BIOETHANOL PRODUCTION BY FRACTIONAL HYDROLYSIS AND CO-CULTURE FERMENTATION

Ph.D. THESIS

by

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A THESIS

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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"BIOETHANOL PRODUCTION BY FRACTIONAL HYDROLYSIS AND CO-CULTURE FERMENTATION"** 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, 2012 to June, 2018 under the supervision of Dr. Sanjoy Ghosh, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

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

(ARCHANA MISHRA)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

(Sanjoy Ghosh)
Supervisor

Date: **29/06/2018**

ABSTRACT

It is widely known that transportation sector is almost entirely dependent on fossil fuels; primarily on petroleum-based fuels (liquefied petroleum gas, gasoline, compressed natural gas and diesel fuel gas). Amount of petroleum availability is depleting day by day; therefore alternatives are needed to produce liquid fuels for reducing the future effects of the shortage in supply of transportation fuels. Term biofuel refers to solid (biochar), liquid (biodiesel, bioethanol, and vegetable oil) or gaseous (biohydrogen, biosyngas, and biogas) fuels that are mainly produced from biomass. They are renewable; most common is bioethanol (petrol additive or gasoline substitute). Bioethanol has the potential to reduce both crude oil consumption, and environmental pollution. Conventional resources for ethanol production (grains majorly) compete directly with human food materials and give rise to food vs. fuel conflict. Hence, it is essential to produce ethanol from various feedstocks and not depend solely on grains and molasses.

Lignocellulosic feedstocks are obtained and harvested from agricultural wastes materials as well as forest residues crops. It consists of cellulose (40-60%), hemicellulose (20-40%), and lignin 10-25% on an average. Typically cellulose and hemicelluloses part comprise 2/3rd of the total dry biomass. Carbohydrate part (cellulose and hemicellulose) of lignocellulosic biomass can be saccharified to obtain soluble sugars and further convert it into ethanol by fermentation. Major obstacles for the commercial lignocellulosic ethanol production include a. Maximum amount of fermentable sugars (hexoses and pentoses) extraction from the lignocellulosic feedstocks b. Suitable microorganisms (more tolerant toward fermentation inhibitors) and fermentation techniques to ferment maximum amount of sugars present in the lignocellulosic biomass hydrolysate for high ethanol yield and productivity, and c. Process integration requirement to minimise the total number of steps involved in overall production. Technological approach improvements and optimisation of various factors have been given priority in various studies. Nevertheless, still, there are some challenges which need to be adequately addressed in the development of a sustainable bioethanol industry.

To contribute in the pool of existing knowledge, we have tried to develop a technique which can convert lignocellulosic biomass into fuel ethanol in just two process steps, with high conversion efficiency and thus hope to bring down the ethanol production cost effectively. The biomass chosen for the present work was kans grass biomass (*Saccharum spontaneum*), a perennial C4 plant with high amount of carbohydrate (65.5%, w/w) compared to other

lignocellulosic biomasses which can be converted to ethanol by suitable process technologies. It grows throughout the year on marginal and wetlands. Once planted, harvesting can be done many times for many years; re-plantation and watering are not required, ensuring the steady supply of raw materials.

For saccharification, a unique technique called as 'fractional hydrolysis' has been developed that gives us pentose and hexose sugars as separate hydrolysate fractions. Different physical and chemical parameters (preheating time, liquid:solid/biomass loading, and number of stages) have been optimised for the fractional hydrolysis process using one-variable-at-a-time (OVAT) approach. The 8-stage fractional hydrolysis process was able to recover 84.88% total reducing sugars from kans grass biomass with minimum toxics (1.27×10^{-2} g furfural and 3.04×10^{-2} g phenolics) using sulphuric acid. To validate these results, the process was extended for other cheap and easily available lignocellulosic feedstocks (wheat straw and sugarcane bagasse) and inorganic acids (hydrochloric acid, phosphoric acid, and nitric acid) up to 30% concentration (v/v). The compositional analysis of all the three selected biomasses in the present work showed high cellulose and hemicelluloses content (63-66%). The fractional hydrolysis technique has been proven independent of feedstock type and resulting in saccharification (%): kans grass 84.88, sugarcane bagasse 82.55, and wheat straw 81.66. Among the acids used, TRS recovery was very less using phosphoric acid whereas nitric acid resulted in maximum sugar recovery, but the high cost makes it economically non-feasible. The results of both HCl and H_2SO_4 were comparable but comparatively lower price of H_2SO_4 makes it as the most suitable reagent for fractional hydrolysis process resulting in maximum sugar recovery with minimum toxics. The fractional hydrolysis process was able to recover xylose and glucose sugar fractions separately and called as xylose-rich fraction (XRF) and glucose-rich fraction (GRF) respectively.

The structural characterisation of raw biomass, biomass after XRF removal, and fully treated biomass showed marked differences during all the analyses. SEM images of biomass showed surface distortion in the form of cracks and pores on the surface compared to the intact surface of raw feedstocks. FESEM gave elemental composition at each stage with a high-resolution images. SPM was used to measure the roughness analyses with 3-D imaging. FTIR spectroscopic analysis of the biomass showed a decrease in the absorption peaks indicating the loss of cellulose and hemicelluloses. XRD analysis was done to measure the changes in crystallinity indices of feedstocks during the course of fractional hydrolysis. TGA provided information about the pyrolysis temperatures of cellulose, hemicelluloses, and lignin present in

the feedstocks along with weight loss %. These results validate the efficiency of 8-stage fractional hydrolysis technique for the maximum sugar recovery with negligible toxics.

Based on these results, further fermentation studies have been carried out on kans grass biomass using 8-stage fractional hydrolysis process with H_2SO_4. As there is no known naturally occurring microorganism that can ferment pentose and hexose sugars simultaneously with the same efficiency, hence getting the separate XRF and GRF is of tremendous advantage. Moreover, fractional hydrolysis technique merges two different steps (pretreatment and hydrolysis) into one. Also, the concentration of toxic compounds in hydrolysate was very low; therefore, after saccharification, hydrolysate fractions can be taken directly for fermentation without any detoxification, thereby cutting down the overall production cost.

In the fermentation part, *Z. mobilis* was selected for the hexose fermentation as it gives high ethanol yield and productivity. Also, *Z. mobilis* is capable of producing almost a theoretical amount of ethanol from glucose, via Entner-Doudoroff pathway under anaerobic condition and tolerates high concentration of ethanol. Among xylose fermenting organisms, *Candida shehatae* or *Scheffersomyces shehatae* has been found to ferment ethanol faster compared to other organisms; also the specific rate of ethanol production was highest among pentose-fermenting yeasts.

The generation of two different sugar fractions as XRF and GRF from the kans grass hydrolysis enabled xylose fermentation first and thereby eliminated lower ethanol tolerance problem of the pentose-fermenting yeasts and catabolite repression of xylose by glucose consumption as the preferred carbon source for *S. shehatae*. It also allowed to maintain the different aeration requirements of two organisms in co-culture system (microaerobic for *S. shehatae* and strictly anaerobic for *Z. mobilis*). Moreover, a single reactor can be used for the fermentation of both the sugars. Initially, 2-step sequential co-culture fermentation was carried out using TRS concentration up to 60 g/L in XRF and 200 g/L in GRF. The process resulted in 55.95 g/L of ethanol with average yield coefficient of 0.41 and productivity of 0.65 g/L/h from the kans grass biomass hydrolysate. It was observed that upon increasing sugar concentration, sugar consumption rate decreases to a large extent. Moreover, ethanol concentration >55 g/L is very difficult to obtain even on increasing glucose up to 200 g/L. Therefore, multi-step successive glucose feeding co-culture system was developed to overcome these problems. A multi-step glucose feeding co-culture system containing *S. shehatae* (for xylose fermentation) and *Z. mobilis* (for glucose fermentation) provided high average ethanol yield, concentration and productivity compared to the previous co-culture system. The average ethanol yield coefficient

and overall volumetric ethanol productivity were found as 0.44 and 0.79 g/L/h respectively, and 79.59 g/L of ethanol concentration (up to stage 4) was achieved by utilising sugar up to 200 g/L.

The significance of the present study revealed that a novel "fractional hydrolysis" recovered the maximum amount of soluble pentose and hexose sugars (84.88% of the total reducing sugars) separately; direct from the lignocellulosic biomass with negligible toxic products generation. Moreover, a single-reactor unique approach of co-culture fermentation using *Z. mobilis* (for GRF fermentation) and *S. shehatae* (for XRF fermentation) by utilising maximum sugars present in the kans grass hydrolysate may be able to reduce the overall bioethanol production cost further with high ethanol yield (0.44) and concentration (79.59 g/L).

Keywords: Lignocellulosics, Fractional hydrolysis, Holocellulose, Reducing sugars, Co-culture fermentation, Bioethanol

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ABBREVIATIONS

1G	1st generation
2G	2nd generation
3G	3rd generation
3-D	3-Dimensional
5-HMF	5-Hydroxymethy furfural
^{o}C	Degree Celsius
ANPR	Advance Notice of Proposed Rulemaking
AFEX	Ammonia Fibre Expansion Method
ARP	Ammonia Recycle Percolation
AU	Arbitary Unit
BET	Brunauer-Emmett-Teller
BJH	Barrett-Joyner-Halenda
CBP	Consolidated Bioprocessing
DDGS	Distillers dried grains with soluble
DNS	3,5-Dinitro salicylic acid
DMC	Direct Microbial Conversion
EDX	Energy-dispersive X-ray spectroscopy
	(L: Lignin; H: Hemicellulose; C: Cellulose)
FID	Flame Ionisation Detector
Fig.	Figure
FESEM	Field Emission Scanning Electron Microscopy
FTIR	Fourier Transform Infrared Spectroscopy
gTME	Global transcription machinery engineering
G	Gram
GC	Gas Chromatography
GHG	Greenhouse gas emisssions
GRF	Glucose-rich fraction
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IMTECH	Institute of Microbial Technology
LAP	Laboratory Analytical procedure
L	Litre

MPa	Mega Pascal
µL	Microlitres
µm	Micrometre
µmol	Micromoles
mg	Milligram
mL	Millilitre
mm	Millimeter
mm	Millimeter
mM	Millimole
min	Minutes
M	Molarity
MT	Metric Tonnes
MTBE	Methyl Tertiary Butyl Ether
MTCC	Microbial Type Culture Collection and Gene Bank
NCIM	National Collection of Industrial Microorganisms
NCL	National Chemical Laboratory
n.d.	Not Detected
nm:	Nanometer
N:	Normality
NREL	National Renewable Energy Laboratory
OD	Optical density
OVAT/OFAT	One-Variable/One-Factor at a Time
PPP	Pentose Phosphate Pathway
RPM	Revolutions Per Minute
ppb	Parts per Billions
RTIL	Room Temperature Ionic Liquids
SAA	Soaking in Aqueous Ammonia
SEM	Scanning Electron Microscopy
SHF	Separate Saccharification and Fermentation
SPM	Scanning Probe Microscopy
SS	*Scheffersomyces shehatae*
SSF	Simultaneous Saccharification and Fermentation
SSCF	Simultaneous Saccharification and Co-fermentation
T	Temperature

TCD	Thermal Conductivity Detector
TFA	Trifluoro Acetic acid
TGA	Thermogravimetric Analysis
TRS	Total reducing sugar
XDH	Xylose dehydrogenase
XI	Xylose isomerase
XK	Xylose kinase
XR	Xylose Reductase
XRD	X-Ray diffractions
XRF	Xylose-rich fraction
VHE	Very High Gravity
ZM	*Zymomonas mobilis*
v/v	Volume by Volume
w/v	Weight by Volume
w/w	Weight by Weight
%	Percentage

CHAPTER 1

Introduction and Review of Literature

1. Introduction

Increase in oil security and environmental concerns, as well as the fossil fuels negative impact on environment, boosted the demand for renewable and eco-friendly energy supply. It's been anticipated long that fuel reserves (fossil fuels) are going to be exhausted in the near future as a result of continuous increase in the consumption of these fuels [1]. Fig. 1.1 evidently confirms these facts by showing energy consumption in the form of different fuel types during last decade. Additionally, the burning of fossil fuels contributes largely to the greenhouse gases (GHG) emissions and global warming that causes temperature and water level rise, loss of diverseness, and pollution [2-4]. Therefore, an alternative fuel must have superior environmental benefits, economy, and net energy gains over energy sources required to produce it [5-6]. Bioethanol is one amongst the foremost promising alternatives to conventional fossil fuels, mainly produced from carbohydrate-rich and renewable sources. Several countries (USA and Brazil majorly, China, North American nation like Canada, and several other European union member states have already declared commitments to reduce fossil fuels dependence through bioethanol programs.

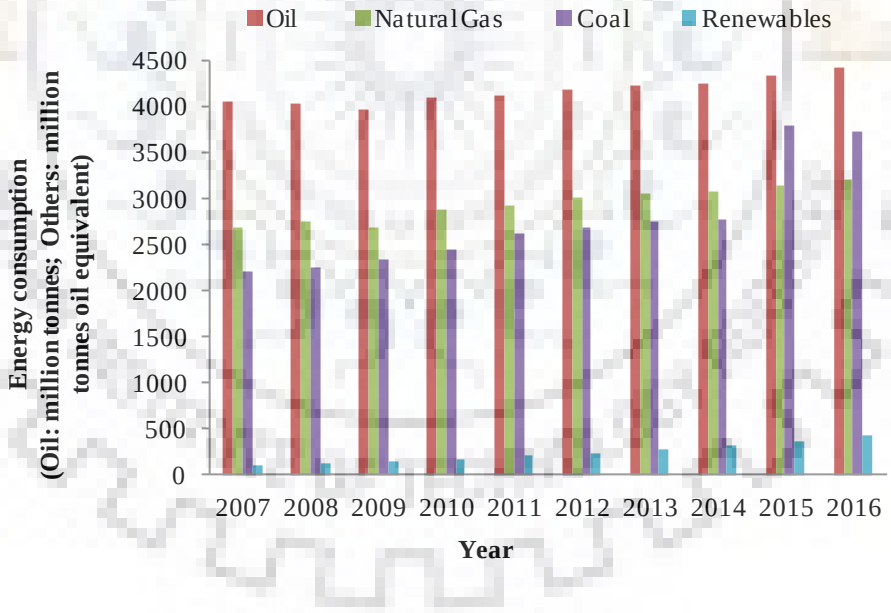


Fig. 1.1. World energy consumption by different fuel types (2007-2016) [7].

In the view of these points, world bioethanol production has been increased over time **(**Fig. 1.2). Fig. 1.2 shows that USA produces the highest quantity of bioethanol (more than half of the world's total ethanol production as estimated in 2017). Status of global bio-refineries contributing to fuel ethanol production in 2016 (operational, under development, and suspended) can be seen from Fig. 1.3.

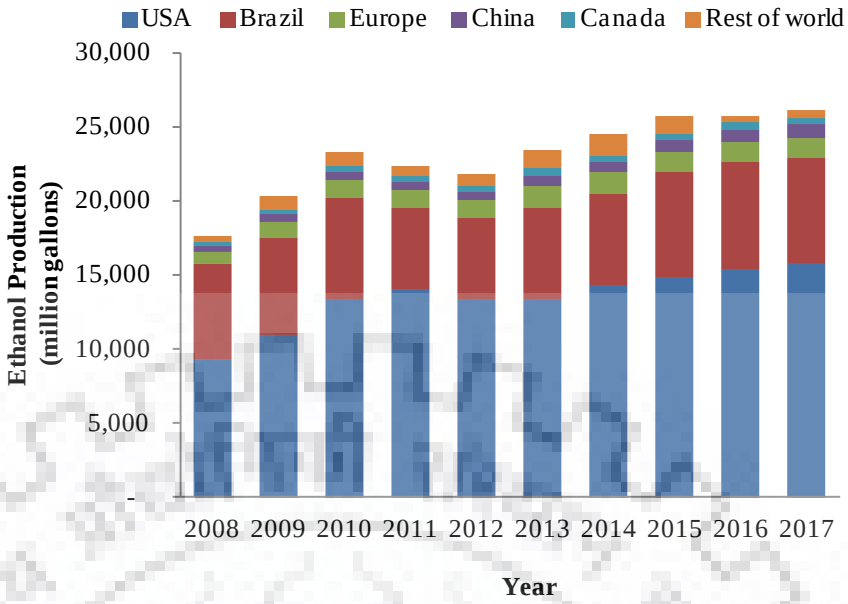


Fig. 1.2. Global ethanol production by region (2008-2017) [8].

Fig. 1.3. Global Biorefineries, 2016; Legend: Blue markers (operational biorefineries), Yellow (in development), and Red (suspended) [9].

1.1 Indian scenario

India's primary energy consumption share is 212.7 million tonnes (5.5%) of global, 3rd biggest after China and USA in the year 2016 [7]. The total primary energy consumption from coal (56.9%), crude oil (29.38%), natural gas (6.23%), hydroelectricity (4.01%), nuclear energy (1.19%), wind power, solar power, and biomass electricity was 723.9 Mtoe in 2016 (Fig. 1.4),

excluding traditional biomass use. Country's dependence on energy imports is expected to exceed 53% of the total consumption. 195.1 million tonnes of crude oil was imported in 2015, which is about 80% of its domestic consumption. Around 31% of total imports are oil imports [10]. Energy consumption in India in different sectors for last ten years has been presented in Fig. 1.5. From the figure, it is visibly seen that energy demand is increasing every year in all sectors. Therefore, to meet its energy demand, the country will have to import huge amounts of energy from other countries [11].

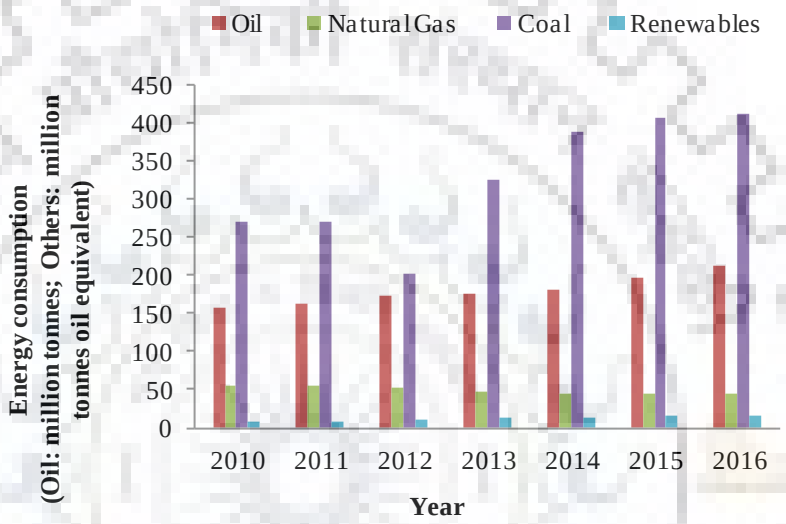


Fig. 1.4. Primary energy consumption in India (2010-2016) by fuel types.

Currently, India is fourth highest greenhouse gas (GHG) emitter [13]. The transportation sector of India accounts for about 13% of total CO_2 emissions, although per capita emissions are less than half of world's average [6,14]. Various technologies in the field of renewable energy have been developed for heat and electricity generation (biomass combustion, hydropower, and windmills). But in the expanding transportation sector, no similar kind of development has happened for alternative energy sources production [15]. Hence, the country needs to find other sustainable and renewable energy generation sources to meet the demand in transportation sector; providing a good market for biofuels.

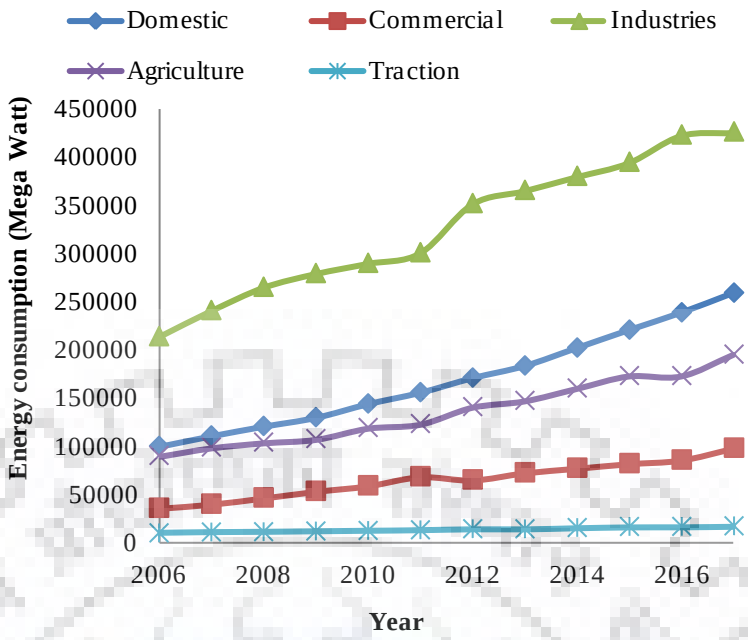


Fig. 1.5. Trend of category-wise energy consumption (2006-2017) [16].

In India, sourcewise installed capacities of various renewable energies during last four years is shown in Fig. 1.6. Wind power sources contribute highest whereas waste to energy source or biofuel-based energy source is still in its primary stage (country's biofuel production accounts for only 1% of global production) [13,17]. Commercially only bioethanol and biodiesel are produced in India [13]. The government of India has set a mandate of 5% blending of renewable biofuel in both petrol and diesel. Currently, diesel biofuel blending is near zero, and petrol blending is around 3% from molasses based ethanol. The annual requirement of ethanol stands at about 500 crore litre in India, but total installed capacity is just about 265 crore litre. Therefore, the target of 20% blending by 2020 which is set by biofuel policy of India look too far unless second generation (2G) ethanol production technologies are successfully demonstrated at commercial level [18].

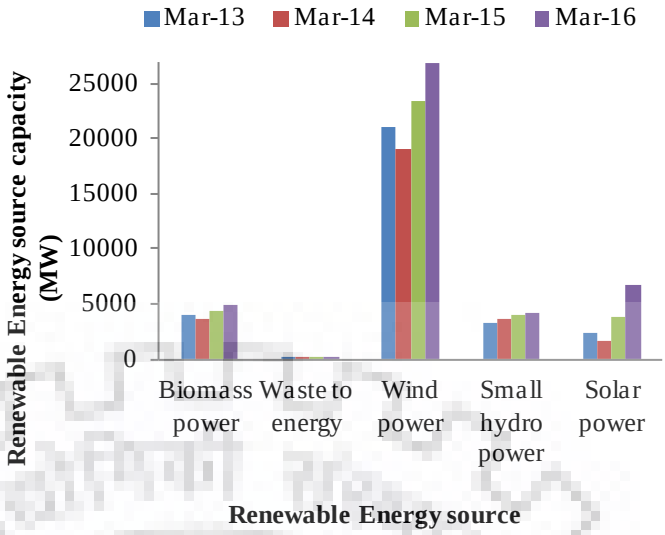


Fig. 1.6. Sourcewise installed capacities of Renewable powers in India [19].

As the productivities of jatropha and sugarcane were very less during past few years, it has become a big challenge for industries to produce a high volume of fuels (ethanol and diesel). Hence to increase yield and productivity, land and other requirements (water and fertilisers) use have to be increased which is not feasible due to large population growth. Sustainable supply of non-edible and non-grazeable biomass is one of the major limitations for ethanol production in India. In terms of renewable energy resources and agricultural produce, India has immense potential with their even distribution compared to fossil fuel reserves. It will surely help in the biomass-based energy development rather than food crops to combat the primary energy demand of country and to maintain the sustainable energy supply.

1.2 Biofuel classification

Traditional energy sources for power generation, cooking, and heating consisted of solid biomass (fuel wood and agro-residues) whereas it was dung-cakes (pellet form) and densified rice straw in the rural areas. Biofuels usually categorised into liquid and gaseous types, produced from biomass using microbes. Liquid fuels are bioethanol, biodiesel, and biobutanol mainly whereas biomethane and biohydrogen come are classified as gaseous fuels (Fig. 1.7).

Biodiesel is a substitute for diesel fuel that can be produced from microalgae, plant vegetable oils, and animal fats. The focus has been shifting towards yeast and oleaginous fungus for microbial lipids production for biodiesel [20]. Biobutanol has superior fuel qualities compared to other alcohols and can be produced from lignocellulosic feedstocks effectively. It can be

used in pure form also unlike ethanol [21]. Biomethane (biogas) is the mixture of methane and carbon dioxide (major proportions) along with very limited quantities of nitrogen, ammonia, and eco-friendly nature hydrogen is considered as the fuel of the future. The potential feedstock for biohydrogen production is carbohydrate-rich plant biomass [22].

