel Feedstock. European Journal of Scientific Research. 29(3), 396-403.

[9] Wyman, C.E., 1994. Ethanol from lignocellulosic biomass: technology, economics and opportunities. Bioresource Technology. 50, 3–16.

[10] Lynd, L.R., Cushaman, J.H., Nichols, R.J., Wyman, C.E., 1991. Fuel ethanol from cellulogic biomass. Science. 251, 1318-1323.

[11] Badger, P.C., 2002. Ethanol from cellulose: a general review. In: Janick J, Whipkey A, editors. Trends in new crops and new uses. Alexandria, VA, ASHS Press, 17–21.

[12] Parikka, M., 2004. Global biomass fuel resources. Biomass and Bioenergy. 27, 613- 620.

[13] Gray, K.A., Zhao, L., Emptage, M., 2006. Bioethanol. Current Opinion in Chemical Biology. 10, 141-146.

[14] Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M. and Kammen, D. M., 2006. Ethanol Can Contribute to Energy and Environmental Goals. Science. 311, (5760), 506-508.

[15] Kataria, R., Chaudhary, G. and Ghosh, S., 2009. Potential of Bioenergy Production from Grasses and its Impact on Environment. Research Journal of Biotechnology. 4, 5-14.

[16] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology. 96, 673-686.

[17] Wooley, R., Ruth, M., Glassner, D., Sheejan, J., 1999. Process design and costing of bioethanol technology: a tool for determining the status and direction of research and development. Biotechnology Progress. 15, 794–803.

[18] Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., and Lee, Y.Y., 2005. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology 96, 1959-1966.

[19] Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphy, R., Templer, R., and Tschaplinski, T., 2006. The path forward for biofuels and biomaterials. Science. 311, 484-489.

[20] Harmsen, P., Huijgen, W., Bermudez, L., Robert, B., 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Food and Biobased Research. 978-90-8585-757-0.

[21] Gray, K.A., Zhao, L., and Emptage, M., 2006. Bioethanol. Current Opinion in Chemical Biology 10, 141-146.

[22] Shang, T.Y., 2007. Value added products from animal manure, Bioprocessing for value-added products from renewable resources, new technologies and applications. Elsevier. 641-651.

[23] Bayer, E.A., Chanzy, H., Lamed, R., Shoham, Y., 1998. Cellulose, cellulases and cellulosomes. Current Opinion in Structural Biology. 8, 548-557.

[24] Zhang, Y.H.P., Himmel, M.E., Mielenz, J.R., 2006. Outlook for cellulase improvement: Screening and selection strategies. Biotechnology Advances. 24, 452-481.

[25] Jeffries, T.W., 1985. Emerging technology for fermenting d-xylose. Trends in Biotechnolgy. 3, 208–12.

[26] Skoog, K, Hahn-Hagerdahl, B., 1988. Xylose fermentation. Enzyme and Microbial Technology. 10, 66–90.

[27] Samson, R., Resource Efficient Agricultural Production (REAP) - Canada (2007) 22. Dan Putnam and Davis, CA 95616, In Proceedings, 2008 California Alfalfa & Forage Symposium and Western Seed Conference, San Diego, CA, 2-4 December (2008)

[28] Breaking the Biological Barriers to Cellulosic Ethanol: A Joint Research Agenda, DOE/SC-0095, June (2006)

[29] Gulati, A., 2006. Agricultural Growth and Diversification since 1991" in Encyclopedia of India, edited by Stanley Wolpert, Gale Thomson, ISBN 0-684-31350-2, 14-17

[30] Logsdon, J.E., 2006. Ethanol, in: R. E. Kirk, D. F. Othmer, et al. (Eds.), Encyclopedia of Chemical Technology. John Wiley & Sons, Inc., New York.

[31] Rosillo-Calle, F. and Walter, A., 2006. Global market for bioethanol: historical trends and future prospects. Energy for Sustainable Development. 10, 20-32.

[32] Rasskazchikova, T. V., 2004. Ethanol as high-octane additive to automotive gasolines: production and use in Russia and abroad. Chemistry and Technology of Fuels and Oils. 40(4), 203-210.

[33] http://www.sfi.mtu.edu/FutureFuelfromForest/CellulosicEthanol.htm

[34] Sun, Y., Che, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technology. 83, 1-11.

[35] Annual Report. 1995 Centre for Ecological Sciences, Indian Institute of Science, Bangalore.

[36] Ravindranath, N.H., Hall, D.O., 1995. Biomass, energy and environment: a developing country perspective from India. Oxford: Oxford University Press.

[37] Ravindranath, N.H., Somashekar, H.I., Nagaraja, M.S., Sudha, P., Sangeetha, G., Bhattacharya, S.C., 2005. Assessment of sustainable non-plantation biomass resources potential for energy in India. Biomass Bioenergy. 29, 178–90.

[38] Pachauri, R.K., Sridharan, P.V., 1998. Solid wastes. In looking back to think ahead. New Delhi: Tata Energy Research Institute. 245–265.

[39] Fengel, D., Wegener, G. 1984. Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter: Berlin.

[40] Banik, R.M., Santhiagu, A., Upadhyay, S.N., 2007. Optimization of nutrients for gellan gum production by *Sphingomonas paucimobilis* ATCC-31461 in molasses based medium using response surface methodology. Bioresource Technology. 98, 792-797.

[41] Azzam, A.M. 1989 Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation. Journal of Environtal Science and Health. 24(4), 421–33.

[42] Dale, B.E., Henk, L.L., Shiang, M., 1984. Fermentation of lignocellulosic materials treated by ammonia freeze-explosion. Developments in Industrial Microbiology. 26, 223–33.

[43] Duff, S.J.B, Murray, W.D., 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresource Technology. 55, 1–33.

[44] Martin, C., Galbe, M., Wahlbom, C.F., Hahn-H¨agerdal, B., Jonsson, L.J., 2002. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*. Enzyme and Microbial Technology. 31, 274–82.

[45] Reshamwala, S., Shawky, B.T., Dale, B.E., 1995. Ethanol production from enzymatic hydrolysis of AFEX-treated coastal Bermuda grass and switchgrass. Applied Biochemistry and Biotechnology. 51/52, 43–55.

[46] Yanase, H., Yamamoto, K., Sato, D., Okamoto, K., 2005. Ethanol production from cellobiose by *Zymobacter palmae* carrying the *Ruminocuccus albus* _-glucosidase gene. Journal of Biotechnology. 118, 35–43.

[47] http://www.engineeringnews.co.za/article/secondgeneration-biofuels-may-offer-a-way-forward-2008-10-31.

[48] McMillan, J.D., 1994. Pretreatment of lignocelluloses biomass. In: HimmelME,Baker JO,OverendRP, editors. Conversion of hemicellulose hydrolyzates to ethanol. Washington, DC: American Chemical Society Symposium. 292–324.

[49] Boopathy, R., 1998. Biological treatment of swinewaste using anaerobic baffled reactors. Bioresource Technology. 64, 1–6.

[50] Cheung, S.W., Anderson, B.C., 1997. Laboratory investigation of ethanol production from municipal primary wastewater. Bioresource Technology. 59, 81–96.

[51] Dewes, T., Hunsche, E., 1998. Composition and microbial degradability in the soil of farmyard manure from ecologically managed farms. Biological Agriculture and Horticulture.16, 251–68.

[52] McKendry, P., 2002. Energy production from biomass (part I): overview of biomass. Bioresource Technology. 83, 37–46.

[53] Fengel, D., Wegener, G., 1984. Chemical composition and analysis of wood. *In* Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin. 26–65

[54] Laureano-Perez, F., Teymouri, H., Alizadeh, Dale, B.E., 2005. Understanding factors that limit enzymatic hydrolysis of biomass characterization of pretreated corn stover. Applied Biochemistry and Biotechnology. 1081–1099

[55] https://myorganicchemistry.wikispaces.com/Cellulose.

[56] Saha, B.C., 2003. Hemicellulose bioconversion. Journal of Industrial Microbiology and Biotechnology. 30, 279–291.

[57] Balaban, M., Ucar, G., 1999. The effect of the duration of alkali treatment on the solubility of polyoses. Turkish Journal of Agriculture and Forestry. 23, 667–671.

[58] Lawther, J.M., Sun, R., Banks, W.B., 1996. Effect of steam treatment on the chemical composition of wheat straw. Holzforschung 50, 365–371.

[59] http://en.wikipedia.org/wiki/File:Hemicellulose.png.