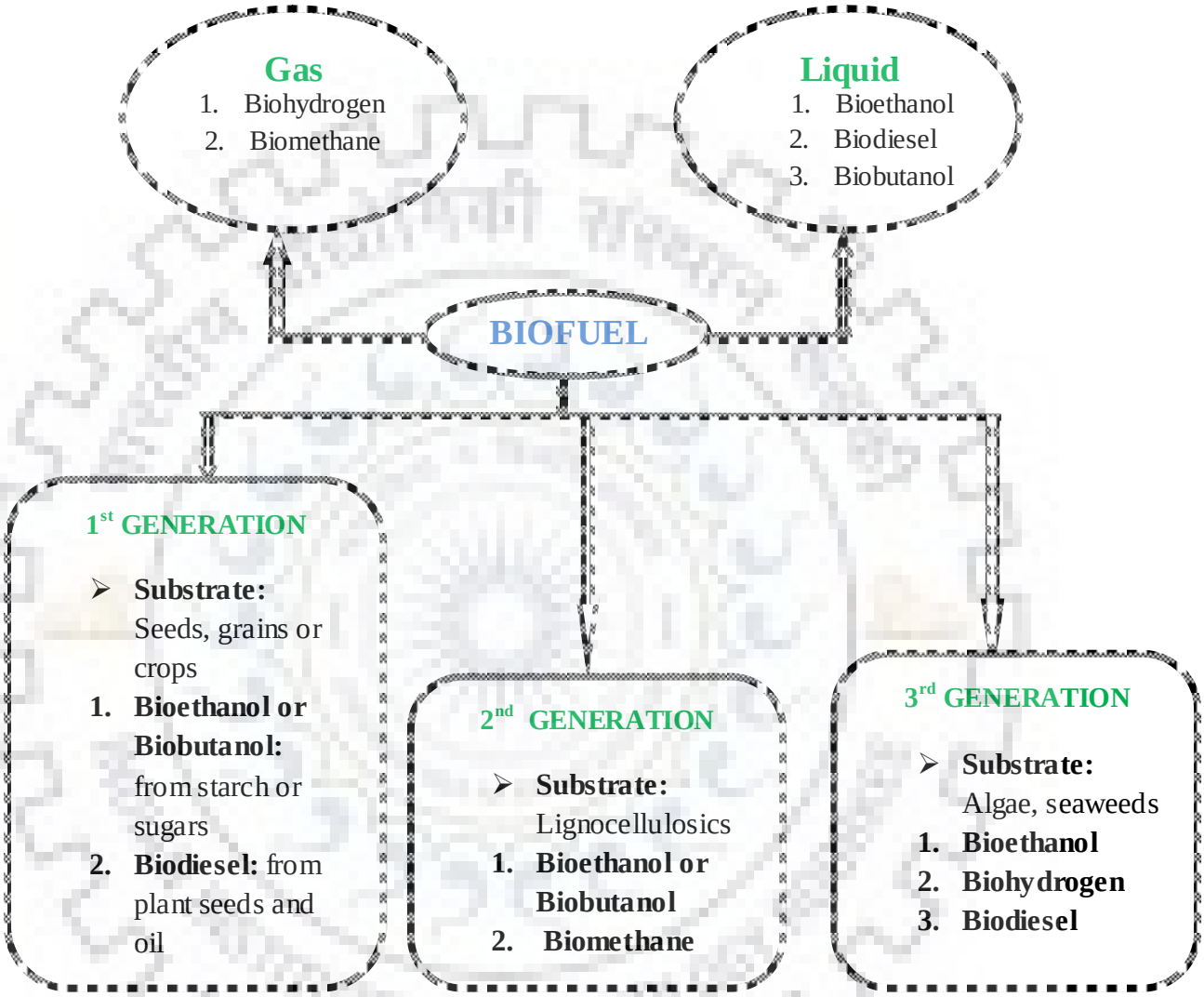


Fig. 1.7. Biofuel classification.

1.3 Bioethanol

Ethanol or Ethyl alcohol (C_2H_5OH) is a promising energy supply over gasoline (C_7H_{17}) with many advantageous properties. 1 L of ethanol can afford only 66% of the energy provided by gasoline; still, ethanol has a higher octane rating (106-110) compared to the gasoline (87-94) (Table 1.1) and it enhances the gasoline performance in case of blending [23]. Additionally, higher octane level of ethanol permits it to burn at a better compression ratio with reduced

burning time and resulting in lesser engine clock. Moreover, ethanol has the better heat of evaporation (1177 kJ/kg) than gasoline (348 kJ/kg) at 60^{o}C, a better laminar flame speed (39 cm/s) whereas it is 33 cm/s for gasoline at 100 kPa pressure and 325 K temperature [24-27]. The high heat of vaporisation (725.4 kJ/kg) confirms the volumetric efficiency of ethanol-gasoline blend is higher than the pure gasoline (heat of vaporisation of gasoline is 223.2 kJ/kg), which improves power output [28].

Bioethanol is an eco-friendly aerated fuel (34.7% oxygen) whereas; there is no oxygen present in the gasoline, resulting in 15% higher combustion efficiency [29] and reducing the particulate and nitrogen oxides emission. Ethanol contains a negligible quantity of sulfur and blending of ethanol-gasoline fuel helps to reduce sulfur content, further formation of sulfur oxide (carcinogenic and contributes to acid rain) [30]. Bioethanol is a safer substitute to methyl tertiary butyl ether or MTBE, which is often considered to use as an octane enhancer for fuel gasoline and added to reduce the amount of carbon monoxide (CO) and carbon dioxide (CO_2) during combustion [31]. MTBE reportedly creates its way into groundwater, contaminating drinking water and inflicts severe harmful results on health [32]. In 2000, the USA Energy Policy Act released an Advance Notice of proposed rulemaking (ANPR) under the Toxic Substance Control Act to reduce the utilisation of MTBE as a gasoline extender [33]. About 45% of total ethanol produced is being used as potable alcohol, 40% for industrial use, and only the remaining part is utilised for petrol blending [34].

Ethanol obtained from sugars and starch sources are termed as 1st generation (1G) bioethanol, whereas lignocellulosic biomass and algae produce 2nd and 3rd generation (2G and 3G) bioethanol respectively. 3rd generation bioethanol is currently confined to the lab-scale research and in the immature stage, whereas lignocellulosic biomasses have shown potential as feedstocks on the industrial scale. Conversion of 1G and 2G ethanol differs considerably during sugar obtainment stage. Starchy crops undergo only through the hydrolysis process for sugar conversion to ethanol, whereas lignocellulosic biomass has to be pretreated before hydrolysis for lignin removal and to alter cellulosic structures for increasing catalyst accessibility.

.

Table 1.1. Fuel Properties comparison [35].

Property	**Ethanol**	**Gasoline**
Chemical formula	C_2H_5OH	C_8H_{15}
Composition (C,H,O) (mass %)	52,13,35	86,14,0
Stoichiometric A/F ratio	9.0	14.6
Oxygen content, mass %	34.7	0
Liquid density (kg/m^3)	790	760
Viscosity (cp)	1.2	0.56
Specific gravity	0.788 (298 K)	0.739 (288.5 K)
Vapor density (relative to air)	1.59	3-4
Saturation pressure at 38°C (kPa)	13.8	31
Flash point (°C)	21.1	-45- to -38
Boiling point (°C)	78.4	25-215
Solubility in water (mL/100 mL of H_2O)	Fully miscible	<0.1
Vapor toxicity	Toxic (even in small dose)	Moderately irritant
Octane number	108	87 (Regular), 88-90(Midgrade) and 91-94 (Premium)
Auto-ignition temperature (K)	707	693
Heat of evaporation (kJ/kg)	725.4	223.2
Lower heating value (MJ/kg)	26.7	43.5
Peak flame temperature (K)	2193	2303
Flammability or explosion limits		
Lower (%)	3.3	1.3
Upper (%)	19	7.6

1.4 Biomass sources for ethanol production

Bioethanol is classified into different generations based on the biomass sources used for its production (Fig. 1.7).

1.4.1 First generation bioethanol

First generation ethanol is obtained from sugar and starch sources. The classification has been done on the basis of sugar obtaining step. Only extraction is required to obtain fermentable sugars in case of sugar crops, whereas starchy crops must be hydrolysed for it.

1.4.1.1 Sugar source: It includes

a. Energy crops: Sweet sorghum, sugar beet, and sugarcane

b. Fruits: Water melon, apple, grape, and dates

c. Sugar refinery wastes: Sugarcane and beet molasses [36].

The major advantages of these crops are high sugar yield as well as low conversion costs, while their seasonal availability limits the use [1]. Each crop has some potential as an ethanol feedstock. Sugarcane (C4 plant) has a high conversion rate of solar radiation into biomass [37], and able to grow in tropical and sub-tropical regions. Sugarcane stalk juice along with molasses (a by-product of sugar refineries) has been used for many years as the promising feedstocks for bioethanol production. In Brazil, cane juice is the primary feedstock (79%), whereas molasses is the major feedstock in India for bioethanol production [38,39]. Availability of readily fermentable sugars has made the cane sugar an ideal feedstock for ethanol. Harvesting time, variety, and maturity are responsible for variations in sugar concentration. Sweet sorghum (C4) plant has high carbon assimilation efficiency to use high water and can accumulate a high level of extractable sugars in its stalks [40]. It can be cultivated in almost all tropical climate and temperate areas and requires low rainfall, unlike sugarcane [41]. Sugar beet produces a root containing a high amount of sugars with 0.5-2 kg average root weight. Sugar concentration depends on the growth conditions and varieties [42]. Molasses is a sugar-rich, dark, and viscous by-product of sugar refinery industries. Traditionally, molasses were used as a binder and feed ingredient, but now used as an attractive raw material for ethanol production. Sugar content and their composition vary according to the sugar extraction processes and composition of initial materials used (sugarcane and beet) [37]. Large amounts of fruits are discarded during harvest and marketing (low quality or unacceptable physical appearance) that make them unfit for human consumption. Fruits contain a large amount of soluble sugars that can be easily fermented by the yeast. However, fresh fruits use for bioethanol production is illogical instead of human consumption. A number of waste fruits have been investigated for ethanol production [43,44].

1.4.1.2 Starch sources

Starchy crops are widely available across the world and used because of their high ethanol yield, long storage period and ease of conversion. Cereals contain 60-80%, tubers and roots 60-90%, legumes 25-50%, and immature green fruits are enriched with ~70% starch [45]. Majorly used starch sources for fuel ethanol production are corn, wheat, sorghum grains, sweet potatoes, cassava, and potatoes [46]. Conversion efficiency and ethanol yield vary with the individual crop (Table 1.2). USA is the largest producer of corn ethanol; in 2014 it has produced 14.3 billion gallons of ethanol and exported around 825 million gallons to 51 countries across the world [47]. Cassava and potato tuber crops are other two important starchy sources for ethanol production. Cassava ranks as the sixth major food crop in developing

countries and can grow in semi-arid conditions with very low rainfall [48]. Another promising character is that soil fertility is not necessary to grow cassava. Potato is a seasonal crop primarily abundant in the northern hemisphere; can be grown in temperate climatic conditions, and one of the important food crops around the world. Sweet potato is a perennial root crop grown in the tropical, sub-tropical regions, and an annual crop in the temperate regions [49]. It has several cultivars based on the starch content (white, orange, and purple). White varieties contain higher amounts of starch (25-40%), larger in size, and less sweet than other varieties which make it unsuitable as a food source crop [50].

Table 1.2. Comparison of 1G feedstocks for bioethanol production [51].

Biomass	**Ethanol yield (L/ton)**
Sugarcane	70-90
Sugarbeet	95-107
Corn	370-470
Sorgum grain	380
Sorgum stalk juice	40-86
Molasses	280
Sweet potatoes	125-170
Cassava	363-455
Potato	80-100
Wheat	376-435

1.4.2 Second generation bioethanol

Lignocellulosic biomasses are used for the second generation ethanol production. An additional step (pretreatment) is required for obtaining the fermentable sugars from the biomass.

1.4.2.1 Lignocellulosic biomass

As discussed previously, bioethanol production is based on the sustainable and widely distributed biomasses compared to fossil fuels. The major drawback of 1G ethanol is threat of food supply limitation affecting the human world population [52]. 2G ethanol production requires advanced technologies and facilities to aid the conversion process for extraction of fermentable sugars [23]. Table 1.3 shows the comparison of first and second generations of bioethanol in several aspects.

Table 1.3. Comparison of 1G and 2G ethanol [53].

	1G	**2G**
Feedstock source	Edible crops (sugarcane and cereals)	Non-edible crops (lignocellulosic, forest residues, municipal wastes)
Cultivating Land	Grows on arable land	Grows on arable and marginal land
Major stages during ethanol production	Sugar extraction, fermentation, distillation	Pretreatment, hydrolysis, fermentation, distillation
Bioethanol yield	Low	Medium
Environment impact	Low contribution to CO_2 mitigation	High contribution to CO_2 mitigation
Most important advantage	Simple conversion process	No "Food vs fuel" conflict
Disadvantage	"Food vs fuel" debate	Recalcitrant structures of the feedstock
Feedstock cost (Per unit of production)	High	Low
Capital cost (Per unit of production)	Low	High
Operating cost (Per unit of production)	Low	High
Total cost (Per unit of production)	Low	High

On a life-cycle basis also, environmental benefits of all the biomass-based ethanol are not equal (Fig. 1.8). As Fig. 1.8 illustrates, sugarcane and cellulosic ethanol emit almost 90% lower GHG upon combustion compared to gasoline, whereas corn-based ethanol offers rather limited benefits (only 18% lesser GHG than gasoline).

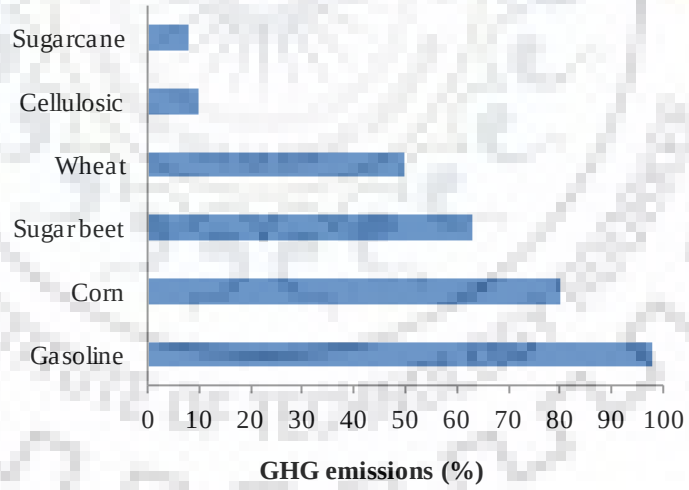


Fig. 1.8. GHG emissions by bioethanol produced from different feedstocks, compared to gasoline [54-55].

Lignocellulosic biomasses have been divided into several categories: a. Energy crops (like perennial grasses) b. Aquatic plants (water hyacinth) c. Forest residues (sawdust, bark thinning residues along with softwood and hardwood) d. Agricultural residues (bagasse, straws, and stovers) and e. Municipal solid wastes. Each category has proven its potential to be used as a

feedstock for bioethanol production as shown in Table 1.4. In general, lignocellulosic biomasses are the largest promising and abundant feedstocks available throughout the world, without necessitating any extra land and removing the food-fuel conflict [53].

Table 1.4. Advantages of lignocellulosic biomass for 2G ethanol production [53].

Lignocellulosic feedstock	**Advantages**	**References**
Agricultural residues	• Easy availability • Valorisation • Eco-friendly management of agro-wastes • Reduce deforestation • Short harvest rotation period	[56-59]
Perennial grasses	• High biomass yield • High cellulose content • Easy to grow and harvest • Potential to cover 50–70% of the total bioethanol feedstocks • Yield can be 0.9- 37 t dry matter/ha • Potential ethanol yield is 160–460 L/ton of biomass	[60-62]
Aquatic plants	• No competition with arable lands • Fast growing, High productivity • Abundant in some parts of the world which makes it a suitable feedstock	[63-64]
Forest residues	• Lower ash content • High density • Harvesting time can be flexible • Unexploited and underutilised • Potential ethanol yield is 220–275 L/ton (for softwood) and 280–285 L/ton (for hardwood)	[65-68]
Municipal solid waste	• Valorisation • Waste management • Potential ethanol yield is 154 L/ton of biomass	[69-71]

Pictorial representation of lignocellulosic biomass of the plant cell wall is shown in Fig. 1.9. Carbohydrate is the principal fermentable component, which includes soluble sugars (mono- and di-saccharides) obtained from cellulose and hemicelluloses hydrolysis. From the Table 1.5, it is clear that carbohydrate contents in these biomasses vary significantly and depend primarily on the biomass type, botanical sources, and crop hybrids. Cellulose and hemicellulose constitute roughly 2/3rd of the total dry biomass weight. Cellulose and hemicelluloses hydrolyse into hexose and pentose sugars, and further degrade into furfural and phenolic compounds (Fig. 1.10).

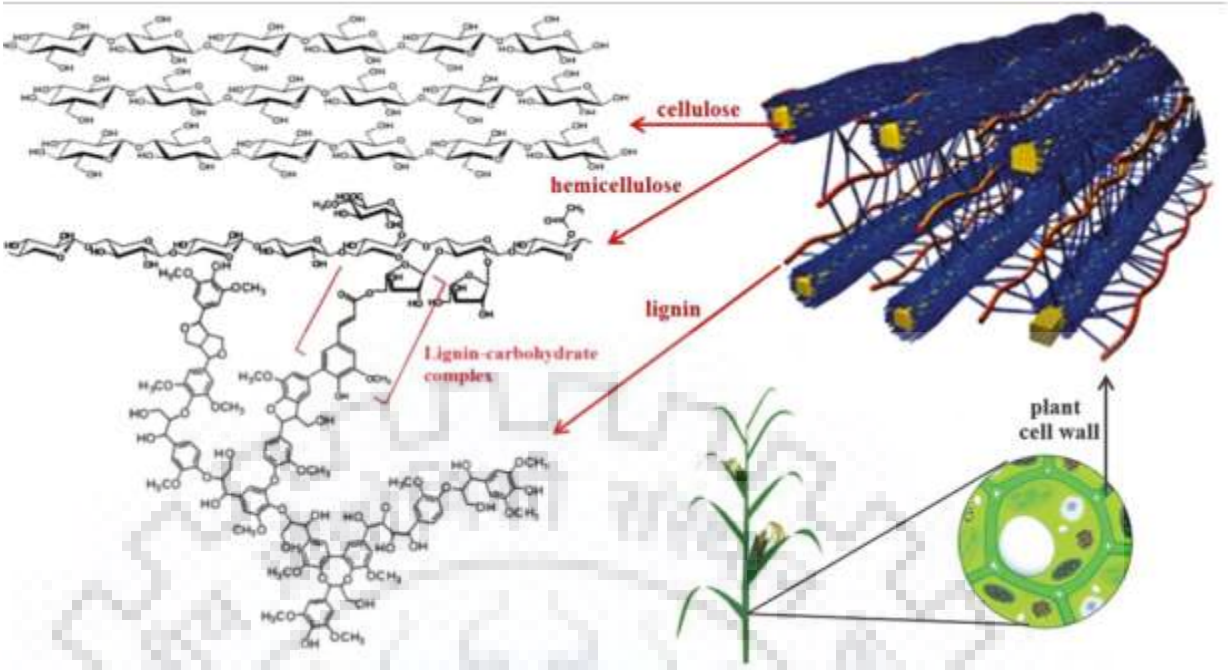


Fig. 1.9. Lignocellulosic biomass structure [72].

Table 1.5. Composition of various lignocellulosic feedstocks.

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Woods: Softwood	27-30	35-40	25-30	[73]
Hardwood	20-25	45-50	20-25	
Grasses: General	25-40	25-50	10-30	[74]
Switchgrass	5-20	30-50	10-40	[73]
Kans grass	35-45	20-30	15-30	[75-76]
Miscanthus	38-40	18-24	24-25	[77]
Municipal solid waste	33-39	9-16	10-14	[62, 78]
Sugarcane bagasse	42-48	19-25	20-42	[77,79]
Sweet sorghum bagasse	34-45	18-27	14-21	[77]
Straws: Rice straw	28-36	23-28	12-14	[77]
Wheat straw	33-38	26-32	17-19	[77]
Barley straw	31-45	27-38	14-19	[77]
Oat straw	31-37	27-38	16-19	[80]
Rye straw	33–35	27–30	16–19	[80]
Rice husk	25–35	18-21	26-31	[81]
Corn stover	38-40	24-26	7-19	[77,82]
Corn cob	42-45	35-39	14-15	[83,70]
Newspapers	40-55	25-40	18-30	[84]

1.4.3 Third generation bioethanol

Higher photosynthetic rate, faster growth, and biomass production compared to other energy crops make algae as one of the potential sources for biofuels production (biodiesel, bioethanol,

isobutene, and biohydrogen) [85-86], but high production cost is the major limitations for commercialisation. Algal biomass harvesting contributes approximately 20-30% of total production cost [87]. Although microalgae are receiving worldwide interest as a potential feedstock and species such as *Chlorella vulgaris*, *Dunaliella salina*, *Spirulina platensis*, and *Haematococcus pluvialis* are cultivated in small and medium scales with a total world production of about 10,000 tons/year, their process economics are yet to be fully addressed [88].

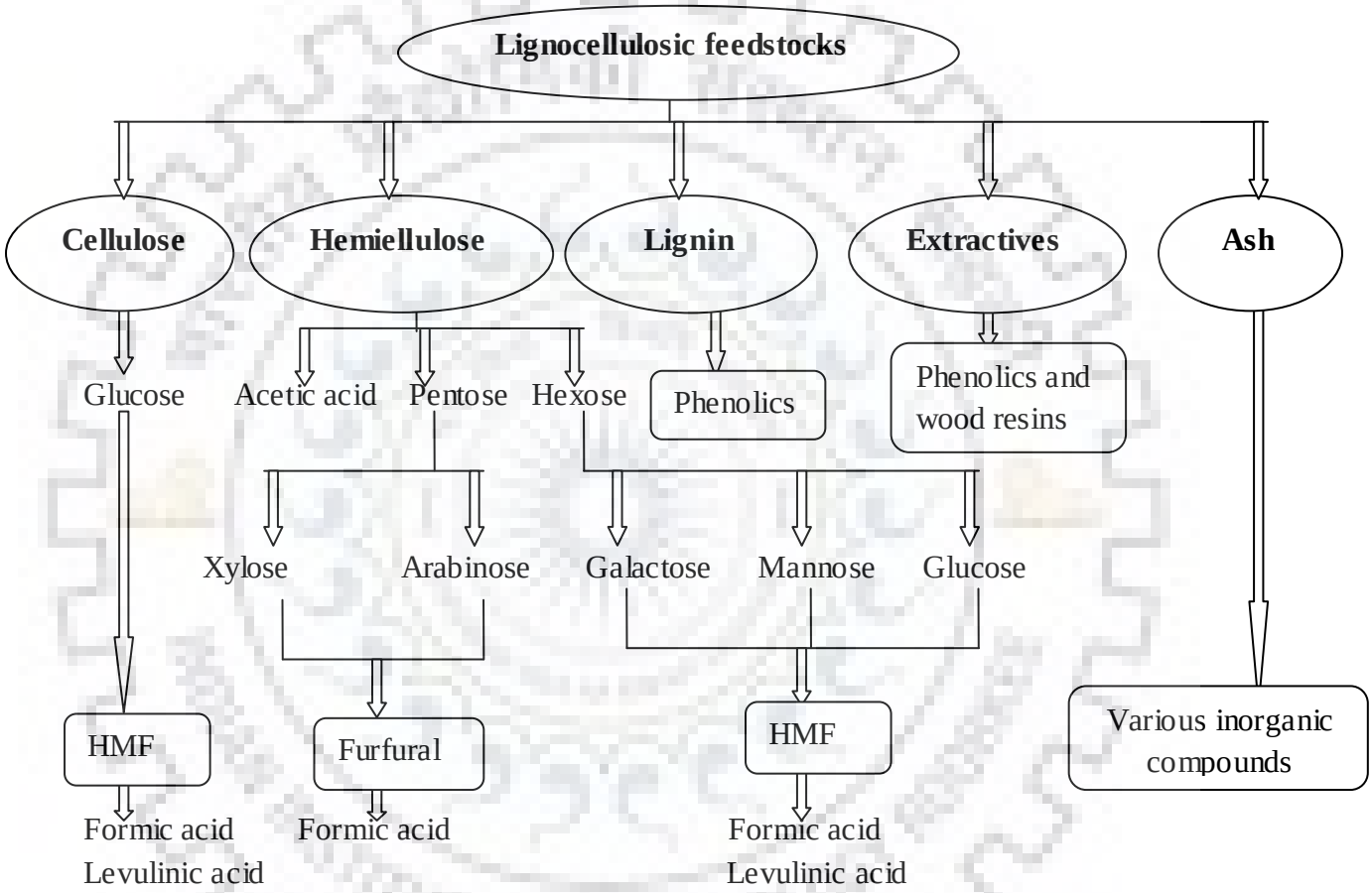


Fig. 1.10. Lignocellulosic biomass composition and their potential degradation products (HMF: 5-hydroxymethyl furfural) [53].

1.5 Carbohydrate content in lignocellulosic biomass

The three major organic compounds namely cellulose and hemicelluloses (carbohydrate or holocellulose), and lignin form the plant cell wall. The holocellulosic part on an average constitutes up to 2/3rd of lignocellulosic biomass and contributes to ethanol production. All the three components are bound to each other through hydrogen bonding and form a heterogeneous

matrix. Chemical bonds also exist between lignin and hemicellulose (arabinose and galactose residues mainly) molecules [89]. Although the ratio of cellulose, hemicellulose, and lignin in a plant cell wall varies from one feedstock to another, but usually it is 4:3:3. Traces of pectin, protein, extractives, and ash are also present in the lignocellulosic biomass. The bonds and linkages present in cellulose, hemicellulose, and lignin molecules are described below in Table 1.6. Cellulose is an unbranched linear chain polymer. The length is determined by the number of glucan units in the polymer, which is called as degree of polymerisation. Degree of polymerisation depends on the type of plants and varies from 100-10000 units. Hemicelluloses are heterogenous polysaccharides and their content varies from 11-37% of the lignocellulosic dry weight. Hemicelluloses can be relatively easily hydrolysed to their monomeric components which consist of xylose, mannose, glucose, galactose, arabinose, and small amounts of rhamnose, glucuronic acid, methyl glucuronic acid, and galacturonic acid [90].

Table 1.6. Bonds and Linkages present in the lignocellulosic biomass [91-92].

	Cellulose	**Hemicelluloses**	**Lignin**
Monomer	Glucose residues	Hexoses (D-galactose, D-glucose and D-mannose), Pentoses (L-arabinose and D-xylose)	Coniferyl alcohol (G-lignin), Sinapyl alcohol (S-lignin), p-coumaryl alcohol (H-lignin)
Intrapolymer linkages	Hydrogen	Ester, Ether	Ether, Carbon-Carbon, Carbon-Oxygen
Interpolymer linkages	• Hydrogen (Cellulose-Hemicellulose; Cellulose-Lignin) • Ether (Cellulose-Lignin)	• Hydrogen (Hemicellulose-cellulose; Hemicellulose-Lignin) • Ester, Ether (Hemicellulose-Lignin)	• Hydrogen (Cellulose-Lignin; Hemicellulose-Lignin) • Ester (Hemicellulose-Lignin), • Ether (Cellulose-Lignin; Hemicellulose-Lignin)
Bond between different monomer units	β -1,4-Glycosidic bonds	• β-1,4-glycosidic bonds (straight chain), • β-1,2, β -1,3, and β-1.6 glycosidic bonds (branched chain)	Ether bond (mainly β -O-4 bond) and C-C bond
Bond between cellulose, hemicellulose, and lignin	No chemical bond	Chemical bond with lignin	Chemical bond with hemicellulose
Nature	Amorphous, crystalline and para-crystalline	Amorphous and small three dimensional (3-D) in homogenous crystalline region	Amorphous
Polymerisation	100-10,000	>200	~4000

Lignin is the most robust, recalcitrant, and complex molecule composed of phenylpropane units linked in a 3-D structure [93]. Generally, softwood contains more lignin than hardwood. Chemical bonds are present between lignin and cellulose, and even hemicellulose. Lignins are extremely resistant to chemical and enzymatic degradation [90,94]. Extractives are wood compounds which are soluble in neutral organic solvents (acetone, alcohol etc.) or water. It represents a minor fraction (1-5%) of lignocellulosic biomass. They contain a large amount of lipophilic and hydrophilic group. Extractives are classified into four groups: (a) Terpenoids and steroids (b) fats and waxes (c) phenolic constituents (d) Inorganic components [90,94]. The composition and structure of lignocellulosic biomass determine its digestibility to subsequent chemical or biological treatments [95-96].

1.6 Potential feedstocks for 2G ethanol production in India

Biomass selection is a vital criterion for the sustainable biomass production; depends mainly upon its CO_2 fixation efficiency, which is determined by the type of photosynthesis occurring in a plant species. The predominant type is C3 photosynthesis found in terrestrial plants, here CO_2 is fixed by Ribulose 1,5-bisphospahte carboxylase/oxygenase or Rubisco. C4 photosynthesis is mediated by phosphoenolpyruvate (PEP) carboxylase which is the evolution of biochemical and morphological modification of C3 type. C4 plants can survive in saline and dry areas along with erratic rainfall, unlike C3 [97]. Increased CO_2 fixation under the extreme conditions lead to enhanced photosynthetic efficiency and resulting in faster carbon capture and higher growth rates. C4 grasses are high biomass producing biomasses with minimum inputs, distributed around the world in hot, humid, and arid climatic conditions. Food crops such as sugarcane (*Saccharum* species) and maize (*Zea mays*) belong to C4 grasses and are provides huge amount of agricultural residues. Among the dedicated biomass crops, switchgrass (*Panicum virgatum*) and miscanthus (*Miscanthus* species) belong to C4 grasses [98]. In India, *Saacharum spontaneum*, a wild sugarcane variety is one of the dedicated energy crops that belong to C4 grasses.

From 26 different varieties of crops, the gross lignocellulosic biomass residue generation in India has been estimated as 686 MT annually. Approximately 545 MT is contributed by cereals, pulses, sugarcane, and oilseeds; whereas horticultural crops contribute only 61 MT to it and 80 MT by other crops (like jute and cotton). Cereals contribute the highest amount of residues (54%) followed by sugarcane (16%). In India, forests cover around 22% land area representing second largest land use of the country. Forest residues (sawdust, chips, and bark)

inherit about 65% of the biomass potential [99-100]. These resources of lignocellulosic feedstocks contain a significant energy value (~150 EJ/year) for biofuel generation. Some of the potential lignocellulosic feedstocks in India for bioethanol production are discussed below.

Sugarcane: It is a perennial grass variety. Its height can reach over 5 m and has potential to store sucrose in its stem [101]. Sugarcane is the largest crop produced worldwide [102], and in Brazil, it is successfully used for ethanol production at commercial level [103]. However, it raises food-fuel arguments especially in a country with huge population like India.

Switch grass: It has high tolerance to poor soils, wide pH range, extensive carbon sequestration, deep and extensive tap root system, resistance to disease and pest, and most importantly good carbohydrate content makes it suitable for ethanol production [104].

Kans grass: Kans or Sarkanda (*Saccharum spontaneum*) is a variety of switchgrass. It is a perennial C4 energy crop that requires less water for growth and grows well in the marginal land [105]. Its growing capacity is very fast because of C4 photosynthetic machinery which fixes CO_2 more than a normal plant and subsequently sequesters a large amount of carbon [106]. The regeneration ability of the grass is also very high and once planted, it can supply biomass for years. Quick colonisation is the main characteristic of this plant which makes it suitable for plantation in waste and marginal lands. It has potential to provide 10 tonnes/ha biomass approximately while other crops give only about 5-6 tonnes/ha/year [107]. In India, the total land area available for energy crops cultivation is about 51.09 Metric hectares producing 510 MT of kans grass per year [108]. Therefore, kans grass can be considered as a promising feedstock for biofuel production.

Wheat straw: India covers 13.2% of the total wheat production of the global and 2nd largest wheat producer after China [109]. Wheat straw is basically a by-product of the wheat crop; therefore amount of straw production is in line with the main crop. 30.5 million hectare area is used for the wheat production in India. In 2013, wheat productivity has been increased up to 3145 kg/hectare [110]. There are two growing seasons of the wheat crop in India; May-September and November-April. To prepare the fields for another crop sowing, farmers often set fire to the standing straws, to clear the field of crop residue, which is one of the major reasons for pollution [111]. Although wheat straw is an important cattle fodder; besides fodder and other uses, Indian states have set some policies for straws to promote research and development in cellulosic ethanol production field. In 2013, Punjab has set up policies for straw management and utilisation to consume 1.5 million tonnes of straw per year by 2027 for the

production of 0.37 million kilo litre ethanol [112], therefore, wheat straw can be considered as a suitable biomass for ethanol production in India.

Sugarcane bagasse: It is the most abundant agricultural residue around the world (540 million tons of biomass per year), and an attractive feedstock for the bioethanol production [113]. Bagasse is the fibrous matter residue left after the juice extraction from sugar cane. For every 10 tonnes of sugarcane crushed, 3 tonnes of bagasse (1400 kg dry bagasse) is produced [114]. The quantity of sugarcane bagasse production is in line with the quantity of sugarcane produced in each country as it is a by-product of the cane sugar industry [115]. In India, sugarcane is grown on around 2.8% of total land used for agriculture. In 2015-16, India has produced around 352 million tonnes of sugar. With huge production of sugarcane, more than 500 sugar mills and being one of the largest sugar exporters, India is considered as a sugar giant and sugarcane bagasse a reliable feedstock for the bioethanol production [116].

1.7 Bioethanol production from lignocellulosic biomass

Second generation bioethanol production process mainly consists of four steps: pretreatment, saccharification or hydrolysis, detoxification, and fermentation.

1.7.1 Pretreatment

Pretreatment is directed to reduce the cellulose crystallinity of lignocellulosic biomass, increase accessible surface area for hydrolysis, and lignin barrier breakage. Cellulose becomes more accessible to hydrolytic agents after pretreatment; facilitating rapid conversion of polymeric carbohydrate into its monomeric fermentable sugars upon pretreatment. Many pretreatment methods exist for biomass solubilisation, fractionation, hydrolysis, and separation of lignocellulosic components (cellulose, hemicelluloses, and lignin). Pretreatment methods have been categorised into physical/mechanical, chemical, physico-chemical, and biological methods. Detailed comparison of various pretreatment routes is presented in Table 1.7.

1.7.1.1 Physical methods

It includes mechanical comminution, extrusion, and ultrasonic treatment of the biomass. Particle size reduction by mechanical comminution (milling, chipping or grinding) makes material handling easy and enhances specific surface area. In the extrusion process, biomass is first treated at very high temperature (>300^{o}C), shearing and mixing is carried out afterwards to modify the cellulose structure (physical/chemical). Biomass is treated with energy by

irradiation during ultrasonic treatment. It is assumed that hydrogen bond of cellulosic crystalline structure breaks upon supply of an adequate amount of energy [117].

1.7.1.2 Chemical methods

It generally includes alkaline, acid, and ozonolysis pretreatment methods. An overview on various types of chemical pretreatment is discussed in this section.

1.7.1.2.1 Acid based pretreatment

Acid based pretreatment is one of the most promising and widely used methods with respect to industrial implementation [118]. Acid causes hemicellulose and lignin solubilisation and improves cellulose accessibility for hydrolysis. Formation of toxic compounds like phenolics, furfural, 5-HMF is the major disadvantage of this process. Acid hydrolysis has been further classified into weak and strong acid hydrolysis.

A. Weak or dilute acid hydrolysis: There are two ways to approach this process

a. High temperature with continuous flow: T>160 ℃ and substrate concentration is 5-10% by weight.
b. Low temperature and batch process: T≤160 °C and substrate concentration is 10-40% by weight.

In this process, dilute sulphuric acid is sprayed onto the lignocellulosic biomass, held at 160-220°C for few minutes. It results in the hydrolysis of hemicellulose, releasing soluble pentose sugars from cell wall matrix into hydrolysate. Hemicellulose removal enhances biomass digestibility and porosity. Complete removal of hemicelluloses gives maximum digestibility [119]. Some organic acids (e.g. maleic acid and fumaric acid) can also be used in the place of inorganic acids [120]. This method has shown good performance in hemicellulosic sugars recovery and widely accepted but the released sugars might be further converted to furan compounds (furfural and 5-HMF) which are toxics to microbial fermentation. Corrosiveness of acids might be an issue using this method. As lignin is not removed from the biomass during this process, it is most suitable for lignocellulosic biomass having low lignin content.

B. Strong acid hydrolysis: Sulphuric acid (H_2SO_4) and Hydrochloric acid (HCl) have been widely used for the treatment of lignocellulosic biomass as they are very powerful reagents for cellulose hydrolysis [121]. There are various advantages of this process: mild temperature requirement, feedstock flexibility, and high sugar yield. Drawbacks are corrosiveness of acids

and recycling of acids is must for the economy. Several industries around the world are in process of commercialising strong acid hydrolysis for second generation bioethanol production.

Dilute acid treatment is more feasible for industrial scale than concentrated acid. Various reactors such as percolation, plug flow, flow through, countercurrent, and shrinking bed are developed for this process. Acid pretreatment methods can either be high temperature and short reaction time or low temperature and long reaction time (30-90 minutes). Various studies have reported high sugar recovery using dilute H_2SO_4, although many other acids such as HCl, HNO_3, H_3PO_4, oxalic acid, formic acid, acetic acid, and maleic acid have also been reported.

1.7.1.2.2 Alkali based pretreatment

Alkali removes lignin part of the lignocellulosic biomass thereby increasing accessibility of the holocellulose for further hydrolysis. Alkali treatment acts by the removal of acetyl group and various uronic acids from hemicelluloses which reduces the holocellulosic accessibility to hydrolysis [95]. Mechanism of alkaline hydrolysis is based on the intermolecular ester bonds saponification which crosslinks xylans and lignin [121]. Reagents used for alkali pretreatments are:

A. Calcium hydroxide ($Ca(OH)_2$ or sodium hydroxide (NaOH): $Ca(OH)_2$ or NaOH are used most widely, salts are formed which can get incorporated in the substrate. Hence, recycling or removal of salts is necessary [122]. Process conditions are relatively mild during the process and reaction time can be very long. Mild conditions prevent lignin condensation and sugar degradation. Addition of oxygen or air improves delignification process significantly [95].

B. Aqueous ammonia: Aqueous ammonia treatment reduces lignin content at elevated temperatures and also removes some hemicelluloses while decrystallising the cellulose present in lignocellulosic biomass. Ammonia pretreatment techniques include:

- Ammonia fibre expansion method (AFEX)
- Ammonia recycle percolation (ARP)
- Soaking in aqueous ammonia (SAA)

In AFEX, liquid ammonia is used for the biomass treatment at temperature (90-100^{o}C) for 30-60 min followed by rapid pressure release [123]. It results in physical disruption of lignocellulosic fibres and cellulose decrystallisation to some extent. The rapid expansion of liquid ammonia causes swelling of biomass, and it either modifies or reduces the effective cellulose and lignin crystallinity [124]. Washing is not compulsory as toxics are not produced

using it. This method is more effective for agricultural residues and herbaceous biomass than woody. In ARP, flow-through column reactor is used for the biomass treatment. Aqueous ammonia flows through the reactor column at high temperature which is packed with lignocellulosic biomass. To prevent flash evaporation, reactor system should be pressurised slightly [125-126]. Solid fraction (rich in cellulose and hemicelluloses) is separated, and the liquid fraction is flown through steam heated evaporator for recovery of ammonia. Ammonia is then recycled back to reactor inlet, and separated fraction is passed into a crystalliser. Washing is carried out to extract the retained sugars in a solid matrix. SAA is carried out at low temperature and removes the lignin efficiently by minimal interaction with hemicelluloses resulting in an increased surface area and pore size. The retained celluloses and hemicelluloses can be further hydrolysed by enzymes or chemicals. Ammonia cost and its recovery drives the main cost of this process [127-128].

NaOH is used most widely used reagent for the alkali pretreatment. $Ca(OH)_2$ has additional advantages of low cost and less safety requirements compared to other alkaline reagents. Also, it can be easily recovered from the hydrolysate by using CO_2 [96]. High downstream process cost makes alkaline pretreatment costly. Further washing of calcium and sodium salts require large amount of water and is difficult to remove.

1.7.1.2.3 Organosolv

In this method, organic or aqueous organic solvent mixtures (ethanol, methanol, acetone, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol) are used in combination with inorganic acid catalyst for delignification [129]. At high temperature, oxalic acid, salicylic acid, and acetylsalicylic acid can also be used with or without the addition of organic acids [130]. For value-added products and high quality lignin extraction, organosolv treatment has been used extensively. Efficient lignin removal leads to ~90% sugar recovery after hydrolysis of the treated biomass. Main drawback of the organosolv process is solvent and catalysts cost (Table 1.7). Solvent recovery can reduce the operational cost considerably [121]. As organic solvents are inflammable, safety measures are required, which is an additional cost making the process non-economical at large scale. Furthermore, removal of organic solvents is needed as they have shown inhibitory effect on enzymatic hydrolysis [96].

1.7.1.2.4 Ionic liquids or Room temperature ionic liquids (RTIL)

Biomass and ionic liquids are used in a ratio of 1:10 (w/w) at the temperature ranging from 100-150^{o}C. Ethanol, methanol, and water can be used as antisolvents for soluble biomass regeneration and then subject it to saccharification. Ionic liquids with small inorganic anions and large organic cations behave like a salt. At room temperature or below, RTILs exist as liquid while at high temperature they have the ability to form hydrogen bonds with cellulose as anions (formate, acetate, chloride or alkyl phosphonate). RTILs can digest 90% of cellulose [131]. RTILs remained in the biomass can interfere with hydrolytic enzymes activity and downstream fermentation processes, affecting sugar yield and ethanol production [132-133]. RTILs can be regenerated by flash distillation from antisolvents [82]. Recycling methods should be energy efficient for industrial scale implementation; also toxicity to microorganisms and enzymes should be considered using this method [134-135]. Additionally, some technique needs to be developed to recover lignin and hemicellulose after extracting cellulose from solutions. These are some of the limitations using RTILs for pretreatment of lignocellulosics. Although no industry employs it as of now, this method has great potential in biorefinery industries despite these flaws.

1.7.1.3 Physico-chemical methods

Combinations of physical and chemical processes for the pretreatment are referred as physico-chemical pretreatment method. Commonly used physico-chemical pretreatment methods are discussed in this section.

1.7.1.3.1 Steam explosion or autohydrolysis

Chipped or milled biomass is treated from 30 s to 20 min to high pressure saturated steam. Pressure is reduced suddenly after steam treatment causing explosive decompression of biomass resulting in lignin matrix disruption and hemicellulose degradation. Here autohydrolysis of acetyl group present in the hemicellulose occurs. Factors influencing the process include particle size, moisture content of the biomass, reaction time, and temperature. Advantages are high sugar recovery, low capital investment, energy efficiency, and positive environmental impact along with possibilities of using larger particle size make this process feasible at industrial scale [136].

1.7.1.3.2 Liquid hot water/hydrothermolysis/hydrothermal treatment/aqueous fractionation/ solvolysis or aquasolv

Rapid decompression occurs in this process without the requirement of any additional catalyst. High pressure and high temperature is used for the maintenance of liquid water. Temperature (170-230^{o}C) and pressure (>5 MPa) is used for 15 minutes during the process usually [82]. The process results in removal of hemicelluloses from the biomass and increases cellulose accessibility. Main advantages are high pentose sugar recovery and low toxics generation, but high water and energy requirement make it industrially non-feasible.

1.7.1.3.3 Ammonia based pretreatment/ Ammonia fibre expansion (AFEX)

This process is already explained briefly in chemical methods section.

1.7.1.3.4 CO_2 explosion

CO_2 is used as a supercritical fluid (exhibits gaseous mass temperature properties and liquid has solvating power) at high pressure in this method. This process is effective in lignin removal from both hardwood and softwood efficiently [137]. To improve the delignification, co-solvents (e.g. ethanol) can be added. Advantages of supercritical CO_2 include low cost, non-inflammability, non-toxicity, easy recovery, and eco- friendly behaviour [138]. Aqueous CO_2 from carbonic acid can increase hydrolysis rate. Overall the process results in cellulose and hemicellulose disruption and increases accessible surface area for enzymatic hydrolysis.

1.7.1.3.5. Oxidative delignification: It has been further classified into

A. Delignification by oxidising agents (hydrogen peroxide or peracetic acid) B. Ozonolysis
C. Wet oxidation

A. Oxidising agents: Most commonly used oxidising agent is hydrogen peroxide. At 25-30^{o}C, 1-2% H_2O_2 is effective in most of the hemicellulose recovery of the biomass and 50% of lignin dissolution [139]. Reactions like electrolytic substitution, side chain displacement, oxidative breakdown of aromatic nuclei, and breakage of alkyl or aryl ether linkage occur during this process.

B. Ozonolysis: Lignocellulosic biomass is pretreated with ozone, an effective oxidising agent. Ozone does not alter holocellulosic part, degrades lignin only by attacking its aromatic ring. Baggase, poplar sawdust, peanut, pine, rye straw, cotton straw, and wheat straw have been

treated using ozone [121,140]. Ozonolysis is performed at room temperature (around 30^{o}C) and pressure usually. Ozone gas is passed through the vessel containing substrate (fixed or packed bed or stirred semi batch reactors) during the process [140-141], no toxics are generated.

C. Wet oxidation: This process is suitable for high lignin-containing biomasses. In this method, biomass is treated with the water and oxygen for 30 minutes at T>120^{o}C [142]. Oxygen pressure, reaction time, and temperature affect overall process [143]. The process catalyses acid formations due to various oxidative and hydrolytic reactions. All the three components (cellulose, hemicelluloses, and lignin) get affected during the process. Hemicelluloses degrade into soluble pentose sugars; cellulose degrades partially whereas lignin undergoes cleavage. Alkaline reagents like Na_2CO_3 enhance the process by hemicellulose solubilisation and reduction in toxics formation [144].

1.7.1.3.6 Microwave

The microwave is a widely used method due to its easy operation and high heating efficiency. Time typically varies from 5-20 min. The process modifies cellulosic ultra-structure and causes hemicellulose and lignin degradation, thereby improving the enzymatic susceptibility of lignocellulosic biomass [145]. Homogeneous heating, energy savings, short reaction time, and minimum toxics generation are the main advantages of this process. To modify the native structure of cellulose with hemicellulose and lignin degradation, it is one of the best methods for pretreatment [146].

Table 1.7. Comparison of different pretreatment routes [51,147-148].

Pretreatment methods	Mode of action (accessible surface area included)	Potential sugar yield	Inhibitor formation	Residue formation	Need for recycling of chemicals	Investment cost	Operational cost	Applicable to various biomass	Advantages	Disadvantages
Mechanical/ milling	-	Low	No	No	No	Moderate	High	Medium range	• Reduction in crystallinity of cellulose and degree of polymerisation • Particle size reduction improve pore size and specific surface area	• High energy • High power requirement
Liquid hot water	• Hemicellulose removal	High	Yes	No	No	Moderate	-	-	• Biomass size reduction not needed • Usually no chemicals required • Corrosion resistant material not required.	• High energy requirement • High power requirement • Toxics generation
Weak acid Or Dilute acid	• Hemicellulose removal • Lignin structure alteration (minor)	High	Yes	Yes	Yes	Moderate to low	Moderate	Medium range	• High sugar recovery • Less inhibitors	• Low reducing sugar concentration • Degradation products generation
Strong acid Or Concentrated acid	• Cellulose and hemicellulose hydrolysis	High	Yes	Yes	Yes	High	Moderate to low	Wide range	• Ambient temperature requirement • High glucose yield	• Acid recovery needed • Requirement of corrosion resistant material • Toxic and hazardous nature of acids
Alkaline	• Lignin removal (major) • Hemicellulose removal	High	No	Yes	Yes	Low		Medium to fewer range	• Reduction in crystallinity of cellulose and degree of polymerisation • Lignin structure disruption	• High cost • Not suitable at industrial scale
Organosolv	• Lignin removal (major) • Hemicellulose removal • depending on solvent used	High	No	Moderate	Yes	High	High	Medium range	• Lignin disruption • Hemicellulose hydrolysis	• Solvent reuse and recovery needed and high cost

Wet oxidation	• Lignin removal (major) • Dissolve hemicelluloses • Cellulose decrystallisation	Moderate to low	No	Moderate	No	Moderate	-	-	• High hemicelluloses and lignin solubilisation • Less inhibitor formation	• High cost of oxygen • High cost of alkaline catalysts
Steam explosion	• Hemicellulose removal (major) • lignin structure alteration	Moderate	Yes	Moderate	No	Moderate	Moderate	Medium to low range	• Solubilisation of hemicellulose and transformation of lignin • Low cost • High glucose and hemicelluloses yield in two-step process	• Toxics generation • Hemicellulose degradation is partial
Ammonia fiber expansion	• Removal of lignin (major) • Hemicellulose removal • Cellulose decrystallisation	High	No	Moderate	-	-	-	Low range	• Less formation of inhibitors • Particle size reduction not needed	• Not suitable for biomass having high lignin content • High cost of chemicals
CO_2 explosion	• Removal of hemicellulose • Decrystallisation of cellulose	Moderate	Moderate	No	No	High	-	-	• Accessible surface area increase • Low cost availability • No formation of inhibitors • Non-inflammable • After extraction, easy recovery • Eco-friendly	• High pressure requirement
Biological (Brown, white and soft rot fungi)	• Hemicellulose removal • Lignin structure alteration (minor)	Low	Yes	-	Yes	-	-	-	• Reduction in the degree of polymerisation of cellulose and hemicelluloses • No chemical required • Mild environmental conditions • Low capital costs • Low energy demand • Low inhibitor formation	• Extremely slow rate of degradation and delignification • Loss of carbohydrates (consumed by microrganisms) • Long residence times (10–14 days)

1.7.1.4 Biological methods

Pretreatment step during the bioethanol production is considered as rate limiting step and controls the final product. Physical, chemical, and physico-chemical pretreatment methods require an extensive amount of energy, and they are not environmental friendly [149]. Biological pretreatment method came into existence to overcome these problems. Xylanase enzyme is used for the hemicellulose pretreatment whereas cellulase is used as a cellulytic enzyme in general. Microorganisms which are used for the biological pretreatment are ubiquitous filamentous fungi mostly, isolated from soil or living plants directly. White rot fungi have been found most effective; common examples include *Ceriporiopsis subvermispora, Ceriporia lacerate, Cyathus stercolerus, Pycnoporus cinnarbarinus, Pleurotus ostreaus, and Phanerochaete chrysosporium*. These organisms produce manganese dependent lignin peroxidise, which degrades lignin present in the biomass. Brown rot fungi attack cellulosic part only while white rot fungi and soft rot fungi attack cellulose and lignin both. Laccases treatment of the biomass has been found effective in prevention of inhibitory compounds formation [150]. Advantages of biological pretreatment methods are no chemical use, low energy requirement, low capital cost and eco-friendly nature (Table 1.7), a major limitation is very slow hydrolysis rate [121]. Therefore, more studies need to be carried out on isolates like basidiomycetes fungi for quick and efficient delignification.