[60] http://plantphys.info/plant_physiology/basiccytology1.shtml.

[61] Brown, R.F., 2003. Biorenewable resources: engineering new products from agriculture. Ames, Iowa: Iowa State Press. ISBN 0-8138-2263-7.

[62] Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M.R., 2005. Features of Promising Technologies for Pretreatment of Lignocellulosic Biomass. Bioresource Technology. 96(6), 673-686.

[63] Palmowski, L., Muller, J., 1999. Influence of the size reduction of organic waste on their anaerobic digestion. In: II International Symposium on Anaerobic Digestion of Solid Waste. Barcelona 15–17 June, 137–144.

[64] Delgenés, J.P., Penaud, V., Moletta, R., 2002. Pretreatments for the enhancement of anaerobic digestion of solid wastes Chapter 8. In: Biomethanization of the Organic Fraction of Municipal Solid Wastes. IWA Publishing, 201–228.

[65] Hartmann, H., Angelidaki, I., Ahring, B.K., 1999. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. In: Mata-Alvarez, J., Tilche, A., Cecchi, F. (Eds), Proceedings of the Second International Symposium on Anaerobic Digestion of Solid Wastes, Barcelona, (1), 129-136.

[66] Karunanithy, C., Muthukumarappan, K., Julson, J.L., 2008. Influence of high shear bioreactor parameters on carbohydrate release from different biomasses. ASABE Paper No. 084109. St. Joseph, Mich.: ASABE

[67] Pan, X., 2008. Role of Functional Groups in Lignin Inhibition of Enzymatic Hydrolysis of Cellulose to Glucose. Journal of Biobased Materials and Bioenergy. 2, 25-32.

[68] Allen, S.G., Schulman, Deborah, Lichwa, J., Antal Jr., M.J., 2001. A comparison of aqueous and dilute-acid single-temperature pretreatment of yellow poplar sawdust. Industrial Engineering and Chemistry Research. 40, 2352–2361.

[69] Li, J., Henriksson, G., Gellerstedt, G., 2005. Carbohydrate reactions during hightemperature steam treatment of Aspen wood. Applied Biochemistry and Biotechnology. 125, 175– 188.

[70] Weil, J.R., Sarikaya, A., Rau, S.L., Goebz, J., Lasisch, C.M., Brwer, M., Hendrickson, R., Tadisch, M.R., 1998. Pretreatment of corn fiber by pressure cooking in water. Appl. Biochem. Biotechnol. 73, 1–17.

[71] Laser, M., Schulman, D., Allen, S.G., Lichwa, J., Antal Jr, M.J., Lynd, L.R., 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresource Technology. 81, 33–44.

[72] Perez, J.A., Ballesteros, I., Ballesteros, M., 2008. Optimization liquid hot water pretreatment conditions to enhance sugar recovery from wheat straw for fuel-ethanol production. Fuel. 87, 3640-3647.

[73] Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology. 96, 2026–32

[74] Kumar, P., Barrett. D.M., Delwiche, M.J., StroeveP., 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Industrial & Engineering Chemistry Research. 48 (8), 3713–3729.

[75] Galbe, M., Zacchi, G., 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. Advanced Biochemical Engineering and Biotechnology. 108, 41–65.

[76] Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S., Viikari, L., 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. Appllied Biochemistry Biotechnology. 117, 1–17.

[77] Olsson, H., Jörgensen, K.B.R., Krogh, Roca, C., 2005. Bioethanol production from lignocellulosic material. S. Dimitriu, Editor, Polysaccharides Structural Diversity and Functional Versatility, Marcel Dekker, New York. 957–993

[78] Martín, M.H., Thomsen, H.H., Thomsem, A.B., 2008. Wet oxidation pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation of clover-ryegrass mixtures. Bioresource Technology. 99, 8777–8782.

[79] Klinke, H.B., Ahring, B.K., Schmidt, A.S., Thomsen, A.B., 2002. Characterization of degradation products from alkaline wet oxidation of wheat straw. Bioresource Technology. 82, 15–26.

[80] Keshwani, D.R., 2009. Microwave Pretreatment of Switchgrass for Bioethanol Production. Thesis Dissertation. North Carolina State University.

[81] Zhu, S., Wu, Y., Yu, Z., Wang, C., Yu, F., Jin, S., Ding, Y., Chi, R., Liao, J., Zhang Y., 2006. Comparison of three microvawe/chemical pretreatment processes for enzymaric hydrolyisis of rice straw. Biosystem Engineering. 93, 279–283.

[82] Sun, R.C., Tomkinson, R.C., 2002. Characterization of hemicelluloses obtained by classical and ultrasonically assisted extractions from wheat straw. Carbohydrate Polymer. 50, 263–27.

[83] Yachmenev, V., Condon, B., Klasson, T., Lambert, A., 2009. Acceleration of the enzymatic hydrolysis of corn stover and sugar cane bagasse celluloses by low intensity uniform ultrasound. Journal of Biobased Material and Bioenergy. 3, 25–31.

[84] Pejin D.J., Mojovi´c L. V., Pejin J.D., Gruji´c O.S., Markov,a S. L., Nikoli´cb S.B. and Markovi´c M.N. 2011Increase in bioethanol production yield from triticale by simultaneous saccharification and fermentation with application of ultrasound. Journal of Chemical Technology and Biotechnology. DOI 10.1002/jctb.2675

[85] Wongjewboot I., Kangsadan T., Kongruang S., Burapatana V. and Pripanapong P., Ethanol Production From Rice Straw Using Ultrasonic Pretreatment. 2010. International Conference on Chemistry and Chemical Engineering (ICCCE 2010), 16-19.

[86] Nikolić S., Mojović L., Rakin M., Pejin D., Pejin J., 2010. Ultrasound-assisted production of bioethanol by simultaneous saccharification and fermentation of corn meal. Food Chemistry Volume 122 (1), 216-222.

[87] Schacht, C., Zetzl, C., Brunner, G., 2008. From plant materials to ethanol by means of supercritical fluid technology. Journal of Supercritical Fluids. 46, 299–321.

[88] Dale, B.E., Moreira, M.J., 1982. A freeze-explosion technique for increasing cellulose hydrolysis. Biotechnology and Bioengineering Symposium.12, 31–43.

[89] Zheng, Y.Z., Lin, H.M., Tsao, G.T., 1998. Pretreatment for cellulose hydrolysis by carbon dioxide explosion. Biotechnology Progress. 14, 890–896.

[90] Gary, B., Elizabeth, Y., Kimberly, B., John, C., Ramachandran, K.B., Ramakrishnan, S., 2011. Chemical and Physicochemical Pretreatment of Lignocellulosic Biomass: A Review, Enzyme Research. doi:10.4061/2011/787532.

[91] Carvalheiro, F., Duarte, L.C., Gírio, F.M., 2008. Hemicellulose biorefineries: a review on biomass pretreatments. Journal of Science and Industrial Research. 67, 849–864.

[92] Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P., 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Industrial Engnineering and Chemical Research. 48, 3713–3729.

[93] Taherzadeh, M.J., Karimi, K., 2008. Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review. International Journal of Molecular Science. 9, 1621–1651.

[94] Sealey, J.E., Runge, T.M., Ragauskas, 2000. Laccase N-Hydroxybenzotriazole Full-Sequence Bleaching with Hydrogen Peroxide and Chlorine Dioxide. A.J., TAPPI J. 83(9), 66.

[95] Kumar, R., Wyman, C.E., 2009. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnology Progress. 25 , 302–314.

[96] Carvalheiro, F., Duarte, L.C., Gírio, F.M., 2008. Hemicellulose biorefineries: a review on biomass pretreatments. Journal of Science and Industrial Research. 67, 849–864.

[97] Wyman, C.E., 1996. Handbook on Bioethanol: Production and Utilization. Taylor Francis, Washington, 417.

[98] Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R., 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresource Technology. 96, 1986–1993.

[99] Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry. 40, 3693–3700.

[100] Cara, C., Ruiz, E., Oliva, J.M., Sáez, F., Castro, E., 2008. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresource Technology. 99, 1869–1876.

[101] Rocha, M.V., Rodrigues, T.H., de Macedo, G.R., Gonçalves, L.R., 2009. Enzymatic hydrolysis and fermentation of pretreated cashew apple bagasse with alkali and diluted sulfuric acid for bioethanol production. Applied Biochemistry and Biotechnology. 155, 407–417.