1.7.2 Saccharification/hydrolysis

The pretreated biomass can be saccharified or hydrolysed through chemicals or enzymes for the generation of reducing sugar molecules.

1.7.2.1 Chemical methods

Soluble monomeric sugars (hexoses and pentoses) from the lignocelluosic biomasses can be released through chemical hydrolysis. Sulphuric acid (H_2SO_4) is the most used reagent for acid hydrolysis, although other inorganic acids like hydrochloric acid (HCl), nitric acid (HNO_3), and phosphoric acid (H_3PO_4), as well as some weak organic acids (trifluoracetic acids or TFA) have also been reported to use [150].

Usually, dilute acid is applied for hemicellulose hydrolysis and cellulose pretreatment. However, both the components can be hydrolysed with dilute acid in a two-stage process. The first stage is carried out at a low temperature usually to maximise hemicellulose conversion and the second stage is conducted at 230-240°C to convert cellulose into glucose. Acid

concentration normally ranged from 0.5-1.5% during the process [46,150]. Concentrated acid hydrolysis can be used for hydrolysing both the components of holocellulose (hemicellulose and cellulose). Acid concentration (41-100%), moderate process temperatures, and longer reaction time are the main features of concentrated acid hydrolysis [151].

Acid hydrolysis is faster, low cost, and results in high reducing sugar yield, however, corrosivity of acids and inhibitors formation (like furfural and 5-HMF) might interfere in the fermentation process.

1.7.2.2 Enzymatic methods

Nowadays, attention is increasing towards microbial enzymatic processes for saccharification of the lignocellulosic biomass due to above mentioned limitations of chemical hydrolysis. Enzymatic hydrolysis can be done under the milder reaction conditions [152]. Cellulase is widely used and most diverse enzyme that hydrolyses β-1,4 linkages of the cellulose molecules present in the lignocellulosic biomass. It can be produced both by bacteria and fungi. At least 11 families of cellulases are known on the basis of their amino acid structure and sequence. Their mechanism of action differs from each other in terms of ability to degrade an insoluble substrate (by diffusion) and moving the cellulosic segment from the insoluble part to its active site. Cellulose is a complex enzyme mixture of exoglucanase, endoglucanase, and β-glucosidase. Endoglucanase attacks low crystallinity regions of the cellulose fiber and creates free chain-ends by randomly attacking the o-glycosidic bonds resulting in release of glucan chains of different lengths. Cellobiohydrolase or exoglucanase acts on the ends of the cellulose chain releasing β-cellobiose as the end product. β-glucosidase is specific towards β-cellobiose disaccharides and hydrolyses it to produce glucose [153]. Degradation of hemicellulose (xylan) requires a complex multiple enzyme systems, referred as xylanase system (hemicellulase). It contains endoxylanase and exoxylanase (act on the main chains of xylans and hydrolyse them into smaller ones), β-xylosidase (attacks xylo-oligosaccharides and produce xylose), α-arabinofuranosidase and α-glucoronisidase (act on the xylan backbone and removes arabinose and 4-o-methyl glucuronic acid respectively), acetyl xylan esterase (attacks the acetyl substitutions on the xylose moieties) and ferulic acid esterase (hydrolyses the ester bonds located between arabinose and ferulic acid substitutions) [154-155]. Several bacterial (*Bacillus, Cellumonas, Thermospora, Microbispora, streptomyces*, and *Erwinia*) and fungal species (*Penicillium, Trichoderma, Humicola, Fusarium*, and *Aspergillus*) are able to produce both

cellulases and hemicellulases [156]. Enzyme reuse or recycling is must to make the enzymatic hydrolysis process economical.

1.7.3 Detoxification of inhibitors

The severe conditions applied during chemical pretreatment and hydrolysis often lead to breakdown of the major lignocellulosic components into various by-products (furfural, 5-HMF, acetic acid, formic acid, levulinic acid, and other phenolics) as outlined in Fig. 1.10. These compounds when accumulate sufficiently high, could be inhibitory for both fermenting organisms and hydrolytic enzymes [157]. The amount and type of inhibitors generated during the pretreatment and hydrolysis depend on the nature of the biomass, reaction conditions (pressure, temperature, time, pH), and addition of catalysts [158]. There are several ways to counteract the toxics generation problems either by removing or neutralising them. These methods are broadly classified into:

a. Physical or physicochemical methods: Evaporation, rota-evaporation, and membrane based separations
b. Chemical methods: Neutralisation, overliming with $Ca(OH)_2$, activated charcoal, and ion exchangers (cationic and anionic)
c. Biological methods: Laccase or peroxidase mediated treatments mostly [158].

An efficient detoxification method should be cheap, selectively remove inhibitors, and easy to integrate [159].

1.7.4 Fermentation

Fermentation is the final step responsible for yield and productivity of the whole ethanol production process. Most commonly used organism is *Sacchromyces cerevisae* due to its wider pH range, cost effectiveness, and less susceptibility [160]. Baker's yeast has been employed over the decades as a starter culture for industrial ethanol production because of its easy availability and low cost. The yeast survival during fermentation is affected by the stressful conditions such as high ethanol concentration, osmotic stress, increase in bacterial contamination, and temperature [161]. Pentose sugar fermentation is the major limitation for industrial level ethanol production. *S. cerevisae* can ferment only hexoses as it lacks pentose-fermenting genes [162]. Yeasts from other genera like *Candida, Pichia, Pachysolen,* and *Schizosaccharomyces* are having the ability to ferment only pentose sugars to ethanol [163]. Successful utilisation of both pentose and hexose sugar is the major priority for an enhanced

ethanol fermentation efficiency. Co-culture system for fermentation provides the opportunity to achieve it and maximises substrate utilisation rate, ethanol yield, and productivity, thereby reducing the process costs.

Overall fermentative reactions of glucose and xylose sugars can be described by the following equations:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy} \quad (1)$$

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 + \text{energy} \quad (2)$$

Based on the stoichiometry, the theoretical ethanol yield is 0.51 g ethanol/g glucose or xylose. Note that alcohol is a byproduct of this fermentation.

1.7.4.1 Co-fermentation for ethanol production

Two different approaches have been adopted by the researchers to utilise both pentose and hexose sugars efficiently for ethanol fermentation. First is the developement of genetically modified strains into which pentose fermentation pathway has been incorporated. Some strains have been genetically modified to ferment both sugars (glucose and xylose) simultaneously, and giving high ethanol yield (examples include recombinant *Escherichia coli*, *Zymomonas mobilis, Klebsiella oxytoca*, and *S. cerevisiae* [164]. Genetically engineered *E. coli* strain KO11 is able to produce alcohol from all hemicellulosic sugars with >95% theoretical yield [165], which presumably reflects the employment of yeast extract and tryptone within the fermentation medium as supplementary carbon sources. Another way is to use two strains simultaneously called as ‘‘co-culture’’ (both the organisms are cultured together and exist simultaneously in the fermentation medium). Co-culture system has the edge over single culture since there is the possibility of a synergistic effect on the metabolic pathways for ethanol production [166]. The best example of multiple microbial culture system is fermentation of traditional beverages or food like salami, sourdough, cheese, yogurt, sauerkraut, beer, wine, and whiskey, where they utilise complex substrates efficiently [166-167]. US Department of Energy (DoE) study states that co-culture bioconversion technique has the potential of becoming a high-payoff opportunity for 2G bioethanol production and it is very plausible [168]. Various biomass hydrolysates have been utilised to study the co-culture for ethanol production [164]. Some studies have shown promising outputs. These include co-culture system of *Z. mobilis* and *P. stipitis* (or *Scheffersomyces stipitis*) [169], *E. coli* strain KO11 and *S. cerevisiae* [170], *Z. mobilis* and *Candida tropicalis* co-culture using agricultural

wastes hydrolysates [171], *S. cerevisiae* and *Pachysolen tannophilis* [172], and co-culture of *P. stipitis* and *S. cerevisiae* [173-174].

Currently, the co-culture system is industrially applied for biogas production, wastewater treatment, soil remediation, and traditional food production (cheese, yogurt, pickles, whiskey, etc.) [166-167]. Additionally, various environmental bioconversion processes are catalysed by mixed cultures in an apparently stable manner in natural systems. Based on insights into how industrial and natural systems operate, the concept of employing a co-culture approach for bioethanol production is followed to mix a pentose- and a hexose-fermenting microbe to ferment glucose and xylose at the same time.

1.7.4.1.1 Interactions between microbes used during co-culture system

To have a stable co-culture system, bound necessities should exist. The two strains should be compatible and able to function properly together. Laplace et al. [175] have studied the compatibility aspects of different strains using petri plate assays of *S. cerevisiae, C. shehatae* (or *Scheffersomyces shehatae),* and *P. stipitis*. The study has shown that none of the *S. cerevisiae* strains inhibited growth of xylose-fermenting yeasts (*P. stipitis* or *C. shehatae*) and none of the *C. shehatae* strains were found to be associated in restrictive impact on the growth of *Saccharomyces* species. Thus, it can be concluded that every strain of *C. shehatae* can be used with *Saccharomyces* (glucose fermenting yeast) species. Pathway for glucose sugar metabolism by *S. cerevisiae* during fermentation towards ethanol production has been shown in Fig. 1.11a. Among the *P. stipitis* strains, five strains have shown killer activity against *Saccharomyces* species, and three of them showed killer activity specifically against *S. cerevisiae.*

Another requirement of successful co-culture system is that the fermentation conditions (pH, temperature, and oxygen requirement) for the two strains ought to be compatible. For example, *Z. mobilis* ferments hexose sugars at neutral pH and 37^{o}C temperature, however, these conditions don't seem to be compatible with those of xylose-fermenting yeasts (*P. stipitis* and *S. shehatae*), which require pH 5 and 30^{o}C temperature. The pH scale and temperature at which *S. cerevisiae* ferments hexoses are compatible with xylose-fermenting organisms. Therefore, a mixture of *P. stipitis* or *S. shehatae,* and *S. cerevisiae* may be utilised in co-culture process for ethanol production. Fig. 1.11b shows the xylose metabolism pathway by the yeast during fermentation for ethanol production.

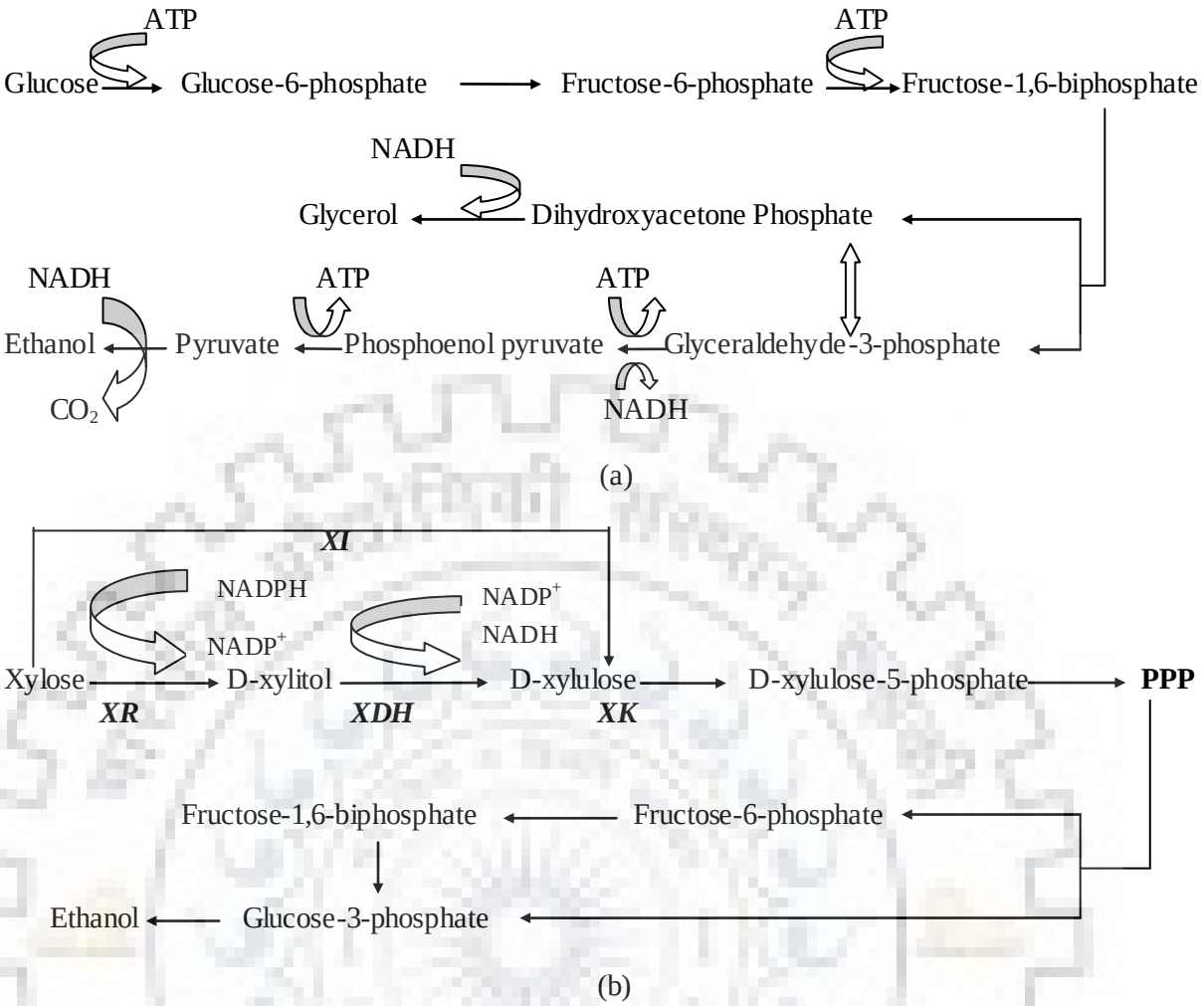


Fig. 1.11. (a) Glucose metabolism by *S. cerevisiae* (b) xylose metabolism by the yeast during fermentation for ethanol production (***XR***: Xylose reductase, ***XDH:*** Xylose dehydrogenase, ***XK:*** Xylose kinase, ***XI***: Xylose isomerase, **PPP:** Pentose phosphate pathway) [176].

Interactions between the organisms play a vital role in co-culture systems compared to pure culture. These interactions result either through direct cell-to-cell communication or by signal substances within the fermentation broth [166]. Stable co-culture can be controlled by metabolic interactions (syntrophic relationships or competition for specific substrates) and interactions between growth promoters or inhibitors (like antibiotics) [177]. Interactions between two organism species could be either positive, negative or neutral [178]. As an example, positive interaction in co-cultures might turn up through oxygen reduction by aerobic organisms, and thereby make anaerobic conditions, promoting anaerobic or microaerophilic organism's growth. This sort of mixed culture provides protection from environmental fluctuations. The negative interaction may happen if two microorganisms contend for a similar resource, like area or a limiting nutrient. Neutral interaction means there is no impact on the two organisms in the co-culture system. For a beneficial co-culture system, positive

interactions between the strains are expected. However, it may not always result in desirable consequences. Therefore, understanding the interactions between organisms during a co-culture system is extremely necessary. However, little or no analysis has been done to this point, primarily due to the complicated nature of systems containing multiple strains. This offers new directions to the future analysis.

1.7.4.1.2 Strains used in current co-culture systems

The principal naturally ethanol producing strains include a variety of bacteria (*Z. mobilis, E. coli, Zymobacter palmae, Bacillus stearothermophilus,* and *Clostridium* species primarily *cellulolyticum, thermocellum, thermosaccharolyticum* or *Thermoanaerobacterium thermosaccharolyticum*), and yeasts (*P. stipitis, S. shehatae, S. cerevisiae, Kluyveromyces marxianus, P. tannophilius* majorly). Among these strains, *S. cerevisiae* is often used for bioethanol production. However, most of the wild strains of yeast don't metabolise xylose; *S. stipitis, S. shehatae,* and *P. tannophilius* are found to be capable of pentose fermentation to ethanol [173,179]. For establishing a co-culture system, a glucose-fermenting and a xylose-fermenting organism should be identified primarily, and then their compatibility and co-fermentation performance studies should be done. To summarise the advantages and limitations of different microbial fermentative strains, Table 1.8 presents potential microbes for second generation ethanol fermentation (bacteria, yeasts, and fungi) that could become avenues to increase ethanol yield and productivity.

Table 1.8. Advantages and limitations of potential ethanol-producing microbes [180].

	Organism	Characteristics	Advantages	Disadvantages	References
Glucose-fermenting organisms	*Saccharomyces cerevisiae*	Facultative anaerobic yeast	•Naturally adapted to ethanol fermentation. • High ethanol yield (90%) • High tolerance to ethanol (up to 10% v/v) and chemical inhibitors • Can be genetically modified easily	•No xylose and Arabinose fermentation. • Cannot survive high temperature during enzyme hydrolysis.	[181-186]
	Zymomonas mobilis	Ethanologenic Gram-negative bacteria	• Very high ethanol yield (97% of the theoretical) • High ethanol tolerance (up to 14% v/v) • High ethanol productivity (five-times more volumetric productivity compared to *S. cerevisiae*) • Can be genetically modified easily •Anaerobic (no additional oxygen required)	• No xylose and Arabinose fermentation. • Low tolerance to inhibitors • Neutral pH range	[187-189]

Xylose-fermenting organisms	*Candida or Scheffersomyces shehatae*	Micro-aerophilic yeast	•Ferment xylose	•Low ethanol tolerance •Low ethanol yield • Does not ferment xylose at low pH	[181,190-192]
	Pichia or Scheffersomyces stiplis	Facultative anaerobic yeast	• High ethanol yield (82%) • Able to ferment most of cellulosic-material sugars including glucose, galactose and cellobiose	• Intolerant to 40 g/L ethanol concentration or above •Does not ferment xylose at low pH •Sensitive to chemical inhibitors. • Re-assimilates formed ethanol	[181,190, 192-194]
	Pachysolen tannophilus	Aerobic fungus	•Ferment xylose	•Low ethanol yield • Does not ferment xylose at low pH	[190,195]
Both glucose- and xylose-fermenting organisms	*Escherichia coli*	Mesophilic Gram-negative bacteria	•Ability to use both pentose and hexose sugars. • Can be genetically modified easily	• Interference of repression catabolism to co-fermentation • Limited ethanol and inhibitors tolerance • Narrow pH and temperature growth range • Organic acids Production • Genetic stability not proven yet	[184,195-196]
	Kluveromyces marxianus	Thermophilc yeast	•Able to grow at a high temperature>52^{o}C • Suitable for SSF/CBP process • Reduces cooling cost •Reduces contamination •Ferments a broad spectrum of sugars. • Can be genetically modified easily	• Excess of sugars affect ethanol yield • Low ethanol tolerance • Poor xylose fermentation; leads mainly to the xylitol formation	[197-199]
	Thermophilic bacteria: •*Thermoanaerobacterium saccharolyticum* •*Thermoanaerobacter ethanolicus* •*Clostridium thermocellum*	Extreme anaerobic bacteria	•Resistance to an extremely high temperature (up to 70^{o}C) • Suitable for SSCF/CBP Processing •Ferment a variety of sugars • Display cellulolytic activity • Can be genetically modified easily	•Low ethanol tolerance	[196,200-203]

It has been seen that the yeast *S. cerevisiae* is ideally used for glucose fermentation along with pentose-fermenting yeast. *P. stipitis* has been used by several researchers as a xylose-fermenting organism in the co-culture system. The foremost used strain combination is *S. cerevisiae* and *P. stipitis* or respiratory-deficient mutant of *S. cerevisiae* [164]. It can be well explained by the fact that the pH and temperature during fermentation by *S. cerevisiae* (for hexose) is compatible with those of *P. stipitis* (for pentose sugar). Air level as low as ~2 mmolL/h is necessary for ethanol production from pentose sugars by the yeasts to keep their viability and NADH balance [164,204-207].

Table 1.9. List of genetically modified strains employed to improve the ethanol yield and productivity [147].

Micro-organism	Strain	Engineered	Key improvements	Substrate	Ethanol data	Reference
Bacteria	*Zymomonas mobilis*	*Zymomonas mobilis* ZM4(pZB5)	Ferment both xylose and glucose	Stillage (residue from starch fermentation)	• 11 g/L (with 10 g/L of glucose supplementation) • 28 g/L (with 5 g/L yeast extract and 40 g/L glucose Supplementation)	[208]
		Zymomonas mobilis AX101	Ferment glucose, xylose and arabinose	Various agricultural wastes	• 3.54 g/L*.h (no acetic acid) • 1.17 g/L*h (in presence of acetic acid)	[209]
	Clostridium thermocellum	*Clostridium thermocellum* DSM1313	Improves ethanol yield	-	• 0.8 g/L at 0.5 g/L of cellobiose	[210]
		Clostridium thermocellum YD01	Improves ethanol yield	Cellobiose	• 1.33 mole-ethanol/mole-glucose equivalent	[211]
		Clostridium thermocellum YD02	Improves ethanol yield	Cellobiose	• 1.28 mole-ethanol/mole-glucose equivalent	[211]
	Escherichia coli	*Escherichia coli* KO11	Ferment both xylose and glucose	Sugarcane bagasse	• 31.5 g/L • Theoretically 91.5% after 48 h of fermentation	[212]
		Escherichia coli FBR5	Ferment xylose	xylose	• 0.5 g/g of xylose	[213]
		Escherichia coli FBR5	Ferment xylose and arabinose	Rice hull	• 2.25% (w/v)	[214]
	Thermoanaero-bacterium saccharolyticum	*Thermoanaero-bacterium saccharolyticum* ALK2	• Improves ethanol yield • Ferment glucose, xylose, mannose and arabinose	-	• 37 g/L	[215]
		Thermoanaerobacter mathranii BG1L1	Improves ethanol yield	Wheat straw	• 0.39-0.42 g/g sugars	[216]
Yeast	*Pichia stipitis*	*Pichia stipitis* A	Adapted at hydrolysate increased concentration	Wheat straw	• 0.41 g/g	[198]
		Pichia stipitis NRRL Y-7124	Adapted at hydrolysate increased concentration	Wheat straw	• 0.35 g/g	[216]
		Pichia stipitis BCC15191	Ferment both xylose and glucose	Sugarcane bagasse	• 8.4 g/L after 24 h fermentation	[217]
	Saccharomyces cerevisiae	*Saccharomyces cerevisiae* D5a	Improved ethanol yield	Rice hull	• 0.58% (w/v) or 100% theoretical yield	[214]
		Saccharomyces cerevisiae 590. E1	Ferment glucose and cellobiose	Whatman paper	• 1.09% from 2% glucose • 1.16% from 2% cellobiose	[218]
		Saccharomyces cerevisiae 590. E1	Ferment cellulose without additional enzymatic hydrolysis process	Corn stover	• 63% theoretical ethanol after 96 h fermentation	[218]
		Saccharomyces cerevisiae RWB 217	Ferment glucose and xylose	2% glucose and 2% xylose	• 0.43 g/g of sugars	[219]
		Saccharomyces cerevisiae RWB 218	Ferment glucose and xylose	2% glucose and 2% xylose	• 0.4 g/g of sugars	[219]
	Candida shehatae	*Candida shehatae* NCL-3501	Co-ferment xylose and glucose	Rice straw	• 0.45 g/g of sugar by autohydrolysis • 0.5 g/g of sugar by immobilised cells	[220]

A respiratory-deficient *Saccharomyces* mutant has been engineered to overcome different oxygen requirements for both glucose and xylose fermentation [221-222]. The more of genetically engineered microorganisms employed in some significant studies to improve ethanol yield is listed in Table 1.9. Another issue with of *P. stipitis* and *S. cerevisiae* co-culture system is low ethanol and sugar tolerance of *P. stipitis* that could induce inhibition of sugar fermentation [223,203]. Delgenes et al. applied continuous culture conditions to deal with this drawback [221-222]. During the continuous mode of fermentation, hexose sugar concentration can be kept significantly low, not to repress pentose sugar utilisation by the xylose-fermenting organisms. *Z. mobilis, S. diastaticus, C. thermocellum*, and *K. marxianus* have been used as a glucose-fermenting organism in some co-culture systems apart from *S. cerevisiae* [169,221-222,224,225-228]. *C. shehatae, C. tropicalis, P. tannophilus, K. fragilis, P. Stipitis,* and *recombinant E. coli* were utilised as xylose-fermenting yeast [170-172,229-232].

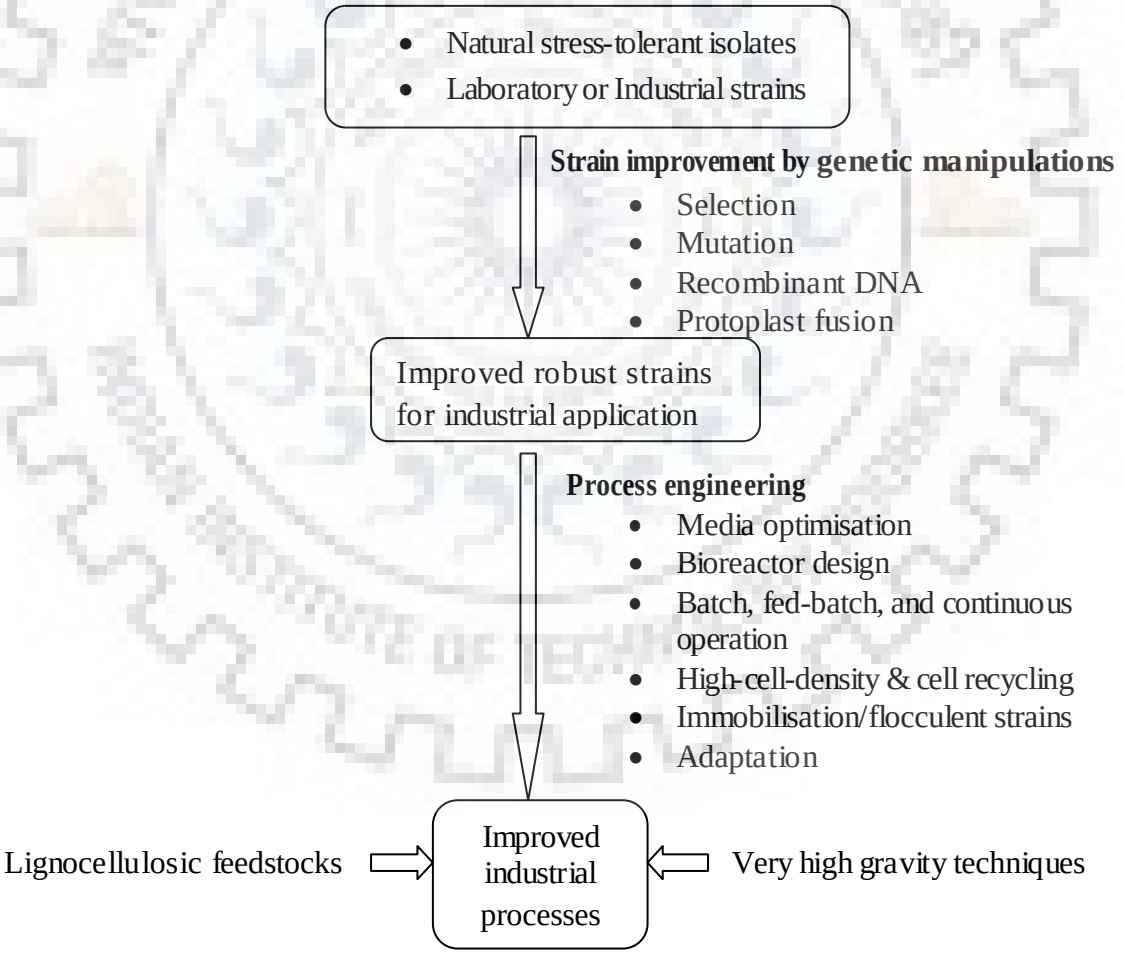


Fig. 1.12. Integration of microbial strains improvement and bioprocess design for an efficient ethanol production process [233].

Several genetic and metabolic engineering tools can be used for the improvement of ethanol-producing microbial strains (Fig. 1.12) [234-235]. Traditional mutagenesis, screening methods, and their combinations can be very useful in microbe improvement [236]. Genome shuffling and global transcription machinery engineering (gTME) have been applied to improve alcohol production by *S. cerevisiae* [237-239]. In parallel to the microbial strain improvements, bioprocess design developments are also very essential to establish an efficient ethanol production system at commercial scale (Fig. 1.12). Use of lignocellulosic hydrolysates as feedstock, very high-gravity (VHG) technologies, and high-cell-density continuous processes are the most significant developments in this direction.

1.7.4.1.3 Fermentation modes

Various fermentation modes (batch, continuous, and fed-batch) have been employed in co-culture systems. Xylose fermentation was conducted from hydrolysate obtained from rice straw by *C. shehatae* in all three modes, and it was observed that fed-batch and continuous co-culture system resulted in higher ethanol yield and volumetric productivity [240]. For an effective co-culture system, choice of fermentation mode should depend on the microbes within the system. Batch and continuous fermentation parameters of glucose and xylose mixture were compared by co-cultivation of a respiratory-deficient mutant of *S. cerevisiae* with *C. shehatae* [241]. The results showed the poor utilisation of xylose in batch mode (6%). However, continuous fermentation resulted in the conversion of both sugars simultaneously.

Batch mode has some limitations despite its simplicity and easily controllable behaviour. First is suppression of xylose fermentation by glucose, especially at the initial stage [242]. Therefore, co-culture system with continuous fermentation mode can be designed, keeping glucose concentration sufficiently low so that xylose utilisation cannot be repressed by the xylose-fermenting organisms [221-222,242]. In the continuous mode, it can be achieved by adjustment of dilution rate and maintenance of low glucose concentration. Another way is the use of *S. cerevisiae* organism which has high fermentative potential allowing fast xylose conversion by generation of a low glucose environment [241]. However, if the amount of ethanol produced exceeds the ethanol tolerance of the xylose-fermenting yeast, it would be a major problem. Ethanol concentration of 30 g/L or more inhibits the xylose fermentation process [228]. Medium-outflown continuous fermentation can avoid this issue of alcohol and other inhibiting substances accumulation in the system [231]. Again, wash out and biphasic or significantly different growth rates of two organisms subject to instabilities [232]. Cell

retention (filtration, immobilisation, and encapsulation) or cell recirculation (centrifugation or using flocculating organisms) has been used to solve this issue.

1.7.4.1.4 Fermentation condition

It depends entirely on the selection of the strains in the co-culture system. Each pair has their own optimum temperature, pH, oxygen requirements, and inoculum size. For glucose and xylose co-fermentation, initial sugar concentration and their proportion affect the fermentation performance. Laplace et al. studied the effects of initial sugar concentration on the fermentation performance of *Z. mobilis*, *S. cerevisiae*, *P. Stipitis*, and *C. shehatae* [170].

Most of the current co-culture systems use fermentation temperature of 30^{o}C, as the optimum temperature of most of the sugar fermenting microbe is 30^{o}C. However, it is not true for *C. Thermocellum*, and *C. thermosaccharolyticum* as they have optimum fermentation temperature of around 60^{o}C [177,223,232,243]. For *Z. mobilis* and *P. stipitis* combination, despite the optimal temperatures conflict, researchers use 30^{o}C as the fermentation temperature. pH 5.0 is used for *S. cerevisiae* and *P. stipitis* co-cultures systems.

Oxygen is also an important parameter along with medium composition, temperature, and pH in co-culture systems. Sequential culture can be used to solve the problem of the different oxygen requirements. The inoculum is ranged from 2-10% (v/v) in current co-culture systems. To evaluate the fermentation performance, ethanol yield ($Y_{p/s}$), volumetric ethanol productivity (Q_p), specific ethanol production rate (q_p), efficiency of substrate utilisation, and various other measures are calculated. Among these, ethanol yield is most useful parameter. The theoretical ethanol yield for both the sugar (glucose and xylose) fermentation is 0.51 g/g [244]. It has been found from the literature that ethanol yield of different co-culture systems ranges from 0.25-0.5 g/g.

1.7.4.1.5 Potential benefits and challenges of co-culture fermentation

Till date, all the co-culture systems for glucose and xylose co-fermentation have been performed at laboratory scale. One of the major challenges of using the co-culture system is low ethanol tolerance of xylose-fermenting yeasts [170,221,227,231]. Selection of more ethanol-tolerant strains and use of ethanol removal systems during co-culture can remove this problem [221]. Another challenge is finding optimum fermentation parameters (pH, temperature, initial substrate concentration, and oxygen). Presence of large amount of inhibitory and toxic compounds in the lignocellulosic hydrolysates also impact co-culture

process. Detoxification (by liming, steam stripping or other methods) before the fermentation adds considerably to the operational costs. Adaptation can be used as an efficient method for increasing inhibitors tolerance for a wide range of yeast strains in the hydrolysate media [245-246]. However, it is only useful when the inhibiting mechanism is known [175]. Carbon catabolite repression also limits the industrial application of co-cultures systems as alcohol produced from glucose sugar may decrease the yield due to the xylose fermentation inhibition. As discussed previously, the continuous mode of fermentation can help in dealing this issue. Although at industrial scale, bioethanol production using co-culture fermentation remains problematic, considerable progress has been made using various strains at laboratory scale. Therefore, co-culture fermentation has a potential of making a great impact in low-cost process development for ethanol.

1.8 Other conversion pathways for lignocellulosics-based ethanol production

As discussed before, sugar is the main component responsible for distinction between production pathways from starch, simple sugar, or lignocellulosic biomass. Sugars can be directly fermented using suitable organisms to produce bioethanol, in case of molasses and sugarcane juice [247], whereas for fermentable sugar production from starchy materials, milling, liquefaction, and saccharification are required. For lignocellulosic feedstock based ethanol production, processes like milling, pretreatment, and hydrolysis are used. The detoxification unit is considered only when toxic compounds are generated in significant quantity during hydrolysis. Schematic representation of ethanol production processes based on all three feedstocks is shown in Fig. 1.13.

The biochemical route for ethanol production has been categorised into:

a. Separate hydrolysis and fermentation (SHF)

b. Integrated technologies:

1. Simultaneous saccharification and fermentation (SSF)
2. Simultaneous saccharification and cofermentation (SSCF)
3. Consolidated bioprocessing (CBP) or Direct microbial conversion (DMC)

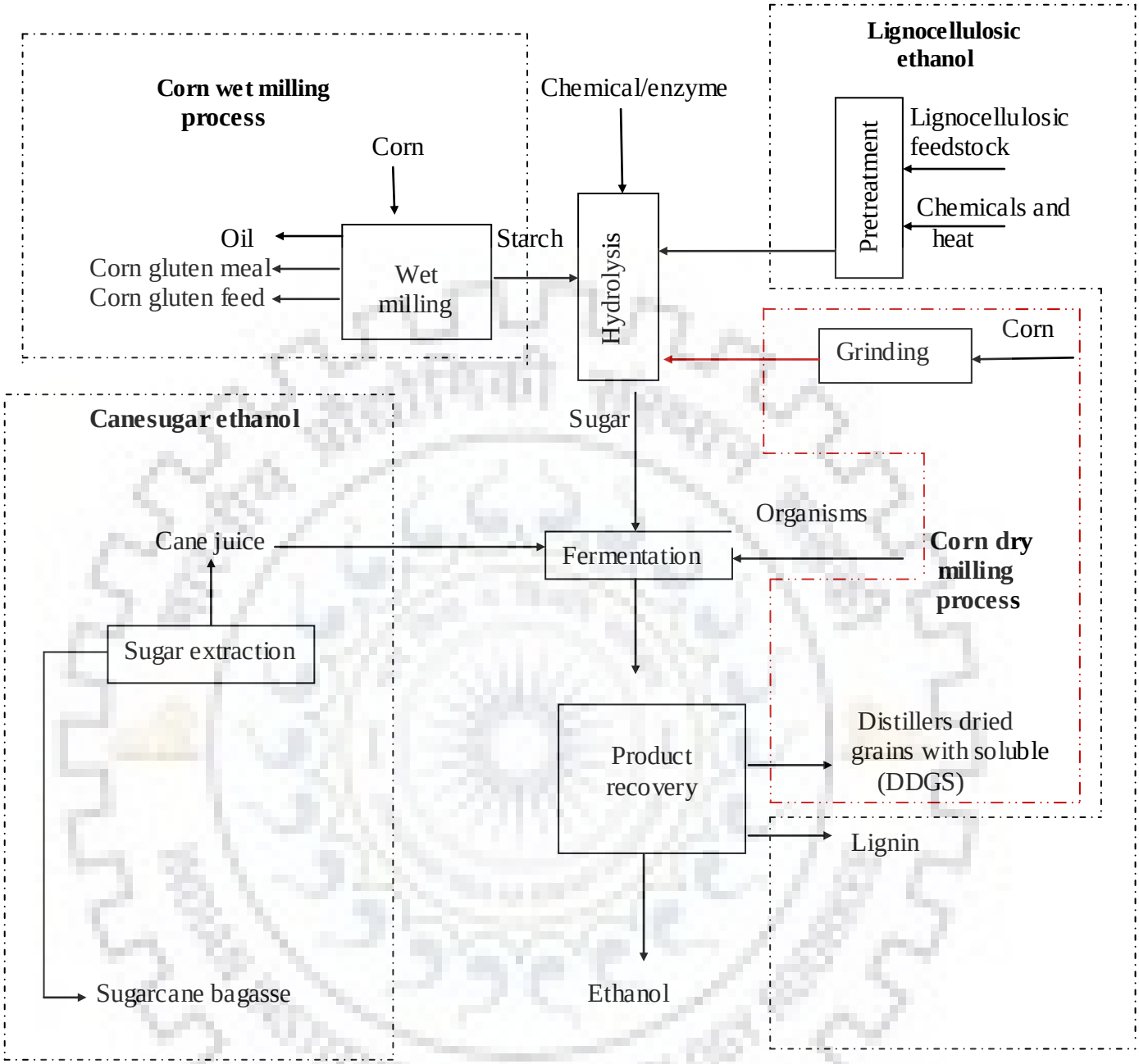


Fig. 1.13. Schematic representation of production of ethanol from corn (corn wet milling, corn dry milling), cane sugar, and lignocellulosic biomass [1,247].

In SHF, hydrolysis (chemical or enzymatic) is performed separately from the fermentation step [248], described in detail in section 1.7. It involves pretreatment (chemical treatment of milled/ground biomass resulting into hydrolysis of hemicellulose and disruption of the lignin associations with the carbohydrate) and hydrolysis (for conversion of cellulosic polymer into its monomeric units) prior to fermentation. Comparison of various pretreatment methods used during SHF has already been presented in Table 1.7. The hydrolysate is then subjected to

neutralisation and detoxified if necessary, which is then fermented to produce ethanol. Lignin (residual insoluble material form) can be burnt for energy generation further [1,249]. In SSF, saccharification and fermentation carried out simultaneously in a single reactor saving overall cost and inhibitor reduction [249-250]. At the same time, process conditions optimisation for enzymatic saccharification and microbial fermentation is the most critical issue using SSF [1,251]. Main advantages and disadvantages of SHF and SSF along with acidic and enzymatic hydrolysis for saccharification (during SHF) have been discussed in Table 1.10.

Table 1.10. SHF and SSF comparison [252-255].

Fermentation route	**Parameters**	**Acid**	**Enzymatic**	**Advantages**	**Disadvantage**
SHF	Hydrolysis condition	Harsh	Mild	• Each step can be operated at different optimal conditions • Separate steps reduces any interference between the steps	• End product inhibition minimises ethanol yield • Chances of contamination due to longer conversion process
	Hydrolysis yield	Low	High		
	Product inhibition	No	Yes		
	By-product formation	Yes	No		
	Catalyst cost	Low	High		
	Hydrolysis time	Short	Long		
	Equipment corrosion	Yes	No		
SSF				• Low Cost • Higher ethanol yields (due to removal of end product inhibition during saccharification) • Requirement of less reactors	• Difference in optimum operating conditions for enzyme hydrolysis and fermentation

SSCF is subjected to the complete assimilation of both pentoses and hexoses which are released during the pretreatment and hydrolysis stage (Fig. 1.14). Mixed culture of microbes or a single microbe which can ferment both the sugars (pentoses and hexoses), is used during SSCF, but hexose-utilising organisms grow rapidly compared to pentose consuming microbes which result in an elevated conversion of hexose sugar [1,256]. In CBP or DMC, ethanol and all the enzymes required for its production are formed in a single reactor by a single microbial community [1,257]. Reaction-reaction integration into ethanol is CBP for the biomass transformation (Fig. 1.14). The major difference between CBP and SSF is that a single microbial community is used in CBP to carry out cellulase production and fermentation both.

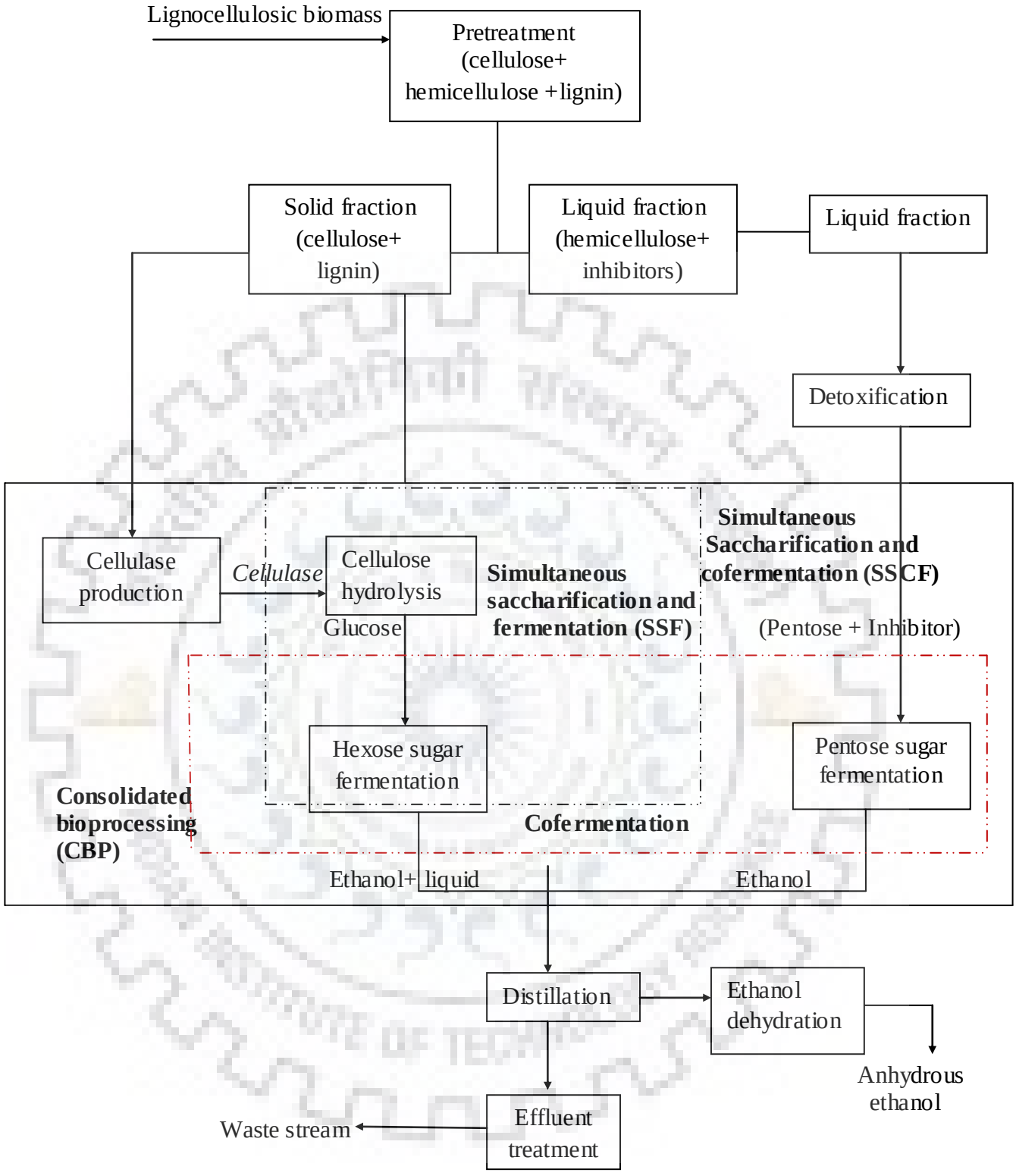


Fig. 1.14. Generic block diagram of bioethanol production from lignocellulosic biomass showing possibilities of various reaction-reaction integrations [1,258].

After fermentation, ethanol produced in the broth is recovered and further purified to get a fuel grade ethanol. Recovery is done by distillation method. Water can be recycled while lignin can be combusted and utilised for heat and electricity generation. Therefore, the overall conversion process of lignocellulosic feedstock to ethanol can be divided into four major steps (pretreatment, hydrolysis, fermentation and product recovery) (Fig. 1.14). Because of the versatility in nature, physicochemical properties of lignocellulosic biomass vary significantly with respect to biomass types, affecting the conversion process. Therefore, it is very difficult to design a uniform conversion method for 2G ethanol production and optimum conditions for all feedstocks [51]. Some recent studies on the second generation ethanol from various lignocellulosic feedstocks are summarised in Table 1.11 discussing major steps followed and their findings.

Table 1.11. Some recent significant studies for 2G ethanol production [51].