[102] Ben-Ghedalia, D., Shefet, G., and Miron, J., 1980. Effect of ozone and ammonium hydroxide treatments on the composition and in vitro digestibility of cotton straw. Journal of the Science of Food and Agriculture. 31, 1337-1342.

[103] Ben-Ghedalia, D., Shefet, G., 1983. Chemical treatments for increasing the digestibility of cotton straw. Journal of Agricuture and Science. 100, 393-400.

[104] Yosef, E., Ben-Ghedalia, D., Miron, J., Huttermann, A., Majcherczyk, A., Milstein, O., Ludemann, H.D., and Frund, R., 1994. Characterization of some cell wall components of untreated and ozone-treated cotton stalks. Journal of Agriculture and Food Chemistry. 42, 86-90.

[105] Quesada, A., Vincent, W.F., Lean, D.R.S., 1999. Community and pigment structure of Arctic cyanobacterial assemblages: the occurrence and distribution of UV-absorbing compounds. FEMS Microbial Ecol. 28, 315–323.

[106] Ben-Ghedalia, D., and Miron, J. 1981. The effect of combined chemical and enzyme treatment on the saccharification and *in vitro* digestion rate of wheat straw. Biotechnology Bioengineering. 23, 823-831.

[107] Papatheofanous, M.G., Billa, E., Koullas, D.P., Monties, B., Koukios, E.G., 1995. Two-stage acid-catalyzed fractionation of lignocellulosic biomass in aqueous ethanol systems at low temperatures. Bioresource Technology. 54, 305–310.

[108] Zhao, X., Cheng, K., Liu, D., 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Applied Microbiology and Biotechnology. 82, 815–827.

[109] Hayes, D.J., 2009. An examination of biorefining processes, catalysts and challenges. Catalysis today. 145, 138–151.

[110] Sánchez, C., 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnology Advances. 27, 185–194.

[111] Kuhar, S., Nair, L.M., Kuhad, R.C., 2008. Pretreatment of lignocellulosic material with fungi capable of higher lignin degradation and lower carbohydrate degradation improves substrate acid hydrolysis and eventual conversion to ethanol. Canadian Journal of Microbiology. 54, 305–313.

[112] Shi, J., Chinn, M.S., Sharma-Shivappa, R.R., 2008. Microbial pretreatment of cotton stalks by solid state cultivation of *Phanerochaete chrysosporium*. Bioresource Technology. 99, 6556–6564.

[113] Itoh, H., Wada, M., Honda, Y., Kuwahara, M., Watanabe, M., 2003. Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. Journal of Biotechnology. 103, 273–280.

[114] Alvo, P., Belkacemi, K., 1997. Enzymatic saccharification of milled timothy (Phleum pretense L.) and alfalfa (Medicago sativa L.). Bioresource Technology 61, 185–198.

[115] Papatheofanous, M.G., Billa, E., Koullas, D.P., Monties, B., Koukios, E.G., 1998. Optimizing multisteps mechanical–chemical fractionation of wheat straw components. Industrial Crops and Products 7, 249–256.

[116] Ballesteros, I., Oliva, J.M., Sa´ez, F., Ballesteros, M., 2001. Ethanol production from lignocellulosic byproducts of olive oil extraction. Applied Biochemistry and biotechnology 91–93, 237–252.

[117] Ballesteros, I., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, M., 2002a. Enzymic hydrolysis of steam exploded herbaceous agricultural waste (Brassica carinata) at different particule sizes. Process Biochemistry 38, 187–192.

[118] Ballesteros, M., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, I., 2004. Ethanol from lignocellulosic materials by a simultaneous saccharification and fermentation process (SFS) with Kluyveromyces marxianus CECT 10875. Process Biochemistry 39, 1843–1848.

[119] Belkacemi, K., Turcotte, G., Savoie, P., Chornet, E., 1997. Ethanol production from enzymatic hydrolyzates of cellulosic fines and hemicellulose-rich liquors derived from aqueous/steam fractionation of forages. Industrial & Engineering Chemistry Research 36, 4572– 4580.

[120] Belkacemi, K., Turcotte, G., Savoie, P., 2002. Aqueous/steam-fractionated agricultural residues as substrates for ethanol production. Industrial & Engineering Chemistry Research 41, 173–179.

[121] De Bari, I., Viola, E., Barisano, D., Cardinale, M., Nanna, F., Zimbardi, F., Cardinale, G., Braccio, G., 2002. Ethanol production at flask and pilot scale from concentrated slurries of steam-exploded aspen. Industrial & Engineering Chemistry Research 41, 1745–1753.

[122] Hamelinck, C.N., van Hooijdonk, G., Faaij, A.P.C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28, 384–410.

[123] McIntosh, S., Vancov, T . 2010. Enhanced enzyme saccharification of Sorghum bicolor straw using dilute alkali pretreatment. Bioresource Technology 101, 6718–6727.

[124] Nakamura, Y., Sawada, T., Inoue, E., 2001. Enhanced ethanol production from enzymatically treated steam-exploded rice straw using extractive fermentation. Journal of Chemical Technology and Biotechnology 76, 879–884.

[125] Negro, M.J., Manzanares, P., Ballesteros, I., Oliva, J.M., Caban˜ as, A., Ballesteros, M., 2003. Hydrothermal pretreatment conditions to enhance ethanol production from poplas biomass. Applied Biochemistry and Biotechnology 105–108, 87–100.

[126] Shevchenko, S.M., Beatson, R.P., Saddler, J.N., 1999. The nature of lignin from steam explosion/enzymatic hydrolysis of softwood. Applied Biochemistry and Biotechnology 77–79, 867–876.

[127] So¨derstro¨m, J., Pilcher, L., Galbe, M., Zacchi, G., 2003. Two-step steam pretreatment of softwood by dilute H2SO4 impregnation for ethanol production. Biomass and Bioenergy 24, 475–486.

[128] Koegel, R.G., Sreenath, H.K., Straub, R.J., 1999. Alfalfa fiber as a feedstock for ethanol and organic acids. Applied Biochemistry and Biotechnology, 105–115.

[129] Laser, M., Schulman, D., Allen, S.G., Lichwa, J., Antal Jr., M.J., Lynd, L.R., 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresource Technology 81, 33–44.

[130] Ogier, J.-C., Ballerini, D., Leygue, J.-P., Rigal, L., Pourquie´, J., 1999. Production d'e´thanol a` partir de biomasse lignocellulosique. Oil & Gas Science and Technology – Revue de l'IFP 54 (1), 67–94 (in French).

[131] Sreenath, H.K., Koegel, R.G., Moldes, A.B., Jeffries, T.W., Straub, R.J., 2001. Ethanol production from alfalfa fiber fractions by saccharification and fermentation. Process Biochemistry 36, 1199–1204.

[132] Dale, B.E., Leong, C.K., Pham, T.K., Esquivel, V.M., Rios, I., Latimer, V.M., 1996. Hydrolysis of lignocellulosics at low enzyme levels: application of the AFEX process. Bioresource Technology 56, 111–116.

[133] Bjerre, A.B., Olesen, A.B., Fernqvist, T., Plo¨ ger, A., Schmidt, A.S., 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. Biotechnology and Bioengineering 49, 568–577.

[134] Parameswaran, B., K.S., Raveendran, S., Kanakambaran, U., Rajeev, K. S., Ashok Pandey. 2012. Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. Renewable Energy, 37,109-116.

[135] Cong Chen , Dorin Boldor, Giovanna Aita , Michelle Walker Ethanol production from sorghum by a microwave-assisted dilute ammonia pretreatment. Bioresource Technology 110 (2012) 190–197

[136] 8. Saoharit Nitayavardhana, Prachand Shrestha, Mary L. Rasmussen, Buddhi P. Lamsal J. (Hans) van Leeuwen, Samir Kumar Khanal, Ultrasound improved ethanol fermentation from cassava chips in cassava-based ethanol plants Bioresource Technology 101 (2010) 2741–2747

[137] Rajendran Velmurugan, Karuppan Muthukumar Ultrasound-assisted alkaline pretreatment of sugarcane bagasse for fermentable sugar production: Optimization through response surface methodology. Bioresource Technology 112 (2012) 293–299

[138] Zhenbin Wang, Xiaoming Lin, Pingping Li, Jie Zhang a,c, Shiqing Wanga, Haile Maa. Effects of low intensity ultrasound on cellulase pretreatment Bioresource Technology 117 (2012) 222–227

[139] Hamelinck, C.N., van Hooijdonk, G., Faaij, A.P.C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28, 384–410.

[140] Hari Krishna, S., Prasanthi, K., Chowdary, G., Ayyanna, C., 1998. Simultaneous saccharification and fermentation of pretreated sugar cane leaves to ethanol. Process Biochemistry 33 (8), 825–830.

[141] Kaar, W.E., Holtzapple, M.T., 2000. Using lime pretreatment to facilitate the enzymic hydrolysis of corn stover. Biomass and Bioenergy 18, 189– 199.

[142] Saha, B.C., Cotta, M.A., 2006. Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. Biotechnology Progress 22, 449–453.

[143] Teixeira, L.C., Linden, J.C., Schroeder, H.A., 1999. Alkaline and peracetic acid pretreatments of biomass for ethanol production. Applied Biochemistry and Biotechnology 77–79, 19–34.

[144] Martinez, A., Rodriguez, M.E., York, S.W., Preston, J.F., Ingram, L.O., 2000. Effects of Ca(OH)2 treatments (''Overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotechnology and Bioengineering 69 (5), 526–536.

[145] Rodrı´guez-Chong, A., Ramı´rez, J.A., Garrote, G., Va´zquez, M., 2004. Hydrolysis of sugar cane bagasse using nitric acid: a kinetic assessment. Journal of Food Engineering 61 (2), 143–152.

[146] Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005a. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnology Progress 21, 816–822.

[147] Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005b. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry 40, 3693–3700.

[148] Schell, D.J., Farmer, J., Newman, M., McMillan, J.D., 2003. Dilutesulfuric acid pre-treatment of corn stover in pilot-scale reactor. Investigation of yields, kinetics, and enzymatic digestibilities of solids. Applied Biochemistry and Biotechnology 105 (1–3), 69–85.

[149] Wooley, R., Ruth, M., Sheehan, J., Ibsen, K., Majdeski, H., Ga'lvez, A., 1999. Lignocellulosic biomass to ethanol process design and eco- nomics utilizing co-current dilute acid prehydrolysis and enzymatic hydrolysis. Current and futuristic scenarios. Technical Report NREL/ TP-580-26157. Golden, CO (USA), National Renewable EnergyLaboratory. 123 p.

[150] Tengerdy, R.P., Szakacs, G., 2003. Bioconversion of lignocellulose in solid substrate fermentation. Biochemical Engineering Journal 13, 169–179.

[151] Mussatto, S.I., Roberto, I.C., 2004. Alternatives for detoxification of diluted-acid lignocellulosic hydrolysates for use in fermentative process: a review. Bioresource Technology. 93, 1–10.

[152] Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pretreatment of biomass. Applied Micriobiology and Biotechnology. 66, 10–26.

[153] Palmqvist, E., Hahn-Hägerdal, B., 2000. Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanism of inhibition. Bioresource technology. 74, 25–33.

[154] Roberto, I.C., Lacis, L.C., Barbosa, M.F.S., Mancilha, I.M., 1991. Utilization of sugarcane bagasse hemicellulosic hydrolysate by *Pichia stipitis*, for the production of ethanol. Process Biochemistry. 26, 15–21.

[155] Delgenes, J., Moletta, R., Navarro, J.M., 1996. Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis* and *Candida shehatae*. Enzyme and Microbial Technology. 19, 220– 225.

[156] Nigam, J.N., 2001. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. Journal of Biotechnology. 87, 17–27.

[157] Heipieper, H.J., Weber, F.J., Sikkema, J., Kewelo, H., de Bont, J.A.M., 1994. Mechanism of resistance of whole cells to toxic organic solvents. TIBTECH 12, 409-415.

[158] Parajo, J.C., Dom_inguez, H., Dom_inguez, J.M., 1998. Biotechnological production of xylitol. Part 3: Operation in culture media made from lignocellulose hydrolysates. Bioresource Technology. 66, 25–40.

[159] Van Zyl, C., Prior, B.A., du Preez, J.C., 1998. Production of ethanol from sugar cane bagasse hemicellulose hydrolysate by Pichia stipitis. Applied Biochemistry and Biotechnology. 17, 357–369.

[160] Lawford, H.G., Rousseau, J.D., 1998. Improving fermentation performance of Recombinant Zymomonas in acetic acid-containing media. Applied Biochemistry and Biotechnology. 70–72, 161–172.

[161] Watson, N.E., Prior, B.A., Lategan, P.M., Lussi, M., 1984. Factors an acid treated bagasse inhibiting ethanol production from Dxylose by Pachysolen tannophilus. Enzyme and Microbial Technology. 6, 451–456.

[162] Olsson, L., Hahn-Hagerdal, B., 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme and Microbial Technology. 18, 312–331.

[163] Jonsson, L.J., Palmqvist, E., Nilvebrant, N.O., Hahn-Hagerdal, B., 1998. Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus Trametes versicolor. Applied Microbiology and Biotechnology. 49, 691–697.

[164] Palmqvist, E., Grage, H., Meinander, N.Q., Hahn-Hagerdal, B., 1999. Main and interaction effects of acetic acid, furfural and phydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnology and Bioengineering. 63, 46–55.

[165] Parajo, J.C., Dom_ınguez, H., Dom_ınguez, J.M., 1997. Improved xylitol production with Debaryomyces hansenii Y-7426 from raw or detoxified wood hydrolysates. Enzyme and Microbial Technology. 21, 18–24.

[166] Larsson, S., Reimann, A., Nilvebrant, N., Jonsson, L.J., 1999. Comparison of different methods for the detoxification of lignocellulose hydrolysates of spruce. Applied Biochemistry and Biotechnology. 77– 79, 91–103.

[167] Silva, C.J.S.M., Roberto, I.C., 1999. Statistical screening method for selection of important variables on xylitol biosynthesis from rice straw hydrolysate by *Candida guilliermondii* FTI 20037. Biotechnology Techniques. 13, 743–747.

[168] Converti, A., Dom_ınguez, J.M., Perego, P., Silva, S.S., Zilli, M., 2000. Wood hydrolysis and hydrolyzate detoxification for subsequent xylitol production. Chemical Engineering Technology. 23, 1013–1020.

[169] Rodrigues, R.C.L.B., Felipe, M.G.A., Almeida e Silva, J.B., Vitolo, M., G_omez, P.V., 2001. The influence of pH, temperature and hydrolysate concentration on the removal of volatile and non-volatile compounds from sugarcane bagasse hemicellulosic hydrolysate treated with activated charcoal before or after vacuum evaporation. Brazilian Journal of Chemical Engineering. 18, 299–311.

[170] Palmqvist, E., Galbe, M., Hahn-H▒agerdal, B., 1998. Evaluation of cell recycling in continuous fermentation of enzymatic hydrolysates of spruce with *Saccharomyces cerevisiae* and on-line monitoring of glucose and ethanol. Applied Biochemistry and Biotechnology 50, 545-551.

[171] Amartey, S., Jeffries, T., 1996. An improvement in Pichia stipitis fermentation of acid-hydrolysed hemicellulose achieved by overliming (calcium hydroxide treatment) and strain adaptation. World Journal of Microbiology and Biotechnology. 12, 281-283.

[172] Alves, L.A., Felipe, M.G.A., Silva, J.B., Silva, S.S., Prata, A.M.R., 1998. Pre treatment of sugar cane bagasse hemicellulose hydrolyzate for xylitol production

by *Candida guilliermondii.* Applied Biochemistry and Biotechnology. 70–72, 89–98.

[173] Ulbricht, R., Northup, S., Thomas, J. 1984. A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. Fundamental and Applied Toxicology. 4, 843–853.

[174] Zaldivar, J., Ingram, L.O. 1999. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. Biotechnology and Bioengineering. 66, 203-210.

[175] Zaldivar, J., Martinez, A., Ingram, L.O. 1999. Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli.* Biotechnology and Bioengineering. 65, 24-33.

[176] Zaldivar, J., Martinez, A., Ingram, L.O.,2000. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli.* Biotechnology and Bioengineering. 68, 524-530.

[177] Jarboe, L.R., Grabar, T.B., Yomano, L.P., Shanmugan, K.T., Ingram, L.O. 2007. Development of ethanologenic bacteria. Advanves in Biochemical Engineering and Biotechnology. 108, 237-261.

[178] Modig, T., Almeida, J., Gorwa-Grauslund, M.F., Lidén G., 2008. Variability of the response of Saccharomyces cerevisiae strains to lignocellulose hydrolysate , Biotechnology and Bioengineering. 100, 423-429.