Feedstock type	Feedstock	Major steps	Key findings	Reference
Corn	Corn stover	• Biological pretreatment using *Ceriporiopsis subvermispora* with 18 d of incubation period	• Lignin degradation: up to 31.59% • Cellulose loss: < 6% • Maximum ethanol yield: 57.8%	[259]
	Corn stover	•Steam pretreatment of SO_2 impregnated biomass at 200 °C for 5 min • SSF using cellulase and β-glucosidase mixture and *S. cerevisiae*	•Maximum ethanol yield: 74% of the theoretical •Maximum ethanol concentration: 125 g/L	[260]
	Corn cob	• Co-generation of lipid and ethanol • Acid hydrolysate was used as substrate for microbial lipid production, and remaining solid residue was hydrolysed enzymatically	•Ethanol yield:131.3 g/kg •Lipid yield: 11.5 g/kg • Conversion efficiency: 71.6% (fermentable sugars into valuable products)	[261]
Straws	Wheat straw	• Dilute acid pretreatment, •Bio-abatement of fermentation inhibitors and •Simultaneous saccharification and fermentation using *Escherichia coli* FBR5	•Effective fermentation time: 83 h •Maximum ethanol concentration: 36 g/L • Maximum ethanol productivity: 0.43 g/L/h • Maximum ethanol yield: 0.29 g/g • Maximum theoretical ethanol yield: 86%	[262]
	Rice straw	• Popping pretreatment technique • Enzymatic hydrolysis	•Optimum enzyme load: 23 FPU/g (cellulose) and 62 U/g (xylanase) • Sugar production: 0.567 g/g of straw after 48 h • Ethanol yield: 0.172 g/g of straw (80.9% of the maximum theoretical yield) after 24 h of fermentation	[263]
	Barley straw	•Treatment with sodium hydroxide in a twin-screw extruder for continuous pretreatment with high biomass loading (up to 20%)	•Maximum ethanol concentration:46 g/L • Maximum ethanol yield:77.4%	[264]
Bagasse	Sugarcane bagasse	• Steam explosion •Phosphoric acid treatment •Enzymatic hydrolysis under high solid loading (18- 22%) and low enzyme load	• Maximum sugar concentration: 76.8 g/L • Total glucan conversion: 69.2%	[265]
	Sugarcane bagasse	•Simultaneous saccharification and fermentation	•Maximum ethanol concentration: 25 g/L • Maximum ethanol productivity: 0.7 g/L/h • Cellulose conversion efficiency: 72%	[266]
	Sweet sorghum Bagasse	•Pretreatment and hydrolysis in a single step using microwave irradiation	•Sugar yield: 820 g/kg of biomass • Ethanol yield:480 g/kg of sugar after 24 h of fermentation	[267]

Woods	Pine (soft wood)	•Extrusion pretreatment method	• Optimum screw speed:150 rpm • Barrel temperature: 180°C •Moisture content: 25% • Maximum cellulose recovery: 65.8% • Maximum hemicellulose recovery: 65.6% • Total sugar recovery: 66.1%	[268]
	Yellow poplar (hard wood)	•Organosolv method for pretreatment using sulfuric acid	•Maximum ethanol concentration: 42.8 g/L) • Optimum temperature: 152°C • Optimum acid concentration: 1.6% • Optimum reaction time: 16 min	[269]`
Grass	Switchgrass	•Stem explosion for combined ethanol and methane production • Comparison of ethanol yield ➢ under steam alone ➢ steam with lime (Calcium hydroxide) ➢ H_2SO_4 pretreatment	•Minimum lignin removal: 12% (with steam alone) • Maximum lignin removal: 35% (steam with lime) • Low lime increased the final ethanol yield •H_2SO_4 enhanced CH_4 yield	[270]
	Cocksfoot grass (*Dactylis glomerata*)	•Wet explosion pretreatment	• Pretreatment at 180-190°C inhibited fermentation completely due to high concentration of inhibitors • Maximum theoretical ethanol yield: 92% (pretreatment conditions:160°C, 15 min, 87 psi oxygen)	[271]
Municipal solid waste		•Pretreatment with fifteen methods followed by hydrolysis	•Maximum glucose yield: 72.80% ➢ Pre-hydrolysis treatment conducted with 1% H_2SO_4 ➢ Steam treatment at 121 °C ➢ Enzymatic hydrolysis with *Trichoderma viride* cellulase at 60 FPU/g of substrate	[272]
Aquatic plants	Water hyacinth	•Alkaline-oxidative pretreatment and saccharification	•Effective pretreatment conditions: 7% NaOH (w/w) and 2% H_2O_2 (w/v) at 100°C • Maximum ethanol yield: 0.35 g/g	[273]
Palm empty fruit bunch		•Dilute H_2SO_4 (1% v/v) treatment at 125˚C for 90 min •NaOH (1% w/v) treatment at 100˚C for 60 min •Enzymatic hydrolysis using cellulase (Novozymes) at 50˚C for 72 h •Fermentation using *S. cerevisiae*	• Maximum ethanol yield: 12.1 g/L (89.1% of the theoretical)	[274]

2. Research gaps

Based on the literature survey, it was found that the overall ethanol production cost is highly sensitive to the feedstock and operating scale. Lignocellulosic biomasses are promising feedstocks because of low costs, high yield, ability to grow in marginal lands with no additional water supply requirement, and wide availability throughout the year. Therefore, various lignocellulosic biomasses are selected for the present study as the ethanol-producing feedstocks. However, there are many research challenges of using lignocellulosic feedstocks for ethanol production which needs to be addressed properly to produce ethanol at commercial scale.

- Improvement in the pretreatment/hydrolysis process is required for the maximum extraction of fermentable sugars available in form of cellulose and hemicelluloses from the biomass.
- Developing suitable fermentation technique and selection of microorganisms that are tolerant to inhibitors and ferment maximum sugars present in the concentrated hydrolysate for better performance.
- Extending process integration to reduce the number of process steps.

3. Objectives of the present study

To contribute in the pool of existing knowledge, in a direction to overcome the above research challenges, present work is designed with the following objectives:

- Development of a suitable technique for extracting the maximum amount of fermentable sugars separately (hexoses and pentoses) with minimum toxics from the lignocellulosic biomass.
- Exploration and evaluation of various inorganic acids and lignocellulosic feedstocks for the developed hydrolysis process.
- Development of suitable co-culture fermentation process to ferment maximum sugars present in the concentrated hydrolysate using *Zymomonas mobilis* and *Scheffersomyces shehatae* with improved fermentation performance.

CHAPTER 2

Materials and Methods

2.1 Materials

Feedstocks, microorganisms, chemicals, and equipments used in the present study have been discussed in this section.

2.1.1 Lignocellulosic biomasses

The wasteland weed, *Saccharum spontaneum* (Kans or Sarkanda), was obtained from outskirts of Roorkee, Uttarakhand (India) from the sides of Ganga canal. Whole plant (leaf sheaths and stems) were cut into small pieces by chopper. The chopped pieces were washed, air dried, milled (Lab willey mill, Meron Lab, New Delhi, India) and screened for the size of approximately 1.56-6.73 mm. Wheat straw and sugarcane bagasse were purchased from local market of Roorkee. Both were air dried without any washing. The feedstocks were milled, and sizes of 1.56-6.73 mm were screened for further studies. The scientific classification of all three feedstocks is given below in Table 2.1.

Table 2.1. Scientific classification of the selected lignocellulosic biomasses [1-3].

Sugarcane bagasse	**Kans grass**	**Wheat straw**
Kingdom: Plantae	Kingdom: Plantae	Kingdom: Plantae
Clade: Angiosperms	Phylum: Magnoliophyta	Clade: Angiosperms
Clade: Monocots	Class: Liliopsida	Clade: Monocots
Clade: Commelinids	Order: Cyperales	Clade: Commelinids
Order: Poales	Family: Poaceae	Order: Poales
Family: Poaceae	Genus: Saccharum	Family: Poaceae
Genus: Saccharum	Species: *S. spontaneum*	Sub-family: Pooideae
Species: *S. officinarum*		Tribe: Triticeae
		Genus: Triticum
		Species: *T. aestivum*

2.1.2 Microorganisms

Zymomonas mobilis MTCC 91 used in the present study was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Growth medium of *Z. mobilis* consisted (g/L): Glucose 20; Yeast extract 10; $MgCl_2$ 1; KH_2PO_4 1; $(NH_4)_2SO_4$ 1: Agar 20. pH was adjusted to 5.5±0.2. The medium was sterilised by autoclaving at 121°C for 20 min, glucose was separately sterilised and mixed

with other medium components in laminar air flow later. The culture was grown at 30±2°C and stored at 4°C on agar plates.

Scheffersomyces shehatae NCIM 3501 was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. Growth medium consisted (g/L): Glucose 10; Malt extract 5; Yeast extract 5; Peptone 5; Agar 20. pH was adjusted to 6±0.2. The medium was sterilised by autoclaving at 121°C for 20 min, sugar was separately sterilised and mixed with other media components in laminar air flow later. The culture was grown at 30±2°C and stored at 4°C in agar plates or slant.

For inoculum preparation, same media were used for both the organisms, except in case of *S. shehatae*, glucose was replaced by xylose in inoculum media with the same concentration. The scientific classification of both the microorganism used for ethanol production is presented in Table 1.2.

Table 2.2. Scientific classification of the selected microorganisms [4-5].

Zymomonas mobilis	***Scheffersomyces shehatae***
Domain:Bacteria	Kingdom: Fungi
Phylum:Proteobacteria	Division: Ascomycota
Class:Alphaproteobacteria	Class: Saccharomycetes
Order: Sphingomonadales	Order: Saccharomycetales
Family: Sphingomonadaceae	Family: Debaryomycetaceae;
Genus: *Zymomonas*	Genus: Scheffersomyces
Species: *Z. mobilis*	Species: *S. shehatae*
	Synonymy: *Candida shehatae*

All the analytical grade chemicals used in the study were procured from HiMedia Laboratories and S D Fine-Chem Limited, India.

2.1.3 Fermentation media

2.1.3.1 *Z. mobilis*

Synthetic fermentation medium of *Z. mobilis* consisted (g/L): Glucose up to 200; Yeast extract 10; $MgCl_2$ 1; KH_2PO_4 1; $(NH_4)_2SO_4$ 1. pH was adjusted to 5.5±0.2. Hydrolysate fermentation medium was prepared by replacing sugar with GRF obtained from Kans grass hydrolysate.

2.1.3.2 *S. shehatae*

Synthetic fermentation medium of *S. shehatae* consisted (g/L): Xylose up to 60; Yeast extract 5; Urea 5; $MgSO_4$ 0.5; KH_2PO_4 1. pH was adjusted to 6.0±0.2. Hydrolysate fermentation medium was prepared by replacing sugar with XRF obtained from Kans grass hydrolysate. The medium was sterilised by autoclaving at 121°C for 20 min, sugar was separately sterilised and mixed with other media components later under aseptic conditions during all the experiments. The temperature was kept 30±2°C with static mode in case of *Z. mobilis*, whereas agitation was 150 rpm (mrc incubator shaker) for *S. shehatae*. The synthetic medium was used to compare the kinetic parameters and fermentation performances.

2.2 Methods

2.2.1 Biochemical compositional analysis of raw biomass (Proximate analysis)

Compositional analysis was done according to the National Renewable Energy Laboratory (NREL) standard procedures published under Laboratory Analytical procedure (LAP) for estimation of total solid content (LAP 001) [6], carbohydrates (glucan and xylan), acid-soluble, and acid-insoluble lignin content (LAP 003-004) [7-9].

2.2.1.1 Total solid and Moisture content

1±0.001 g of the biomass was taken in a dried and prewighed (using SHIDMADZU, ATY224 weighing balance) crucible, and kept in the muffle furnace at 105ºC. Final weight was noted down at a regular interval until the constant weight was obtained. The total solid and moisture content of the biomass was calculated based on the equations.

$$Total\ solid\ (\%, w/w) = \frac{Final\ weight(sample+crucible) - Crucible\ weight}{Initial\ weight\ of\ sample} \times 100 \qquad \text{(Eq. 2.1)}$$

$$Moisture\ content\ (\%, w/w) = \frac{Initial\ weight(sample+crucible) - Final\ weight(sample+crucible)}{Initial\ weight\ of\ sample} \times 100 \qquad \text{(Eq. 2.2)}$$

2.2.1.2 Ash content

The initial known dry weight of the biomass was taken in a crucible and kept in muffle furnace (Yamato, FO 100) at 550ºC for 6 h. After 6 h incineration, the sample was cooled down and placed in a desiccator, and weight of the crucible along with ash content was recorded. The ash content of the substrate was calculated based on the dry weight basis and illustrated in the formula given in Eq. 2.3.

$$Ash\ content\ (\%, w/w) = \frac{Initial\ weight(sample+crucible) - Final\ weight(sample+crucible)}{Initial\ weight\ of\ sample} \times 100 \quad \text{(Eq. 2.3)}$$

2.2.1.3 Acid insoluble lignin estimation

Lignin content was estimated using LAP 003, proposed by Templeton & Ehrman 1995 [7]. 0.3±0.001 g of sample was weighed (W_1) and placed in a test tube. 3±0.01 mL of 72% Sulphuric acid (98% w/w H_2SO_4) was added and mixed thoroughly. Test tube was placed in a water bath at 30°C for 2 h and stirred at every 15 min interval. After 2 h, sample was transferred in a flask and diluted to 4% acid concentration by adding 84 mL deionised water. The flask was covered and kept in an autoclave at 121°C for 1 h. Sample was then allowed to cool down to room temperature and filtered using whatman filter paper. The solid residue was washed using deionised water repeatedly and 20 mL filtrate was stored at 4°C for further analysis. The residue was kept for in a crucible for drying at 105ºC and weighed until the constant weight (W_2) was obtained. After that, the crucible containing solid residue was kept in a furnace at 550ºC for 4 h of incineration. After cooldown, the weight of crucible containing ash was recorded as W_3. The total insoluble lignin was calculated using equation 2.4.

$$Acid\ insoluble\ lignin\ (\%, w/w) = \frac{W_2 - W_3}{W_1 \times Total\ solid\ (\%)} \times 100 \quad \text{(Eq. 2.4)}$$

2.2.1.4 Acid soluble lignin estimation

Acid soluble lignin was determined using the method given by Ehrman 1996 in LAP 004 [8]. The absorbance of filtrate obtained in previous section was recorded at 205 nm against 4% H_2SO_4 solution, and equation 2.5 was used for the calculation.

$$Acid\ insoluble\ lignin\ (\%, w/w) = \frac{\frac{A}{b \times a} \times Dilution\ factor \times \frac{V}{1000}}{W_1 \times Total\ solid\ (\%)} \times 100 \quad \text{(Eq. 2.5)}$$

Where A = Absorbance at 205 nm

b = Path length of cell (1 cm)

a = Absorptivity (110 L/g*cm)

V = Filtrate volume (87 mL)

W_1 = Initial biomass weight

2.2.1.5 Glucan and Xylan estimation

The filtrate stored during the acid insoluble lignin estimation was neutralised using $Ca(OH)_2$ between pH 6-7 and filtered again to remove any precipitate. Total reducing sugar (TRS) includes glucose and xylose and the estimation was done according to DNS method given by Miller 1959 [10], xylose by phloroglucinol method by Eberts et al. 1979 [11]. Approximately 90% of the total glucan represents cellulose content of lignocellulosic biomass whereas, 90% of hemicellulose represents xylan content. Total glucan and xylan contents were determined using the following equations:

$$\text{Total glucan} = \text{Glucose} \times 1.11 \quad \text{(Eq. 2.6)}$$

$$\text{Total xylan} = \text{Xylose} \times 1.11 \quad \text{(Eq. 2.7)}$$

These estimations (section 2.2.1.1-1.2.1.5) can be termed as the proximate analyses of lignocellulosic biomass.

2.2.2 Elemental composition analysis of raw biomass (Ultimate analysis)

The elemental analyses (C, H, N, S) of the biomass were done according to the method proposed by Jimenez and Ladha 2008 [12]. Elements were estimated through an Elementar or elemental analyser (varioMICRO CHNS analyser). Helium was used as a carrier gas during the combustion by oxygen gas whereas combustion and reduction tube were operated at 1150°C and 850°C respectively. Composition of the elements were analysed through a thermal conductivity detector (TCD), which is set at 60°C. The dried lignocellulosic biomass samples were weighed in the range of 2-4 mg and packed in small tin boats loaded in the autosampler. Elematal compositions were recorded from the instruments software interface after combustion and reduction process in terms of % (w/w). Elemental oxygen (%, w/w) was obtained after substituting total CHNS value from 100.

2.2.3 Physical properties of the biomass

2.2.3.1 Bulk density

Bulk densities of the selected lignocellulosic feedstocks were determined according to the method described by Zhang Y. et al 2013 [13]. An empty beaker (500 mL) was weighed to the

nearest 0.01 g (W_1), then beaker was filled with the biomass and it was compacted slightly to ensure absence of any large void spaces. The container and the sample were weighed as W_2. The wet bulk density of the sample was calculated from equation 2.8.

$$\text{Bulk density, } \rho_b \text{ (g/cm}^3\text{)} = \frac{W_2 - W_1}{Volume\ of\ the\ sample\ (cm^3)} \qquad \text{Eq. 2.8}$$

2.2.3.2 Porosity

20 g of sample (W_1) was taken and transferred to a 1 L graduated cylinder. The volume of the sample was measured (V). The porosity of biomass was calculated from the following Eq. 2.9.

$$Porosity\ (\epsilon) = \frac{V(m^3) - \left[\frac{W_1(kg)}{Bulk\ density\ of\ the\ biomass(kg/m^3)}\right]}{\left[\frac{W_1(kg)}{Bulk\ density\ of\ the\ biomass\left(\frac{kg^3}{m}\right)}\right]} \qquad \text{Eq. 2.9}$$

2.2.3.3 Size distribution

Size distributions of the selected lignocellulosic feedstocks were determined using the sieve analysis technique described by Zhang Y. et al 2013 [13]. The particle size distribution was determined using five standard sieves (1.18-6.73 mm sieve size). Samples were shaken manually in each sieve for 10-15 min. The particles collected in each sieve were weighed and percent passing through each sieve is recorded.

2.2.4 Analytical methods

2.2.4.1 TRS estimation

TRS was estimated by DNS method proposed by Miller 1959 [10]. Dinitro salicylic acid (DNS) reagent is prepared as per the composition: DNS (10 g/L), Phenol (2g/L), sodium Sulphite (0.5 g/L), NaOH (10g/L), Rochelle's salt (40% w/v). DNS method is widely used for TRS estimation, as it is rapid, sensitive, and adoptable for handling for the large number of samples.

300 µL sample and 300 µL deionised water were taken in the test tube. 600 µL of DNS reagent is added in it along with 200 µL of Rochelle's salt. The mixture was kept in boiling water bath for 5 min and cooled down to room temperature. It was then diluted 10 times before recording the absorbance at 540 nm (eppendorf, BioSpectrometer). The calibration curve for TRS is shown in Fig. 2.1 using glucose as a standard compound.

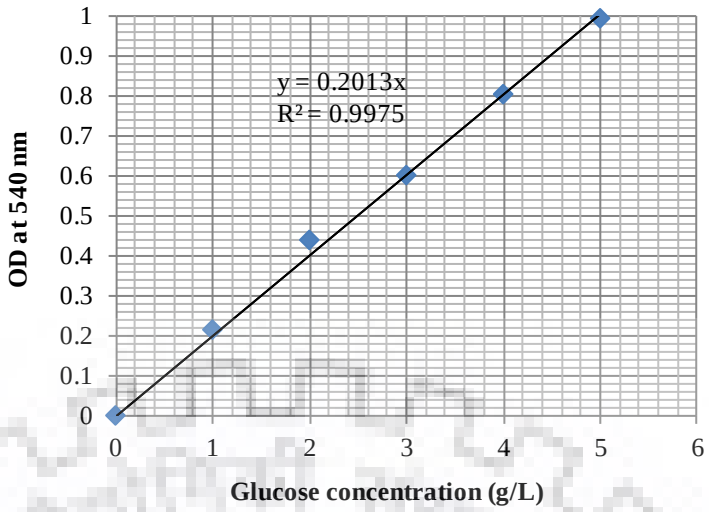


Fig. 2.1. Calibration curve of glucose for TRS estimation.

2.2.4.2. Xylose estimation

Xylose estimation was done by phloroglucinol method proposed by Eberts et al. 1979 [11]. Phloroglucinol reagent was prepared as per the composition (Glacial acetic acid: 100 mL, HCl: 10 mL, phloroglucinol: 0.5 g). 80 µl of sample was taken in the test tubes, and 2 mL of phloroglucinol reagent wass added to it. Mixture was kept in the boiling water bath for 4 min. After cooling it down to the room temperature, absorbance was measured at 540 nm against the blank. A calibration curve was prepared for further calculations (Fig. 2.2).

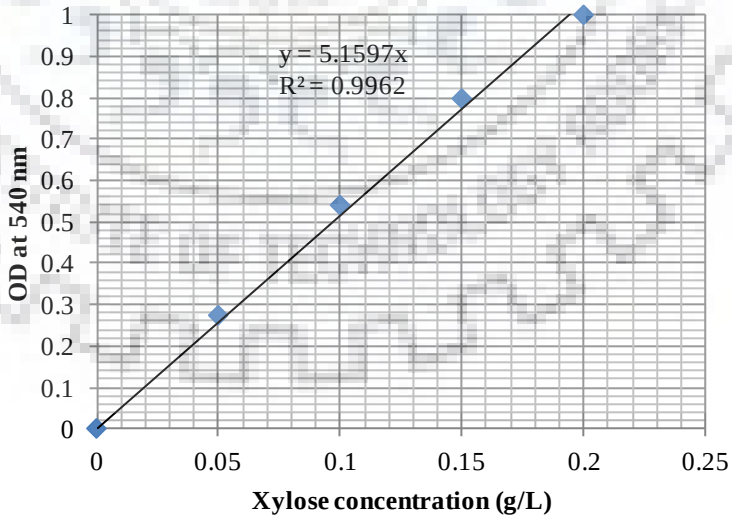


Fig. 2.2. Calibration curve for xylose estimation.

2.2.4.3 Furfural estimation

Furfural mainly derives from the degradation of xylose during hydrolysis and needs to be estimated due to its toxicity to the fermentation process. The estimation was done according to the method given by Al-Showiman 1998 [14]. 1 mL of sample was taken in a test tube, 1 mL of 90% aniline and 0.25 mL of 37% HCl were added to it. Volume was made up to 5 mL and kept in the dark for 15 min at room temperature. The absorbance was measured at 540 nm against the blank. A calibration curve was prepared for the further calculations (Fig. 2.3).

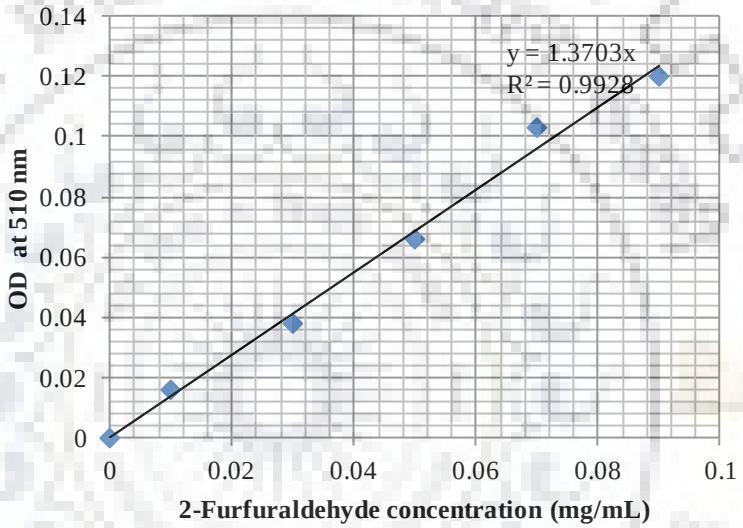


Fig. 2.3. Calibration curve for furfural estimation.

2.2.4.4 Total phenolics estimation

Total phenolics estimation was done using modified Folin-Ciocalteu method [15]. 0.5 mL sample, 0.5 mL Folin-Ciocalteu's reagent, 5 mL distilled water, and 1.5 mL of 20% sodium carbonate were added and mixed well in a test tube. Volume was made up to 10 mL and kept for 2 h of incubation at room temperature; absorbance was recorded at 760 nm against the blank. A calibration curve was prepared using vanillin as a standard compound for total phenolics estimation as shown in Fig. 2.4.

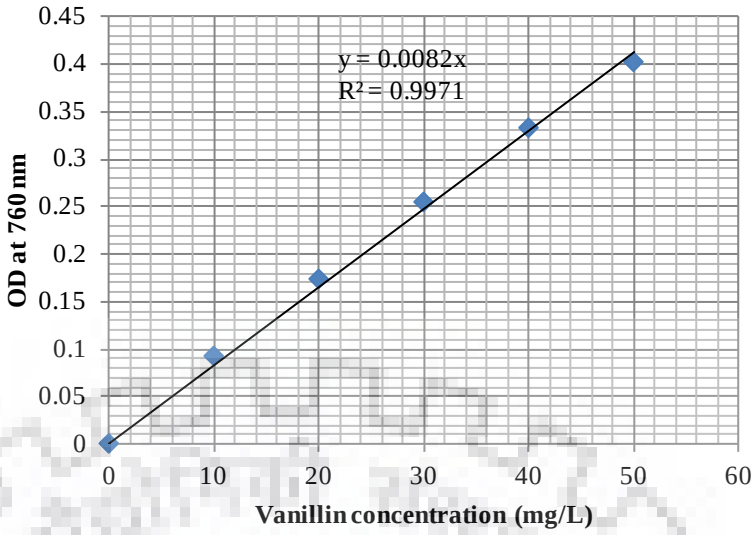


Fig. 2.4. Calibration curve for total phenolics estimation.

2.2.4.5 Ethanol estimation

Analysis of ethanol was done by using Gas Chromatography using flame ionisation detector and solgel wax caplillary column (Dani, Master GC). Samples were filtered using membrane filter (pore size of 0.2 µm) prior to estimation. 2 µL of the sample was injected to the injector manually at an injector temperature of 210°C, detector temperature 210°C, and oven temperature of 150°C. Nitrogen gas was used as a carrier gas at 1 mL/min along with hydrogen gas at 30 mL/min and zero air at 300 mL/min. Further calculations were done from the calibration curve (Fig. 2.5).

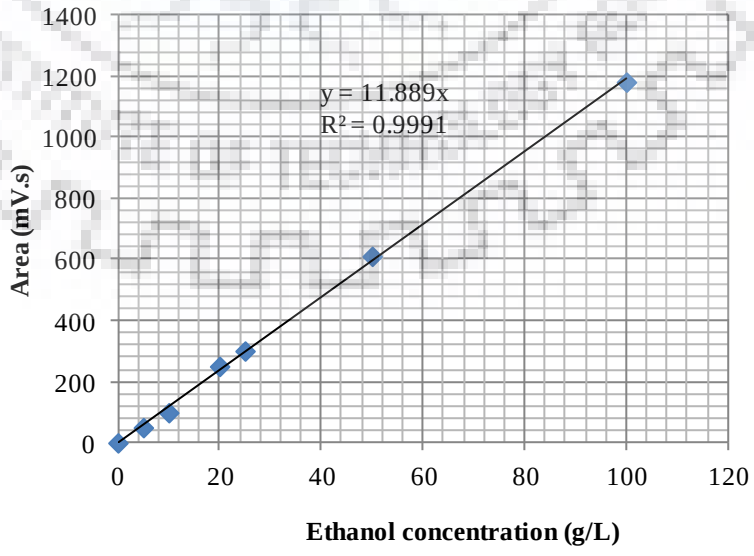


Fig. 2.5. Calibration curve for ethanol estimation.