[179] Tian, S., Zhou G., Yan F., Yu Y., Yang X., 2009. Yeast strains for ethanol production from lignocellulosic hydrolysates during in situ detoxification. Biotechnology Advances. 27, 656-660

[180] Panda, T., Naidu, G.S.N., Sinha, J., 1999. Multiresponse analysis of microbiological parameters affecting the production of pectolytic enzymes: a statistical view. Process Biochemistry. 35, 187-195.

[181] Rahu, R.l., Malav, P.N., Gummadi, S.N., 2007. Production of thermostable α-amylases by solid state fermentation: a review, American Journal of Food Technology. 2, 1-11.

[182] Coughlan, M.P., Ljungdahl, L.G., 1988. Comparative biochemistry of fungal and bacterial cellulolytic enzyme system. In: Aubert JP, Beguin P, Millet J, editors. Biochemistry and genetics of cellulose degradation. London: Academic Press. 11–30.

[183] Jorgensen, H., Eriksson, T., Borjesson, J., Tjerneld, F., Olsson, L. 2003. Purification and characterization of five cellulases and xylanase from *Penicillium brasilianum* IBI 20888. Enzyme and Microbial Technology. 32, 851-861.

[184] Bisaria, V.S. 1991. Bioprocessing of agro-residue to glucose and chemicals. In: Martin AM, editor. Bioconversion of waste materials to industrial products. London: Elsevier. 210–3.

[185] Duff, S.J.B., Murray, W.D., 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresource Technology. 55, 1–33.

[186] Sternberg, D., 1976. Production of cellulase by *Trichoderma*. Biotechnol Bioeng Symp. 35–53.

[187] Fan, L.T., Gharpuray, M.M., Lee, Y.H., 1987. Cellulose hydrolysis biotechnology monographs. Berlin: Springer. 57.

[188] Hu, H.L., J. van den Brink, B.S. Gruben, H.A.B. Wösten, J.D. Gu, de Vries R.P., 2011. Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* with each other and other fungi. International Biodeterioration and Biodegradation. 65, 248-252.

[189] de Vries, R.P., Visser, J., 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiology and Molecular Biology Reviews. 65, 497-522.

[190] Battaglia, E., Benoit, I., van den Brink, J., Wiebenga, A., Henrissat, B., Coutinho, P.M., de Vries, R.P., 2011. Carbohydrate-active enzymes from the zygomycete fungus *Rhizopus oryzae*: a highly specialized approach to carbohydrate sources degradation depicted at genome level. BMC Genomics 12, 38

[191] Mach, R.L., Zeilinger, S., 2003. Regulation of gene expression in industrial fungi: *Trichoderma*. Applied Microbiology and Biotechnology. 60, 515-522.

[192] Lejeune, R., Nielsen, J., Baron, G.V., 1995. Morphology of *Trichoderma reesei QM* 9414 in submerged cultures. Biotechnology and Bioengineeing. 47, 609- 615.

[193] Ilmen, M., Saloheimo, A., Onnela, M.L., Penttila, M.E., 1997. Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. Applied and Environtal Microbiology. 63(4), 1298-1306.

[194] Muthuvelayudham, R., Viruthagiri, T., Deiveegan, S., 2004. Enhanced cellulose production using a mutant strain of *Trichoderma reesei* growing on lactose in batch culture. Chemical Engineering congress.

[195] Muthuvelayudham, R., Viruthagiri, T., 2003. Production of Cellulase protein using mutants of *Trichoderma reesei*. International Congress of Indian Pharmacy Graduates.

[196] Muthuvelayudham, R., Viruthagiri, T., Selvapandian, T., 2005. Biosynthesis of cellulase protein on substrate like cellulose, xylose and lactose using *Trichoderma reesei*. Journal of Engineering and Technology. 118-121.

[197] Bisaria, V.S., Mishra, S., 1989 Regulatory aspects of cellulase biosynthesis and regulation. CRC Critical Reviews in Biotechnology. 9, 61-103.

[198] Muthuvelayudham, R., Deiveegan, S., Viruthagiri, T., 2006. Triggering of Cellulase Protein Production Using Cellulose With Lactose by *Trichoderma reesei*. Asian Journal of Microbiology, Biotechnology and Environmeental Science. 8(2), 33-35.

[199] Aguiar, C.L., 2001. Biodegradation of the cellulose from sugarcane bagasse by fungal cellulase. Ciência e Tecnologia de Alimentos. 2, 117 – 121.

[200] Kapoor, V., Singh, R., Banerjee, R., Kumar, V., 2011. Application of Response Surface Methodology (RSM) for Optimization of Physico-chemical Parameters for the Production of Endoglucanase by Trichoderma ressei Rut C-30 using Agro-residue, *by Dynamic Biochemistry, Process* Biotechnol and Mol. Bio (in press).

[201] Banerjee, R., Pandey, A., 2002. Bioindustrial application of sugarcane bagasse A Technological perspective. International Sugar Journal. 1, 3-7.

[202] Das, M., Banerjee, R., Bal, S., 2008. Multivariable parameter optimization for endoglucanase production by Trichoderma reesei Rut C30 from Ocimum gratissimum seed. Brazilian Archives of Biology and Technology. 51, 35-41.

[203] Olsson, L., Christensen, TMIE., Hansen, K.P., Palmqvist, E.A., 2003. Influence of the carbon source on production of cellulases, hemicellulases and pectinases by *Trichoderma reesei* Rut C – 30. Enzyme and Microbial Technology. 33, 612 – 619.

[204] Thygesen, A., Thomsen, A.B., Schmidt, A.S., Jfrgensen, H., Ahring, B.K., Olsson, L., 2003. Production of cellulose and hemicellulose degrading enzymes by filamentous fungi cultivated on wet-oxidized wheat straw. Enzyme and Microbial Technology. 32, 606-615.

[205] Hamelinck, C.N., van Hooijdonk, G., Faaij, A.P.C., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28, 384–410.

[206] Ojumu, T.V., Ogunkunle, O.A., 2005. Production of glucose from lignocellulosic under extremely low acid and high temperature in batch process, auto-hydrolysis approach. Journal of Applied Sciences. 5 (1), 15–17.

[207] de Groot, M.J.L., Levisson M., van den Dool, C., P vanKuyk, A., Wösten, H.A.B., Ruijter, G.J.G., de Vries, R.P., 2007. Regulation of the pentose catabolic pathway of *Aspergillus niger*. Food Technology and Biotechnology. 45, 134-138.

[208] Zhang, Y.H.P., Lynd, L.R., 2004. Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulose systems. Biotechnology and Bioengineering. 8 (7), 797–824.

[209] Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorious, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiology and Molecular Biology Reviews. 66 (3), 506–577.

[210] Kim, E., Irwin, D.C., Walker, L.P., Wilson, D.B., 1998. Factorial optimization of a six-cellulase mixture. Biotechnology and Bioengineering. 58 (5), 494–501.

[211] Goel S.C., Ramachandran K.B., 1983. Comparison of the rates of enzymatic hydrolysis of pretreated rice straw and bagasse with celluloses.Enzyme and Microbial Technology. 5, 281-4.

[212] Goel S.C., Ramachandran K.B., 1983. Studies on the adsorption of cellulase on lignocellulosics. Journal of Fermentation Technology. 61, 281-6.

[213] Srivastava, A.K., Volesky, B., 1990. Updated model of the batch acetone-butanol fermentation. Biotechnology Letters. 12, 693-698.

[214] Srivastava, S., Srivastava, A.K. 2005. Studies on phosphate uptake by *Acinetobacter calcoaceticus* under aerobic conditions. Enzyme and Microbial Technology. 36, 362-368.

[215] Dien, B.S., Cotta, M.A., Jeffries, T.W., 2003. Bacteria engineered for fuel ethanol production: current status. Applied Microbiology and Biotechnology. 63, 258–66.

[216] U.S. Department of Education Annual Plan.,(2003) Department of Education, Washington, DC, USA.

[217] Sreenath, H.K., Jeffreis, T.W., 2000. Production of ethanol from wood hydrolysate by yeasts. Bioresource Technology. 72, 253–60.

[218] Skotnicki, M.L., Warr, R.G., Goodman, A.E., Lee, K.J., Rogers, P.L., 1982. High-productivity alcohol fermentations using *Zymomonas mobilis*. Biochemical Society Symposium.19, 53–86.

[219] Okamoto, T., Taguchi, H., Nakamura, K., Ikenaga, H., Kuraishi, H., Yamasato, K., 1993. *Zymobacter palmae* gen. nov., sp. nov., a new ethanol-fermenting *Peritrichous* bacterium isolated from palm sap. Archives in Microbiology. 21, 333–7.

[220] Okamoto, H., Taguchi, H., Nakamura, K., Ikenaga, H., 1994. Production of ethanol from maltose by *Zymobacter palmae* fermentation. Agricultural and Biological Chemistry 58, 1328–9.

[221] Jin, Y.S., Jeffries, T.W., 2004. Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*. Metabolic Engineering. 6, 229–38.

[222] Ligthelm, M.E., Prior, B.A., du Preez, J.C. 1988. The oxygen requirements of yeasts for the fermentation of d-xylose and d-glucose to ethanol. Applied Microbiology and Biotechnology. 28, 63–8.

[223] Skoog, K., Jeppsson, H., Hahn-Hagerdal, B., 1992. The effect of oxygenation on glucose fermentation with *Pichia stipitis*. Applied Biotechnology and Biochemistry. 34/35, 369–75.

[224] Bjorling, T., Lindman, B., 1989. Evaluation of xylose-fermenting yeasts for ethanol production from spent sulfite liquor. Enzyme and Microbial Technology. 11, 240–6.

[225] Hahn-Hagerdal, B., Jeppsson, H., Olsson, L., Mohagegi, A., 1994. An interlaboratory comparison of the performance of ethanol producing

microorganisms in a xylose-rich acid hydrolysate. Applied Microbiology and Biotechnology. 41, 62–72.

[226] Sanchez, B., Bautista, J., 1988. Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii*. Enzyme and Microbial Technology. 10, 315–8.

[227] van Zyl, C., Prior, B.A., Du Preez, J.C. 1991. Acetic acid inhibition of d-xylose fermentation by *Pichia stipitis*. Enzyme and Microbial Technology. 13, 82–6.

[228] Zhu J-J, Yong Q, Xu Y, Chen S-X, Yu SY. Adaptation fermentation of Pichia stipitis and combination detoxification on steam exploded lignocellulosic pre-hydrolyzate. Nat Sci 2009;1:47–54.

[229] Gupta R, Sharma KK, Kuhad RC. Separate hydrolysis and fermentation (SHF) of Prosopis juliflora, a woody substrate, for the production of cellulosic ethanol using Saccharomyces cerevisiae and Pichia stipitis NCIM 3498. Bioresour Technol 2009;100:1214–20.

[230] Huang C-F, Lin T-H, Guo G-L, Hwang W-S. Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of Pichia stipitis. Bioresour Technol 2009;100:3914–20.

[231] Telli-Okur M, Eken-Sarac¸ o˘glu N. Fermentation of sunflower seed hull hydrolysate to ethanol by Pichia stipitis. Bioresour Technol 2008;99:2162–9.

[232] Cheng K-K, Cai B-Y, Zhang J-A, Ling H-Z, Zhou Y-J, Ge J-P, et al. Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochem Eng J 2008;38:105–9.

[233] Stoutenburg RM, Perrotta JA, Amidon TE, Nakas JP. Ethanol production from a membrane purified hemicellulosic hydrolysate derived from sugar maple by Pichia stipitis NRRL R-7124. Bioresources 2008;3:1349–58.

[234] Agbogbo FK, Wenger KS. Production of ethanol from corn stover hemi-cellulose hydrolysate using Pichia stipitis. J Ind Microbiol Biotechnol 2007;34:723–7.

[235] Chandel AK, Kapoor RK, Kuhad RC. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by Candida shehatae NCIM 3501. Bioresour Technol 2007;98:1947–50.

[236] Agbogbo FK, Coward-Kelly G, Torry-Smith M, Wenger KS. Fermenta-tion of glucose/xylose mixtures using Pichia stipitis. Process Biochem 2006;41:2333–6.

[237] Nigam JN. Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis. J Ind Microbiol Biotechnol 2001;26:145–50.

[238] Nigam JN. Ethanol production from wheat straw hemicellulose hydrolysate by Pichia stipitis. J Biotechnol 2001;87:17–27.

[239] Nigam JN. Development of xylose-fermenting yeast Pichia stipitis for ethanol production through adaptation on hardwood hemicellulose acid prehy-drolysate. J Appl Microbiol 2001;90:208–15.

[240] Eken-Saracoglu N, Arslan Y. Comparison of different pretreatments in ethanol fermentation using corn cob hemicellulosic hydrolysate with Pichia stipitis and Candida shehatae. Biotechnol Lett 2000;22:855–8.

[241] Fenske JJ, Griffin DA, Penner MH. Comparison of aromatic monomers in lignocellulosic biomass prehydrolysates. J Ind Microbiol Biotechnol 1998;20:364–8.

[242] Abbi M, Kuhad RC, Singh A. Bioconversion of pentoses sugars to ethanol by free and immobilized cells of Candida shehatae (NCL-3501): fermentation behavior. Process Biochem 1996;31:555–60.

[243] Moniruzzaman M. Alcohol fermentation of enzymatic hydrolysate of exploded rice straw by Pichia stipitis. World J Microbiol Technol 1995;11:646.

[244] Kuhad RC, Gupta R, Khasa YP, Singh A. Bioethanol production from Lantana camara (red sage): pretreatment, saccharification and fermentation. Biore-sour Technol 2010;101:8348–54.

[245] Ergun M, Mutlu SF (2000) Application of a statistical technique to the production of ethanol from sugar beet molasses by Saccharomyces cerevisiae. Bioresour Technol 73:251–255

[246] Wright, J.D. et al. (1988) Simultaneous saccharification and fermentation of lignocellulose: process evaluation. Appl. Biochem. Biotechnol. 18, 75–90

[247] Moniruzzaman M,. Dien BS, Skory CD, Chen ZD, Hespell RB, Ho NWY. Fermen-tation of corn fibre sugars by an engineered xylose utilizing Saccharomyces yeast strain. World J Microbiol Technol 1997;13:341–6.

[248] Sonderegger M, Jeppsson M, Hahn-Hägerdal B, Sauer U. Molecular basis for anaerobic growth of Saccharomyces cerevisiae on xylose, investigated by global gene expression and metabolic flux analysis. Appl Environ Microbiol 2004;70:2307–17.

[249] Chou Y-C, Zhang M, Mohagheghi A Finkelstein M. Construction and evaluation of a xylose/arabinose fermenting strain of Zymomonas mobilis. In: Abstracts in 19th symposium on biotechnology for fuels and chemicals, Evans K,. 1997.

[250] Hari Krishna, S., Prasanthi, K., Chowdary, G., Ayyanna, C., 1998. Simultaneous saccharification and fermentation of pretreated sugar cane leaves to ethanol. Process Biochemistry. 33 (8), 825–830.

[251] Tengborg, C., Galbe, M., Zacchi, G., 2001. Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steampretreated softwood. Biotechnology Progress.17, 110–117.

[252] Foody, B., Tolan, J.S., Bernstein, J.D., Foody Sr., P., 2000. Pretreatment process for conversion of cellulose to fuel ethanol. Patent US6090595.

[253] Bertilsson, M., Olofsson, K., Liden, G., 2009. Prefermentation improves xylose utilization in simultaneous saccharification and co-fermentation of pretreated spruce. Biotechnology for Biofuels. 2(8)

[254] Alkasrawi, M., Eriksson, T., Borjesson, J., Wingren, A., Galbe, M., Tjerneld, F., Zacchi, G., 2003. The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol. Enzyme and Microbial Technology. 33, 71–78.

[255] Saha, B.C., Iten, L.B., Cotta, M.A., Wu, Y.V., 2005. Dilute acid pretreatment, enzymatic saccharification, and fermentation of rice hulls to ethanol. Biotechnology Progress. 21, 816–822.

[256] Preez, Du., Prior, B.A., Monteiro, A.M.T., 1984. The effect of aeration on xylose fermentation by *Candida shehatae* and *Pachysolen tannophilus*. Applied Microbiology and Biotechnology. 19, 261–266.

[257] Hahn-Hagerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Liden, G., Zacchi, G., 2006. Bioethanol the fuel of tomorrow from the residues of today. Trends in Biotechnology. 24, 549–56.