2.2.4.6 Biomass estimation

2.2.4.6.1 *S. Shehatae*

Microbial biomass growth estimation of *S. shehatae* was done turbidometrically by recording absorbance at 600 nm. The fermentation broth samples collected were diluted in the ratio of 1:5 with 1N HCl to dissolve the calcium salts attached to yeast biomass. It was then centrifuged at 5000 rpm (SCILOGEX centrifuge, D2012) for 10 min. Pellets were re-suspended in deionised water, and absorbance was recorded. For dry weight calculations, pellet obtained was dried and weighed until the constant weight was obtained [16]. A calibration curve was prepared using OD vs. Biomass dry weight for the cell mass estimation (Fig. 2.6).

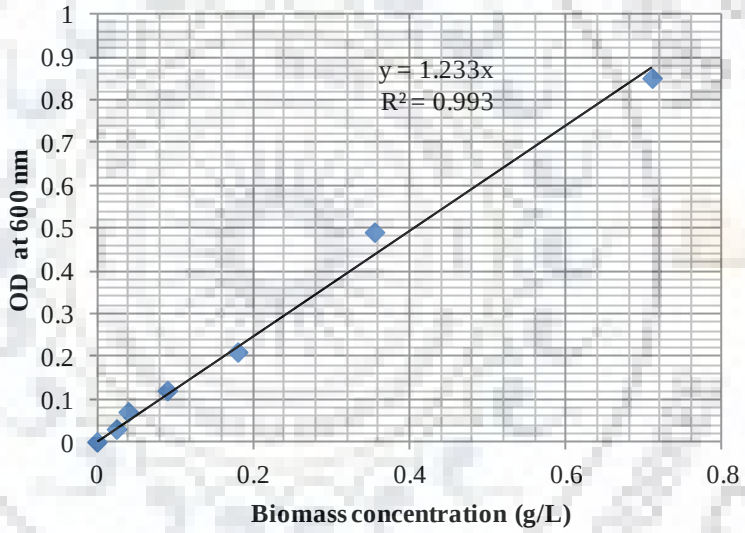


Fig. 2.6. Calibration curve for biomass estimation of *S. shehatae*.

2.2.4.6.2 *Z. mobilis*

The fermentation broth samples collected were centrifuged at 5000 rpm for 10 min. Pellets were re-suspended in minimum amount of deionised water and absorbance was recorded at 600 nm. For dry weight calculations, pellet obtained was dried and weighed until the constant weight was obtained [16]. A calibration curve was prepared using OD vs. Biomass dry weight for the cell mass estimation (Fig. 2.7).

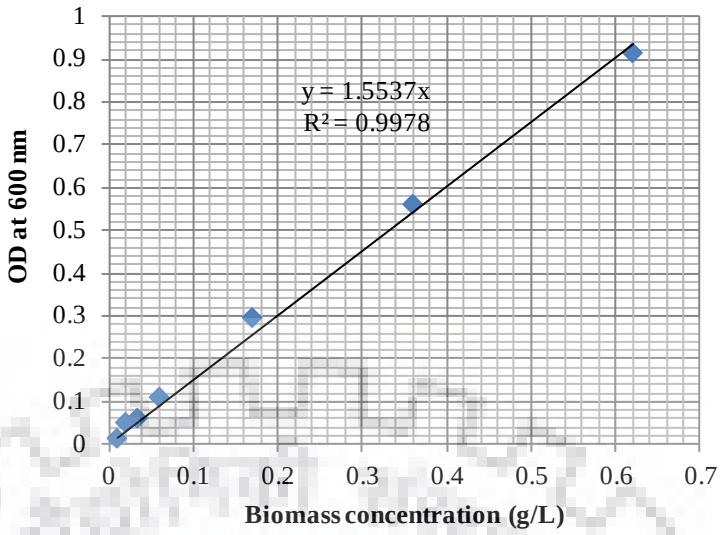


Fig. 2.7. Calibration curve for biomass estimation of *Z. mobilis*.

2.2.5 Structural analysis of the biomass

2.2.5.1 Scanning Electron Microscopy (SEM)

The changes in structural architecture of the biomass, after removal of each fraction in comparison to raw substrate were studied by the SEM images. Dried samples were coated with an adhesive containing gold using a vacuum sputter-coater, mounted on a metallic stub to improve conductivity of the samples and viewed under the machine (LEO 435 VP).

2.2.5.2 Field emission scanning Electron Microscopy (FE-SEM)

The changes in chemical structure of biomass, after xylose-rich fraction (XRF) removal and fully treated biomass were studied using FE-SEM with high resolution images. Here also, dried samples were coated with an adhesive containing gold using a vacuum sputter-coater, mounted on metallic stub to improve conductivity of the samples and viewed under the machine (Carl Zeiss Ultra Plus). The machine had special Energy-dispersive X-ray spectroscopy (EDX) attached to study the texture analysis and approximate elemental composition of the biomass.

2.2.5.3 Scanning probe microscopy (SPM)

Scanning probe microscopy covers several related technologies for imaging and measuring surfaces on a fine scale, down to the level of molecules and groups of atoms. SPM technologies share the concept of scanning an extremely sharp tip (3-50 nm radius of curvature) across the object surface. The tip is mounted on a flexible cantilever, allowing the tip to follow the surface

profile. SPM (INTEGRA, Model-MD-MDT- INTEGRA) was used to study the variations in average roughness of the surface of biomass with 3-D imaging after xylose-rich fraction (XRF) removal and fully treated biomass along with untreated biomass.

2.2.5.4 X-Ray Diffraction (XRD)

The alteration in degree of crystalinity of the biomass after XRF removal and complete treatment was determined through XRD (Bruker, D8-Advance) studies using CoKα radiation (α = 0.179 nm) at 30 mA and 40 kV. The samples were scanned in the range of $2\theta = 5^o$ to 90^o with a speed of 1^o/min and the percentage crystallinity was calculated from equation 2.10 [17].

$$\text{Crystallinity Index (\%)} = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \qquad \text{Eq. 2.10}$$

For cellulose:

I_{002} = Maximum peak intensity (2θ) between 22^o and 23^o

I_{am} = Minimum peak intensity (2θ) between 18^o and 19^o

2.2.5.5 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is an established tool for the structural characterisation. The changes in peak intensities after XRF removal, and complete hydrolysis of all the feedstocks were studied by FTIR by using KBr pellet technique in the spectral range of 450-4050 cm^{-1} with a resolution of 0.5 cm^{-1} [18]. In case of a liquid sample, a drop of the sample was placed onto a KBr pellet, air-dried and then analysed by FTIR.

2.2.5.6 Thermogravimetric analysis (TGA)

Lignocellulosic biomass can be characterised to study their thermal conversion behaviour. Valuable information such as ignition temperature, burn-out temperature, peak temperatures and fixed residues (%) can be obtained from TGA curve. It also gives useful data on specific temperatures where various heterogeneous reactions occur throughout the pyrolysis of biomass during TGA. In the present study, changes in thermal conversion behaviour of all the biomasses after XRF removal and complete hydrolysis were studied using TGA (EXSTAR, SII 6300 EXSTAR). The maximum temperature was 1000^oC and nitrogen gas was used as a carrier gas at 10^oC flow rate.

2.2.5.7 Inductively coupled plasma mass spectrometry (ICP-MS)

Heavy metals estimation in the hydrolysate fractions (XRF and GRF) was done using the ICP-MS (Perkin Elmer, ELAN DRC-e). The hydrolysate fractions obtained were nitrified using 1% HNO_3 and membrane filtered (0.22 µm membrane) prior to the analysis. The filtered hyrrolysate used in ICP-MS for heavy metals and micronutrient estimation at ppb level.

2.2.5.8 Calorific value estimation

The Energy density of the biomass was measured by using standard bomb calorimeter (MAC, MCW-506, New Delhi, India). The solid biomass was dried overnight at 105^{o}C to remove the moisture content and then compressed to form small pellets. The weight of the biomass was taken before being compressed to pellet. The energy density of the biomass was determined based on the temperature difference in the presence of excess pressure (400 psi) and oxygen. The energy density of the samples is calculated according to the Eq. 2.11.

$$\text{Calorific value } \left(\frac{\text{MJ}}{\text{kg}}\right) = \frac{\text{W}\left(\frac{\text{KJ}}{^{o}\text{C}}\right) \times (T_2\text{-}T_1)(^{o}\text{C})}{\text{M (g)}} \qquad \text{Eq. 2.11}$$

W= Water equivalent of calorimeter (9.748 kJ/^{o}C or 2330 Cal/^{o}C)
M= Mass of the sample, and
(T_2-T_1)= Rise in the temperature

2.2.5.9 Surface area analysis

Biomass porosity of the samples was analysed through Quantachrome Instruments pore and surface area analyser. The biomass samples were vacuum degassed for 3 h at room temperature prior to analysis. The degassed biomass samples were then kept in the multipoint Brunauer-Emmett-Teller (BET) analyser for analysis. Surface area of the pores was calculated according to the formula given in Eq. 2.12. Obtained adsorption and desorption isotherms were used for pore volume and pore size calculation using Quantachrome NovaWin software.

$$\text{Surface area } (m^2/g) = \frac{1}{(W((Po/P)-1)} \qquad \text{Eq. 2.12}$$

Where W = Sample weight

Po = Equilibrium pressure on the planar surface

P = Equilibrium pressure in pores with radius r

2.3 Processes

2.3.1 Characterisation of feedstocks

All the three feedstocks selected for the present study were characterised using the aforementioned method for proximate analysis (cellulose, hemicellulose, lignin, ash, and moisture). The ultimate elemental analysis was done to know the C, H, N, S, O elements composition.

2.3.2 Development of fractional hydrolysis technique for the saccharification of lignocellulosic biomass

A novel acid-based fractional hydrolysis technique was developed for the saccharification to obtain soluble sugars (glucose and xylose) separately, direct from the lignocellulosic biomass.

2.3.2.1 Experimental setup

A glass (Borosil) column was designed of 700 mm height with 70 mm internal diameter for the treatment of dry biomass up to 50 g. The column was provided with a narrow mouth at the top to load the biomass and acid inside. A glass rod of 25 mm internal diameter and 500 mm height was centrally inserted from the bottom of outer main column. The inner rode was provided with small holes of size 2 mm all around. It was closed at the top and open from bottom for steam insertion (at 100^{o}C) with a valve. The bottom opening was connected to the steam generator. One opening was provided at the side of the outer column's bottom with a valve for hydrolysate collection and other at the side of the top section for exhaust steam collection. The exhaust steam opening was attached to the condenser and cooling water unit for steam condensation. Cast iron stands were used to hold the fractional hydrolysis column and condenser. Fig. 2.8 shows the experimental setup of the fractional hydrolysis process for saccharification of lignocellulosic biomass.

2.3.2.2 Operation

All the parts of fractional hydrolysis unit were assembled according to the setup. Different concentrations of acid (up to 30%) were prepared. 50 g of dried lignocellulosic biomass was loaded in the column. The bottom steam insertion valve and top opening connected to the condenser were opened. Steam was inserted from the bottom of inner rod for preheating of biomass. After preheating, bottom valve was closed and desired acid concentration was added from the top mouth of the column. After the addition of acid, mouth was closed and bottom

valve was opened for steam insertion again. The process was repeated every time acid was added. Hydrolysates were collected at 30 min interval after each level of acid concentration addition. The liquid fractions collected were analysed for TRS, xylose, furfural and phenolics. Care should be taken while assembling the unit and during the opening and closing of valves; excess or low pressure may damage the column.

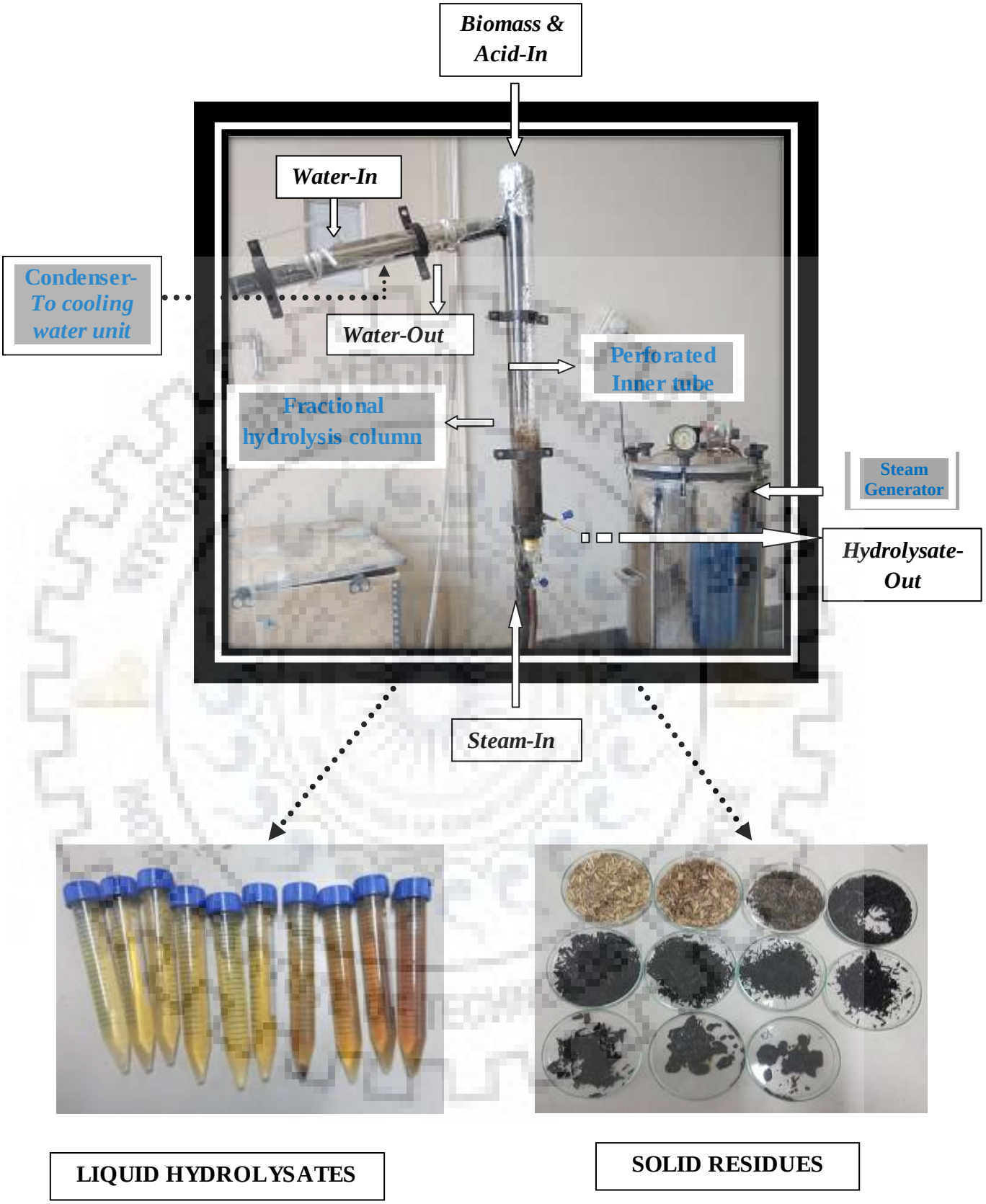


Fig. 2.8. Experimental setup of fractional hydrolysis technique for saccharification of lignocellulosic biomass.

2.3.2.3 Effect of the process variables on fractional hydrolysis

The one-factor at a time (OFAT) approach was used to study the effect of process variables on fractional hydrolysis process. 100^{o}C temperature and acid range (1-30% v/v) was maintained during all the experiments. Physical and chemical parameters which were studied for the maximum TRS release with minimum toxics are:

A. Preheating time: Preheating time was varied from 30-60 min.
B. Biomass loading (Liquid:Solid): Biomass loading was varied from 2-12%.
C. Number of stages: Number of stages was varied using the acid concentration up to 30% (v/v) to minimise the acid use for maximum saccharification and minimum toxics genaration.

For all the experiments, TRS was calculated in each fraction and added to find the saccharification (%). Results were compared to find the optimum condition.

2.3.2.4 Evaluation of various inorganic acids for the fractional hydrolysis process

Conventionally used strong inorganic acids for the acid hydrolysis of lignocellulosic biomass were evaluated for the fractional hydrolysis technique. In the present study, four different strong inorganic acids (HCl, H_3PO_4, HNO_3, and H_2SO_4) were tested in the 7- and 8-stage (number of stages were optimised previously) fractional hydrolysis process for maximum sugar recovery with minimum toxics. The process conditions were: 45 min preheating time, Biomass loading 10% and temperature 100^{o}C. An overview of the methodology followed is shown in Fig. 2.9.

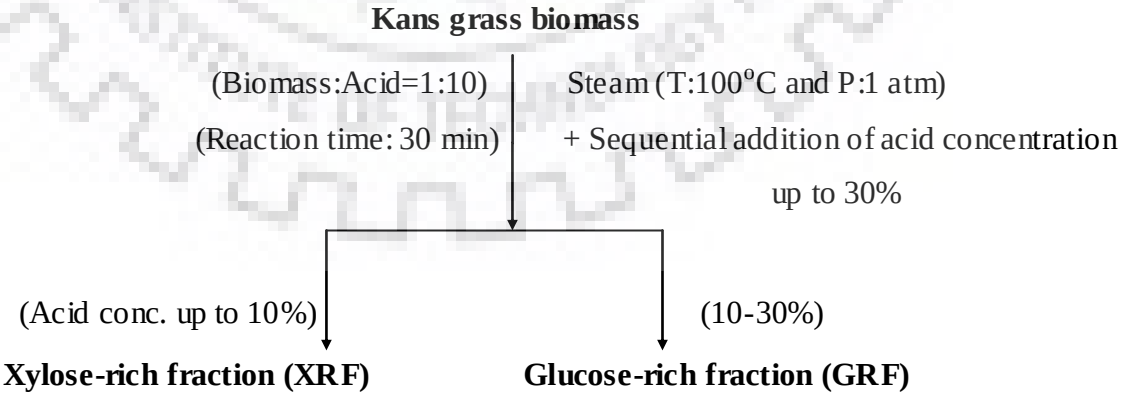


Fig. 2.9. Overview of the fractional hydrolysis process during exploration of different inorganic acids.

2.3.2.5 Evaluation of different lignocellulosic feedstocks for the fractional hydrolysis process

Three different lignocellulosic biomass is selected (kans grass, sugarcane bagasse, and wheat straw) for the study because of their geographically more even distribution and higher polysaccharide content compared to other feedstocks. All the feedstocks were evaluated using and 8-stage fractional hydrolysis process for maximum sugar recovery with minimum toxics. The process conditions were same as previous section. An overview of the methodology followed is represented in Fig. 2.10.

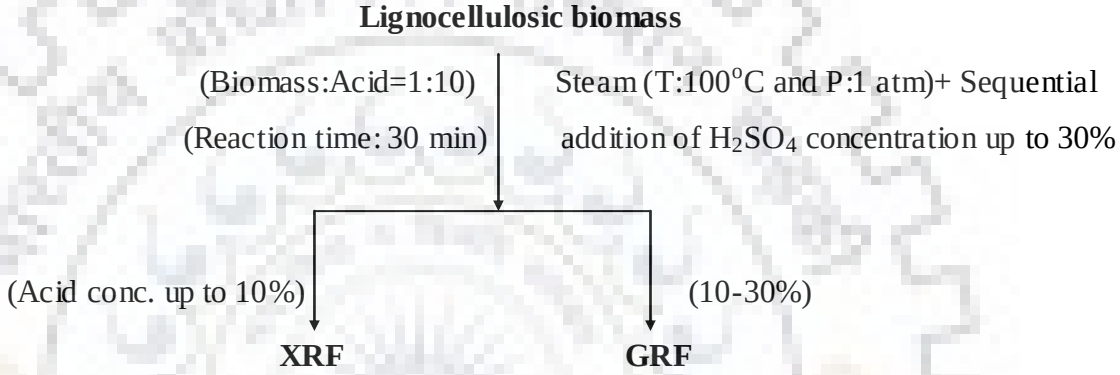


Fig. 2.10. Overview of the fractional hydrolysis process during exploration of different inorganic acids.

2.3.3 Conditioning of hydrolysate production medium

After analysis of the sugars and toxics in each fraction, first four fractions were mixed together as they were enriched with xylose sugar, called as XRF and last four fractions were called as GRF. The pH of both the fractions was raised to 2.0 with NaOH and then 6±0.5 using $Ca(OH)_2$. Both the fractions were filtered to remove the precipitate. Then fractions were concentrated by evaporation to achieve sugar concentration up to 60 g/L in XRF and up to 200 g/L in GRF. These sugar streams were supplemented with other nutrients for fermentation in later stage. XRF was supplemented with nutrients mentioned in section 2.1.3.2 whereas GRF was supplemented with nutrients mentioned in section 2.1.3.1. Synthetic medium was prepared to compare the kinetic parameters with same composition but without any toxics.

2.3.4 Mono-culture fermentation

2.3.4.1 XRF fermentation by *S. shehatae*

Batch fermentation of XRF was conducted using *S. shehatae* culture in 250 mL Erlenmeyer flask with a working volume of 50 mL. 10% v/v inoculum of 18 h old culture was used with 1×10^7 cells/mL during all the experiments. Fermentation temperature was kept 30°C with agitation of 150 rpm. Samples were collected at every 4 h interval to estimate residual TRS, ethanol and biomass in the medium. Experiments were conducted in duplicate.

2.3.4.2 GRF fermentation by *Z. mobilis*

Batch fermentation of GRF was conducted using *Z. mobilis* culture in 250 mL Erlenmeyer flask with a working volume of 50 mL. 10% v/v inoculum of 10 h old culture was used with 1×10^8 cells/mL during all the experiments. Fermentation temperature was kept 30°C without agitation. The mouth of the flask containing fermentation medium was closed with paraffin wax to provide anaerobic condition. Samples were collected at every 3 h interval to estimate residual TRS, ethanol and biomass in the medium. The experiments were conducted in duplicate.

2.3.5 Toxics effect on microbial growth during fermentation

2.3.5.1 Effect of furfurals on *S. shehatae* (NCIM 3501) growth

The effect of furfurals on the *S. shehatae* growth was estimated by adding 2-Furfuraldehyde (0.1-0.5 g/L) in the growth media of microbe using xylose sugar (20 g/L), while in the control flask, no furfural was added. The fermentation conditions were same as XRF fermentation (section 2.3.4.1). The specific growth rate was calculated from the slope of ln(x/xo) vs. Time during exponential growth phase. The relative specific growth (%) rate was calculated by considering specific growth rate of microbe in the control flask as 100, and then reduction in specific growth rate (%) was calculated by subtracting it from 100.

2.3.5.2 Effect of phenolics on *S. shehatae* growth

The effect of phenolics on the *S. shehatae* growth was estimated by adding vanillin (0.1-2.0 g/L) in the growth medium of microbe using xylose sugar (20 g/L), while in the control flask, no vanillin was added. The fermentation conditions were same as XRF fermentation (section 2.3.4.1). The specific growth rate calculations were same as above (section 2.3.5.1).

2.3.5.3 Effect of 5-HMF on *Z. mobilis* (MTCC 91) growth

The effect of 5-HMF on the *Z. mobilis* growth was estimated by adding 0.1-2.0 g/L HMF in the growth medium of microbe using glucose sugar (20 g/L), while in the control flask, no 5-HMF was added. The fermentation conditions were same as GRF fermentation (section 2.3.4.2). The specific growth rate calculations were same (section 2.3.5.1).

2.3.5.4 Effect of phenolics on *Z. mobilis* growth

The effect of phenolics on the *Z. mobilis* growth was estimated by adding vanillin (0.1-2.0 g/L) in the growth medium of microbe using glucose sugar (20 g/L), while in the control flask, no vanillin was added. The fermentation conditions were same as GRF fermentation (section 2.3.4.2). The specific growth rate calculations were same (section 2.3.5.1).

2.3.6 Effect of ethanol concentration/ethanol tolerance limit of microbes

2.3.6.1 Ethanol tolerance limit of *Z. mobilis*

The effect of ethanol concentration on the *Z. mobilis* growth was estimated by adding exogenous ethanol (2-12%) in the fermentation medium of microbe using glucose sugar (60 g/L), while in the control flask, no ethanol was added. The fermentation conditions were same as GRF fermentation (section 2.3.4.2). Samples were collected at every 3 h interval to estimate residual TRS, ethanol, and biomass in the media. Experiments were conducted in duplicate.