[258] Ho, N.W. and Tsao, G.T. 1995. Recombinant yeasts for effective fermentation of glucose and xylose. PCT/US94/12861

[259] Ehrman,T., 1994. Chemical Analysis and Testing Laboratory Analytical Procedures LAP-001. Standard Method for Determination of Total Solids in BiomassNational Renewable Energy Laboratory, Golden:CO.

[260] Ehrman, T., 1994. Chemical Analysis and Testing Laboratory Analytical Procedures LAP-005. Standard Method for Ash in Biomass. National Renewable Energy Laboratory, Golden:CO.

[261] Templeton, D., Ehrman, T., 1995. Chemical Analysis and Testing Laboratory Analytical Procedures LAP-003. Determination of Acid-Insoluble Lignin in Biomass. National Renewable Energy Laboratory, Golden:CO.

[262] Ehrman, T., 1996. Chemical Analysis and Testing Laboratory Analytical Procedures LAP-005. Determination of Acid-Soluble Lignin in Biomass. National Renewable Energy Laboratory, Golden:CO.

[263] Han, J.S., Rowell, J. S., 1997. Paper and Composites from Agro-based Resources, 83–134.

[264] Shukla, V.K.S., Sharma, J.P., 1975. Spectrophotometric determination of furfural in mg quantities using its molybdic acid complex. Journal of Analytical Chemistry. 276,300.

[265] Van Zyl, C., Prior, B.A., du Preez, J.C., 1998. Production of ethanol from sugar cane bagasse hemicellulose hydrolysate by Pichia stipitis. Applied Biochemistry and Biotechnology. 17, 357–369.

[266] Muthuvelayudham, R., Viruthagiri, T., 2006. Fermentative production and kinetics of cellulose protein on Tricoderma reesei ysing sugarcane bagasse and rice straw. African Journal of Biotechnology. 5 (20), 1873-1881.

[267] Xiao, Z., Storms, R., Tsang, A., 2005. Microplate-based carboxymethylcellulose assay for endoglucanase activity. Analytical Biochemistry. 342, 176, 17.

[268] Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23, 257–270.

[269] Ghosh, T.K., 1987. Measurement of cellulase activities. Pure Applied Chemistry. 59, 257–268.

[270] Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducingsugar. Analytical Chemistry. 31, 426–428.

[271] Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Chemistry. 72, 248–254.

[272] Eberts, T.J., Sample, R.H., Glick, M.R., Ellis, G.H., 1979. A simplified, colorimetric micro-method for xylose in serum or urine, with phloroglucinol. Clinical Chemistry. 25, 1440–1443.

[273] Blake, D.A., McLean, N.V., 1989. A Colorimetric Assay for the Measurement of D-GlucoseConsumption by Cultured Cells. Analytical biochemistry.177, 156-60.

[274] Nigam, J.N., 2002. Bioconversion of water-hyacinth (*Eichhornia crassipes*) hemicellulose acid hydrolysate to motor fuel ethanol by xylose–fermenting yeast. Journal of Biotechnology. 97, 107–116.

[275] Shafaghat, H., Najafpour, G.D., Rezaei, P.S., Sharifzadeh, M., 2009. Growth kinetics and ethanol productivity of Saccharomyces cerevisiae PTCC 24860 on various carbon sources. World applied science journal. 7 (2), 140-144.

[276] Chandel, A.K., Kapoor, R.K., Singh, A. & Kuhad, R.C., (2007). Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresource Technology. 98, 1947–1950.

[277] Hu, G., Heitmann, J.A., Rojas, O.J., 2008. Feedstock pretreatment strategies for producing ethanol from wood, bark and forest residues. Bio-resources 3 (1), 270–294.

[278] Keshwani, D., Cheng, J., 2009. Switchgrass for bioethanol and other value-added applications: A review. Bioresource Technology. 100, 1515–23.

[279] Grethlein, H.E., Converse, A.O., 1991. Common aspects of acid prehydrolysis and steam explosion for pretreating wood. Bioresource Technology. 36, 77-82.

[280] Torget, R., Hsu, T.A., 1994. Two-temperature dilute acid prehydrolysis of hardwood xylan using a percolation process. Appllied Biochemistry and Biotechnology. 45, 5-23.

[281] Ingram LO, Lai X, Moniruzzaman M, Wood BE,York SW. Fuel ethanol production from lignocellulose using genetically engineered bacteria. In: 1997.

[282] Esteghlalian A, Hashimoto AG, Fenske JJ, Penner MH. 1997. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. Bioresource Technology. 59, 129-136.

[283] Nguyen, Q.A., Tucker, M.P., Boynton, B.L., Keller, F.A., Schell, D.J., 1998. Dilute acid pretreatment of softwoods. Applied Biochemistry and Biotechnology. 70, 77–87.

[284] Roberto, I.C., Mussatto, S.I., Rodrigues, R.C.L.B., 2003. Dilute-acid hydrolysis for optimization of xylose recovery from rice straw in a semi-pilot reactor. Industrial Crops Product. 7, 171–176.

[285] McMillan, J.D., 1994. Pretreatment of lignocellulosic biomass. ACS Ser 566:292–324.

[286] Kumar, R., Mago, G., Balan, V., Wyman, C.E., 2009. Physical and chemical characterization of corn stover and polar solids resulting from leading pretreatment technologies. Bioresource Technology. 100, 3948–3962.

[287] Karimi, K., Emtiazi, G., Taherzadeh, M.J. 2006. Ethanol production from dilute-acid pretreated rice straw by simultaneous saccharification and fermentation with Mucor indicus, Rhizopus oryzae, and Saccharomyces cerevisiae. Enzyme and Microbial Technology. 40, 138–44.

[288] Jeffries, T.W., Jin, Y.S., 2000. Ethanol and thermotolerance in the bioconversion of xylose by yeasts. Advanced and Applied Microbiology. 221–268.

[289] Torget, R., Hatzis, C., Hayward, T.K., Hsu, T.A., Philippidis, G.P., 1996. Optimization of reverse-flow, 2-temperature, dilute-acid pretreatment to enhance biomass conversion to ethanol. Applied and Biochemistry and Biotechnology. 58, 85–101.

[290] Cara, C., Ruiz, E., Oliva, J.M., Saez, F., Castro, E., 2007. Conversion of olive tree biomass into fermentable sugars by dilute acid pretreatment and enzymatic saccharification. Bioresource Technology. 99, 1869–1876.

[291] Roberto, I.C, Lacis, L.S., Barbosa, M.F.S, Demancilha, I.M, 1991. Utilization of sugarcane bagasse hemicellulose hydrolysate by *Pichia stipitis* for the production of ethanol. Process Biochemistry. 26, 15–21.

[292] Limtong, S., Sumpradit, T., Kitpreechavanich, V., Tuntirungkij, M., Seki, T., Yoshida, T., 2000. Effect of acetic acid on growth and ethanol fermentation of xylose fermenting yeast and *Saccharomyces cerevisiae.* Kasetsart Journal (Nat. Sci). 341, 64-73.

[293] Sindhu, R., Binod, P., Nagalakshmi, S., Janu, K.U., Sajna, K.V., Kurien, N., Sukumaran, R.K., Pandey, A., 2010. Formic acid as a potential pretreatment agent for conversion of sugarcane bagasse to ethanol. Appllied Biochemistry and Biotechnology. 162, 2313–2323.

[294] Bak, J.S., Ko, J.K., Han, Y.H., 2009. Improved enzymatic hydrolysis yield of rice straw using electron beam irradiation pretreatment. Bioresource Technology, 100, 1285-1290.

[295] Ko, J.K., Bak, J.S., Jung, M.W., Lee, H.J., Choi, I.G., Kim, T.H., Kim, K.H., 2009. Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes. Bioresource Technology. 100, 4374–4380.

[296] Fox, D.J., Gray, P.P., Dunn, N.W., Marsden, W.L., 1989. Comparison of alkali and steam (acid) pretreatments of lignocellulosic materials to increase enzymic susceptibility: evaluation under optimised pretreatment conditions. Journal of Chemical Technology and Biotechnology. 44, 135–146.

[297] MacDonald, D.G., Bakhshi, N.N., Mathews, J.F., Roychowdhury, A., Bajpai, P., Moo-Young, M., 1983. Alkali treatment of corn stover to improve sugar production by enzymatic hydrolysis. Biotechnology and Bioengineering. 25, 2067– 2076.

[298] Sharma, S.K., Kalra, K.L., Grewal, H.S., 2002. Enzymatic saccharification of pretreated sunflower stalks. Biomass and Bioenergy. 23, 237–243.

[299] Silverstein, R.A., Chen, Y., Sharma-Shivappa, R.R., Boyette, M.D., Osborne, J.A., 2007. comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresource Technology. 98, 3000–3011

[300] Soto, M.L., Domínguez, H., Núñez, M.J., Lema, J.M., 1994. Enzymatic saccharification of alkali-treated sunflower hulls. Bioresource Technology. 49, 53–59.

[301] Adsul, M.G., Ghule, J.E., Shaikh, H., Singh, R.K., Bastawde,B., Gokhale, D.V., Varma, A.J., 2005. Enzymatic hydrolysis of delignified bagasse polysaccharides. Carbohydrate Polymer. 62, 6-10

[302] Palonen, H., Tjerneld, F., Zacchi, G., Tenkanen, M., 2004. Adsorption of *Trichoderma reesei* CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. Journal of Biotechnology. 107, 65–72

[303] Hui, W., Radhakrishnan, S., Fei, Y., 2011. Effect of Acid, Alkali, and Steam Explosion Pretreatments on Characteristics of Bio-Oil Produced from Pinewood. Energy Fuels. 25 (8), 3758–3764.

[304] Zawadzki, M., Runge, T., Ragauskas, A.J., 1999. Investigation of ortho- and para-Quinone Chromophores in Alkaline Extraction Stage Residual Lignins. In ACS Symposium Series 742, Lignin: Historical, Biological, and Materials Perspectives, Eds. Glasser, W.G.; Northey, R.A.; Schultz, T.P., ACS, Washington, 254, 505.

[305] Zawadzki, M., Runge, T., Ragauskas, A.J., 2000. Facile Detection of Ortho- and Para- uinone Structures in Residual Lignin by 31P NMR Spectroscopy. Journal of Pulp Paper science. 26, 102.

[306] Chang, V.S., Holtzapple, M.T., 2000. Fundamental factors affecting biomass enzymatic reactivity. Applied Biochemistry and Biotechnology. 84, 5-37.

[307] Pourquie, J., Desmarquest, J.P., 1999. Scale up of cellulases production by Trichoderma reesei. In: coughlan MP (ed) Enzyme system for lignocellulose degradation. Elsevier, Landon, 283-292.

[308] Warzywoda, M., larbre, E., Pourquie, J., 1992. Production and characterization of cellulolytic enzymes from *Trichoderma reesei* grown on various carbon sources. Bioresource Technology. 39, 125-130.

[309] Chaudhuri, B.K., Sahai, V., 1993. Production of cellulase using a mutant strain of *Trichoderma reesei* growing on lactose in batch culture. Applied Microbiology and Biotechnology. 39, 194–196.

[310] Szczodrak, J., 987. The enzymatic hydrolysis and fermentation of pretreated wheat straw to ethanol. Biotechnology and Bioengineering. 32, 771–776.

[311] Lee, Y.H., Fan, L. T., 1982. Kinetic studies of enzymatic hydrolysis of insoluble cellulose: analysis of the initial rates. Biotechnology and Bioengineering. 24, 2383–2406.

[312] Soto, M.L., Dominguez, H., Nunez, M.J., Lema, J.M., 1994. Enzymatic Saccharification of alkali-treated sunflower hulls. Bioresource Technology. 49, 53–59.

[313] Ishihara, M., Toyama, S., 1990. Enzymatic degradability of chemically pretreated bagasse., Science bulletin, vol. 37. Okinawa: College of Agriculture, University of the Ryukyus: 99. 2733.

[314] Nigam, J.N., 2001. Ethanol production from hardwood spent sulfite liquor using an adapted strain of Pichia stipitis. Journal of Industrial Microbiology and Biotechnology 26, 145-50.

[315] Huang, C.F., Lin, T.H., Gout, G.L. and Hwang, W.S., 2009. Enhanced ethanol production by fermentation of rice straw hydrolysate without detoxification using a newly adapted strain of *Pichia stipitis*. Bioresource Techology. 17, 3914-3920.

[316] Díaz, M.J., Ruiz, E., Romero, I., Cara, C, Moya, M. and Castro, E., 2009. Inhibition of *Pichia stipitis* fermentation of hydrolysates from olive tree cuttings. World Journal of Microbiology and Biotechnology. 25, 891–99.

[317] Nigam, J.N., 2001. Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis*. Journal of Biotechnology. 87, 17–27.

[318] Cheng. K.K, Cai, B.Y., Zhang, J.A., Ling, H.Z., Zhou, Y.J., Ge Jing-P. and Xu J.M. Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochemical Engineering Journal. 38, 105-09.

[319] Kumar, A., Singh, L.K., Ghosh, S. 2009. Bioconversion of lignocellulosic fraction of water-hyacinth (Eichhornia crassipes) hemicellulose acid hydrolysate to ethanol by *Pichia stipitis*. Bioresource Technology. 100, 3293–97.

[320] Zaldivar, J., Roca, C., Caroline, L. F., Hahn-Ha¨gerdal, B., Olsson, L., 2005. Ethanolic fermentation of acid pre-treated starch industry effluents by recombinant Saccharomyces cerevisiae strains. Bioresource Technology. 96, 1670–1676.

[321] Gupta, R., Sharma, K.K., Kuhad, R.C., 2009. Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by Saccharomyces cerevisiae and *Pichia stipitis*-NCIM 3498. Bioresource Technology. 100, 1214–1220.

[322] Chandel, A.K., Singh, O.V., Venkateswar, R.L., Chandrasekhar, G., Narasu, M.L., 2011. Bioconversion of novel substrate Saccharum spontaneum, a weedy material, into ethanol by *Pichia stipitis* NCIM 3498. Bioresource Technology. 102, 1709-1714.

[323] Badal, C.S., Michael, A., 2010. Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol. New Biotechnology. 27, 10-16.

[324] Bertilsson, M., Olofsson, K., Liden, G., 2009. Prefermentation improves xylose utilization in simultaneous saccharification and co-fermentation of pretreated spruce. Biotechnology for Biofuels. 2-8.

List of publications in journals

Kataria Rashmi, Chaudhary Gaurav and Ghosh Sanjoy "Potential of Bioenergy Production from Grasses and its Impact on Environment". *Research journal of biotechnology*, 4, 5-14 (2009).

Rashmi Kataria and Sanjoy Ghosh "Saccharification of Kans grass using enzyme mixture from *T.reesei* for bioethanol production". *Bioresource technology*, 102, 9970–9975 (2011).

Rashmi Kataria and Sanjoy Ghosh "NaOH pretreatment and enzymatic hydrolysis of *Saccharum spontaneum* for reducing sugars production" (**Accepted** for publication in *Energy resources Part A: Recovery, Utilization, and Environmental Effects*. (DOI number 10.1080/15567036.2010.551268).

Rashmi Kataria and Sanjoy Ghosh "Bioconversion of dilute sulphuric acid hydrolysate of *Saccharum spontaneum* to ethanol by *Pichia stipitis*" *Biomass and bioenergy* (under review, submitted June 2010).

Rashmi Kataria and Sanjoy Ghosh. "Utilization of Kans grass as a potential substrate for ethanol production by Acid hydrolysis." Under comunication

List of Abstract/paper published in conferences

Rashmi Kataria and Sanjoy Ghosh: "A Comparative study of physicochemical and biological methods for production of fermentable sugars from certain variety in switch grass". 49th AMI conference organized in DU, Delhi, India (November 18-20, 2008).

Rashmi Kataria, Chaudhary Gaurav and Sanjoy Ghosh entitled with "Potential for Bioenergy Production and its Impact on Environment". First International biotechnology conference ISBT-2008 held at Gangtok, Sikkim, India (28th to 30th December 2008).

Rashmi Kataria and Sanjoy Ghosh : Fourth International conference on Plant and environmental Pollution held at NBRI Lacknow "Alkali pretreatment and Enzymatic hydrolysis of Kans grass to fermentable sugars for bioethanol production" (8-11 December 2010).

Lalit Kumar Singh, Gaurav Chaudhary, Rashmi Kataria and Sanjoy Ghosh: "Production of reducing sugars from lignocellulosic biomass for fuel ethanol production". National conference on 'Water, Energy and Biodiversity' jointly organized by The Institute of Engineers (India), Tripura State Centre, Tripura and National Institute of hydrology, Roorkee. at Agartala, Tripura (August 20-22, 2011).