2.3.6.2 Ethanol tolerance limit of *S. shehatae*

The effect of ethanol concentration on the *S. shehatae* growth was estimated by adding exogenous ethanol (1-8%) in the fermentation medium of microbe using xylose sugar (60 g/L), while in the control flask, no ethanol was added. The fermentation conditions were same as XRF fermentation (section 2.3.4.2). Samples were collected at every 4 h interval to estimate residual TRS, ethanol, and biomass in the media. The experiments were conducted in duplicate.

2.3.7 Co-culture fermentation using *Zymomonas mobilis* and *Scheffersomyces shehatae*

2.3.7.1 Two-step sequential co-culture system

Fractional hydrolysis generated two different sugar streams i.e. XRF and GRF. Therefore, for maximum utilisation of both the xylose and glucose sugars, co-culture systems were developed. The two step process strategy was conducted in a bioreactor (eppendorf BioFlo 320 bioreactor, capacity 3 L) with fermentation media containing kans grass biomass hydrolysate. In the 1st

stage, the fermentation medium (except XRF) was sterilised in a bioreactor whereas XRF solution was sterilised separately at 121°C for 20 min in an autoclave. XRF was added to the bioreactor before inoculum addition. The first step fermentation was carried out at 150 rpm of agitation and 0.05 vvm aeration. The process was carried out till the exhaustion of xylose sugar. 2nd stage was initiated by stopping the agitator and sparging the N_2 gas for 2 h after the addition of GRF medium and *Z. mobilis* inoculum (10%, v/v) to the bioreactor. The samples were drawn at a regular interval (8 h for stage 1 and 4 h for stage 2) for biomass, residual TRS (g/L) and ethanol production (g/L) analyses. Agitator was set on at 250 rpm for few minutes during stage 2 before taking samples to homogenise the broth contents. This co-culture strategy was conducted initially at low sugar concentration (20 g/L TRS in XRF+40 g/L TRS in GRF) and later at higher concentration (60 g/L TRS in XRF+200 g/L TRS in GRF). To compare the results, all the experiments were conducted using synthetic xylose and glucose sugars in place of XRF and GRF.

2.3.7.2 Multi-step successive glucose feeding co-culture system

Entire fermentation process was carried out in a single reactor with sequential addition of microbes and their fermentation media. XRF fermentation was initiated prior using *S. shehatae* (step 1) due to its low ethanol tolerance and catabolite repression by GRF. 2nd stage was initiated by stopping the agitator and sparging the N_2 gas for 2 h after the addition of GRF medium and *Z. mobilis* inoculum (10%, v/v) to the bioreactor. The *Z. mobilis* culture was introduced only after the consumption of xylose sugar by the microbe. From stage 2-5, step feeding of glucose (up to 200 g/L) was done instead of adding 200 g/L TRS in a single step (section 2.3.7.1). The schematic representation for multi-step successive glucose feeding co-culture system is shown in Fig. 2.11.

Fermentation conditions were kept same (section 2.3.7.1) during both XRF and GRF fermentation. In step 2, GRF containing 100 g/L of TRS was introduced to the bioreactor and process was carried out till the exhaustion of sugars. From stage 3 onwards, glucose feeding was done at shorter time interval by excluding the growth lag phase of *Z. mobilis* occurred during stage 2. GRFs with 100, 150, 200 g/L of TRS were added aseptically during stage 2,3, and 4 respectively and TRS level ~50 g/L was maintained at the onset of each stage (stage 2-4). An aliquot of the sample was withdrawn before and after the glucose feeding at each stage to estimate the residual TRS, biomass, and ethanol production. To compare the results, all the experiments were conducted using synthetic xylose and glucose sugars in place of XRF and

GRF. This co-culture strategy was conducted initially at flask (with a total working volume of 250 mL) and bioreactor level using synthetic medium, and later in the kans grass hydrolysate.

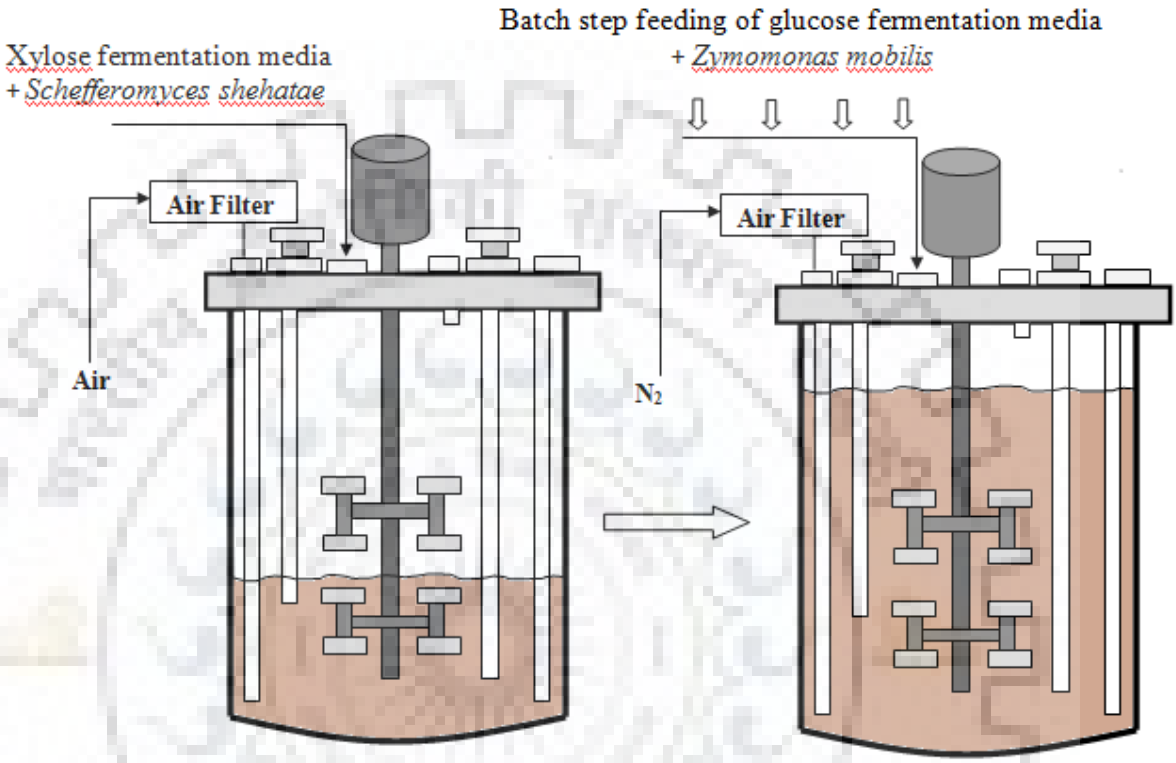


Fig. 2.11. Schematic representation of adopted multi-step successive glucose feeding co-culture system.

CHAPTER 3

Development of fractional hydrolysis technique

3.1 Introduction

The inevitable demand of excess energy due to diminishing resources, environmental problems, and energy security has resulted in the requirement for improvements in the existing renewable energy technologies. Biofuels have the potential to become as one of the alternatives to meet this demand and reduce the greenhouse gas emissions. The foremost thought behind the biofuel production is to utilise the unlocked biomass energy along with various biochemicals generation where biomass stands for an abundant carbon-neutral source. The exploitation of various lignocellulosic feedstocks would be helpful in addressing various social needs. The 2G ethanol production has gained huge interest over the last decade [1-2] that involves three major steps (biomass delignification or pretreatment, saccharification or hydrolysis, and fermentation), either separately or combined.

Saccharification of lignocellulosic biomass is a complicated process. Both cellulose and hemicellulose present in the biomass need to be hydrolysed for commercial ethanol production, which can be done using an acid or enzyme. Generally, the enzymatic hydrolysis produces hydrolysate containing less inhibitory compounds than the acid hydrolysate. But, low specific activity, the requirement of various saccharification steps, and relatively slower hydrolysis rate of enzymatic process make the acid hydrolysis more commercial [3-5]. Concentrated acid-catalysed hydrolysis is one of the most frequently used methods due to its competitiveness, effectiveness for a broad range of biomass, and moderate temperature requirement. Usually, in the acid hydrolysis, lignocellulosic feedstocks are treated initially at milder conditions with dilute acid for pretreatment (hemicelluloses solubilisation and breakdown), and then with concentrated acid for complete saccharification. This process usually involves long total hydrolysis time and toxics concentration in the hydrolysate are quite high which necessitates the need of an additional detoxification step [4,6].

In the present work, a unique technique called as 'fractional hydrolysis' has been developed using sulphuric acid, that gives us pentose and hexose sugars as separate hydrolysate fractions. As there is no known naturally occurring microorganism that can ferment pentose and hexose sugars simultaneously with the same efficiency, hence getting the separate xylose-rich fraction (XRF) and glucose-rich fraction (GRF) is of tremendous advantage. Moreover, this technique merges two different steps (pretreatment and hydrolysis) into one. Also, the concentration of toxic compounds in hydrolysate was very low; therefore, after saccharification, hydrolysate

fractions were taken directly for fermentation without any detoxification, thereby cutting down the overall production cost.

3.2 Results and Discussion

3.2.1 Biochemical characterisation of lignocellulosic feedstocks

The biochemical compositional analysis is a prerequisite for any lignocellulosic feedstock biomass to term as a potential lignocellulosic substrate in terms of total carbohydrate content. Biomass composition is the key factor which decides its potential to be served as a feedstock for biofuels production. The biochemical composition illustrated that selected feedstocks are rich in cellulose and hemicellulose, which makes them the suitable candidates for bioethanol production. Along with initial carbohydrate analysis, lignin, moisture and ash content were also estimated. Cellulose, hemicellulose, and lignin content of all the investigated feedstocks were in close proximity with reported in the literature [7-10]. Differences in seasonal, geographical variations and analytical methods used for analysis may be responsible for the variations.

Kans grass (Saccharum spontaneum) biomass is known as one of the cultivar of switchgrass. Cellulose, the major component of lignocellulosics, was found maximum (44% of dry wt.) in the kans grass biomass. Cellulose, hemicellulose, and lignin content reported in the present study was little different than some of the previous studies [7,11]; may be due to the growth phase of the biomasses at the time of harvesting, seasonal, and geographical variation. The total solid content of the kans grass was found to be 94.0 % (w/w) with 6% (w/w) moisture content. Total carbohydrate content (%, w/w) were calculated as: kans grass 65.5, bagasse 64.17, and wheat straw 65.97. Total lignin content (%, w/w) were: kans grass 28.49, bagasse 27.18, and wheat straw 27.6. The total carbohydrate content and thus available reducing sugars for conversion to ethanol in kans grass biomass was higher than other reported grass which makes it a potential resource for ethanol production. The holocellulosic content (cellulose and hemicellulose) was found to be maximum (65.97%) in wheat straw although it is comparable in all three selected feedstocks (63-66%) for the present study (Table 3.1). Lignin content of the selected feedstocks were comparable to the acid-insoluble lignin content of hardwoods (18-25%) rather than that of herbaceous species and agricultural residues (10-20%) [12]. However, in all the three feedstocks, holocellulosic content was very high. The results for proximate analysis of feedstocks are summarised in Table 3.1.

Table 3.1. Proximate analysis of feedstocks.

	Kans grass	Bagasse	Wheat straw
Total solids (% w/w)	94.0	91.28	93.57
Cellulose (% w/w)	44.0	37.8	40.57
Hemicellulose (% w/w)	21.5	26.37	25.4
Acid-insoluble lignin (% w/w)	26.37	24.08	25.2
Acid-soluble lignin (% w/w)	2.12	3.1	2.4
Ash (% w/w)	3.35	4.3	6.5
Moisture (% w/w)	6.0	8.72	6.43

The ultimate analysis of lignocellulosic biomass reveals it's potential to be serving as a robust feedstock for ethanol production. In fact, it is one of the major criteria from which one can select the feedstock for biofuel generation. The ultimate analysis provides information about the relative abundance of the individual elements present in feedstocks. The Ultimate analysis or elemental compositional analysis (Table 3.2) indicates the degree of cross linking and occurrence of high molecular weight compounds in the feedstocks. During saccharification, C-C and C-O bonds of lignin, which hold together the mono-lignols or lignin precursors of lignin molecule, are cleaved. The CHNS elemental analysis showed high carbon content in feedstocks (% w/w): kans grass 40.03, bagasse 39.91, and wheat straw 41.97. Hydrogen, nitrogen and sulphur contents were almost in a similar range in the feedstocks. Oxygen content was calculated after substituting the total value of CHNS (%) from 100. The results indicate the presence of the above elements in biomass with a high degree of cross linking (Nanda *et al.*, 2014). C/N ratio of the feedstock greatly influences anaerobic digestion; optimum ratio should be 25-30:1 [13]. From Table 3.2, it is quite evident that C/N ratio of kans grass biomass is optimum, which makes it most suitable among selected feedstocks for further hydrolysis experiments. Moreover, the density and porosity of the feedstocks were determined which are useful in hydrolysis reactor design (Table 3.2). Porosity ensures the biomass digestibility and its accessibility to hydrolytic agents.

Table 3.2. Proximate analysis and some physical properties of feedstocks.

Ultimate analysis			
	Kans grass	**Bagasse**	**Wheat straw**
C-content (%)	40.43	39.91	41.97
H-content (%)	5.79	5.34	5.58
O-content (%)	52.18	54.26	52.02
N-content (%)	1.44	0.28	0.31
S-content (%)	0.16	0.22	0.33
Physical properties			
Bulk density (kg/m^3)	280.2	239.9	421.7
Bulk porosity	2.33	4.0	4.66

3. 2.2 Size distribution of feedstocks

Size distribution analysis of the feedstocks was done by sieve analysis technique (1.18-6.73 mm). Mean size was calculated as 3.96 mm with a standard deviation of 2.78 mm for all three biomasses. Maximum percent of particle was distributed between 1.18-2.36 mm (kans grass: 29.24%, sugarcane bagasse: 30.61%, and in case of wheat straw it was 31.85%). The correlation between percent passing of grains used in fractional hydrolysis experiments and sieve size is shown in Fig. 3.1. Cumulative percent less than 5.6 mm size was 87.75 (bagasse), 76.43 (kans grass), and 68.41 (wheat straw) whereas for 4.75 mm it was 73.57, 60.43, and 50.41 respectively. Cumulative percent less than 2.36 mm size was 44.05 (bagasse), 44.67 (kans grass), and 33.89 (wheat straw) whereas for 1.18 mm it was 13.43, 15.27, and 2.04 respectively.

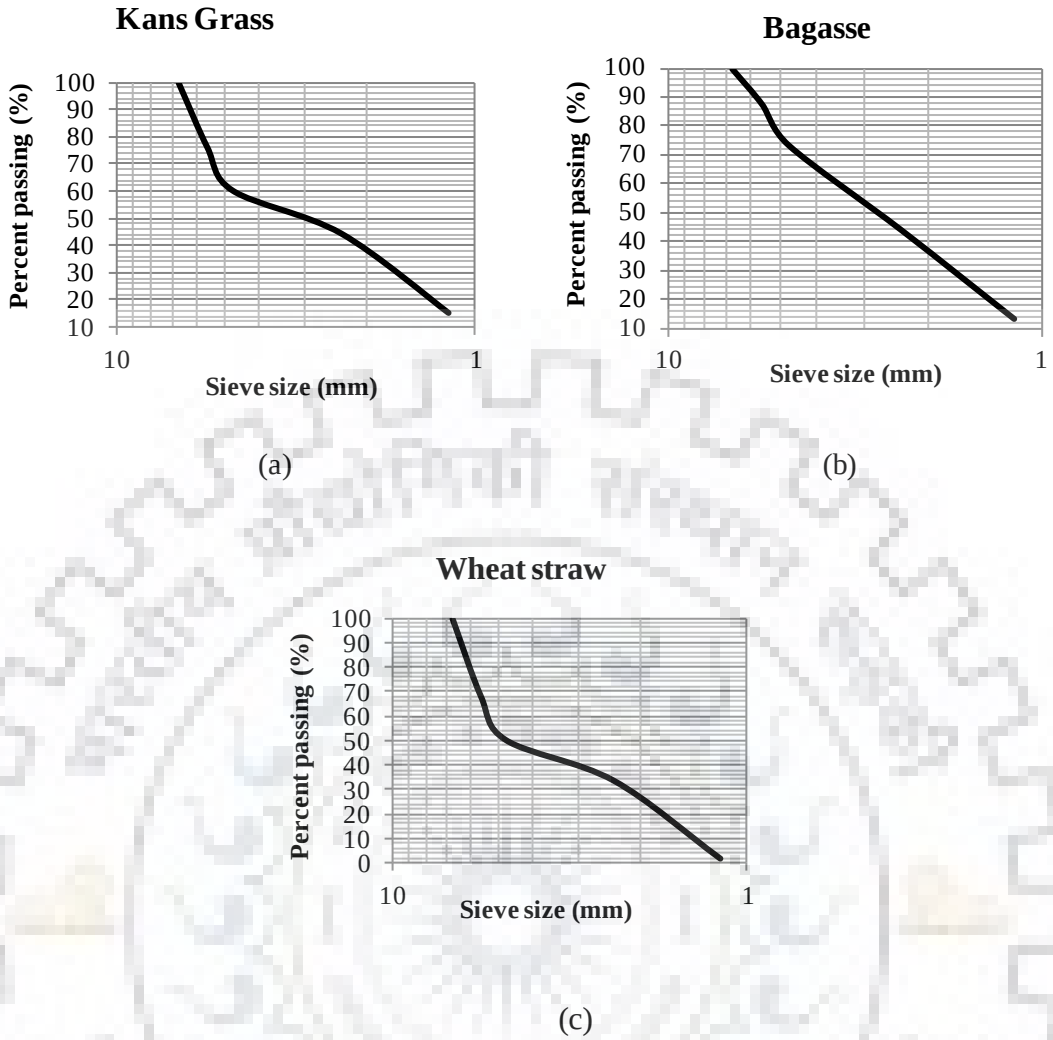


Fig. 3.1. Size distribution of lignocellulosic feedstocks used during fractional hydrolysis experiments (a) Kans grass (b) Sugarcane bagasse (c) Wheat straw.

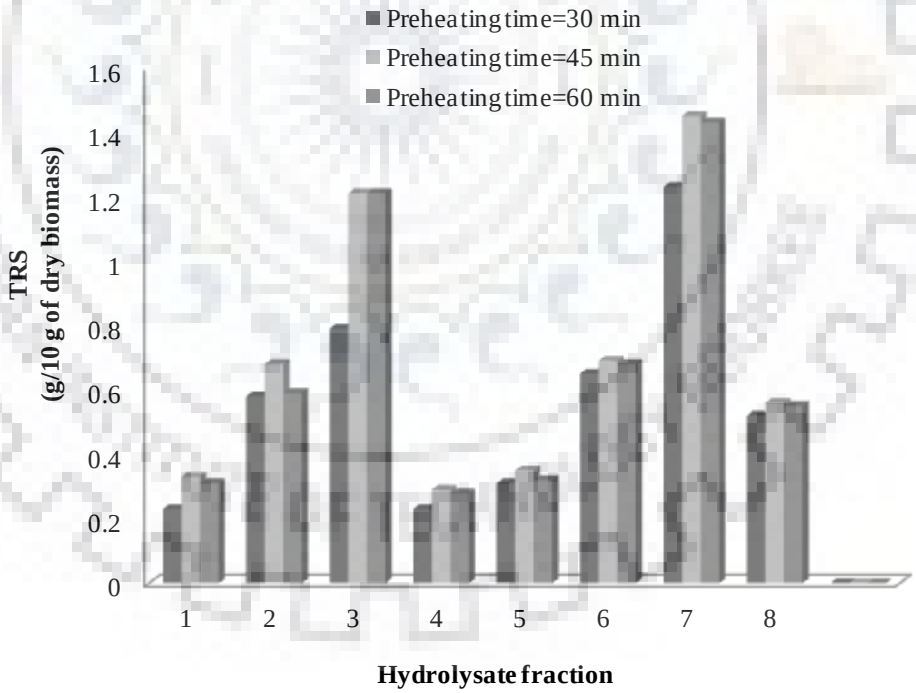
3.2.3 Fractional hydrolysis

The present study is focused mainly on the development of a suitable technique for maximum TRS recovery with minimum toxics generation from the lignocellulosic biomass. For this purpose, a new acid and steam-based "fractional hydrolysis" technique was developed. The technique was focused on getting hexose and pentose sugars separately, direct from the lignocellulosic biomass. The fractional hydrolysis process was developed using H_2SO_4 (concentration up to 30%) and kans grass biomass. One-variable-at a time (OVAT) approach was used to study the effect of various parameters on sugar recovery and toxics formation. Effect of preheating time, biomass loading, and number of stages (up to 30% acid concentration) were studied for the maximum TRS recovery with minimum toxics. Because of the complex structure of lignocellulosics, it requires some preheating before the addition of

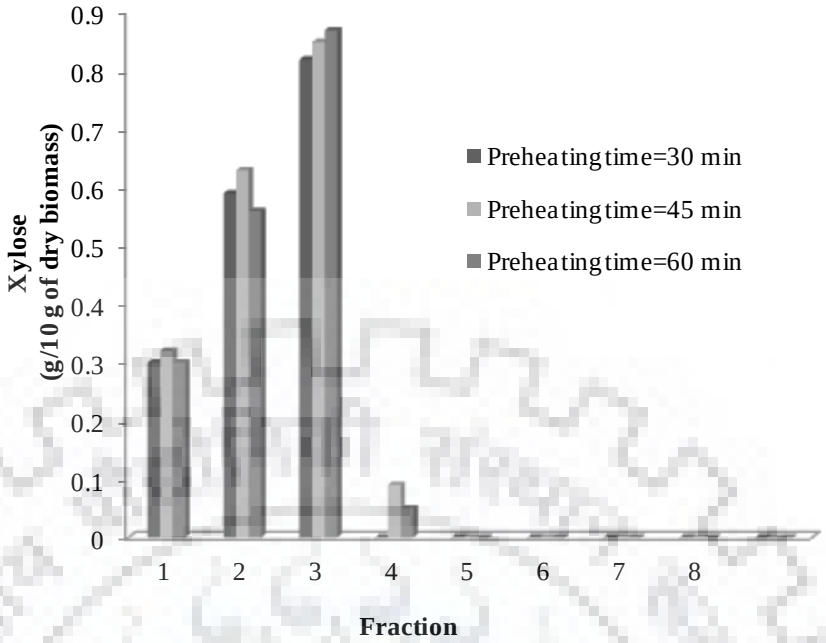
chemical reagent for saccharification. Therefore, the study of preheating time effect on fractional hydrolysis process was pre-requisite. Furthermore, biomass loading effect (Liquid:Solid) and different strategies within H_2SO_4 concentration range (1-30 %) were tried to minimise acid use and make the process more cost-effective. Hemicelluloses are lower molecular weight compounds compared to cellulose and have branches with short lateral chains, therefore, xylose-rich fractions was obtained initially upon fractional hydrolysis. Data on sugar recovery (TRS and xylose) and toxics (furfural and phenolics) formation were calculated per 10 g of dry kans grass biomass for the fractional hydrolysis experiments.

3.2.3.1 Effect of preheating time

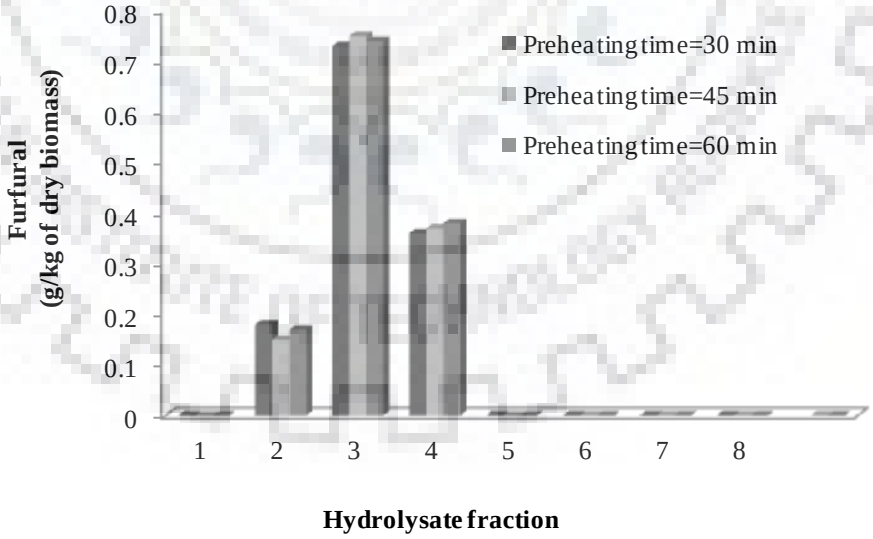
Preheating time was varied from 30-60 min for this study. Effect of preheating time on sugar recovery (TRS and xylose) and toxics generation (furfural and phenolics) in each hydrolysate fractions is shown in Fig. 3.2 (a-d). Recovered sugars and toxics generated are shown at each stage (1-8).



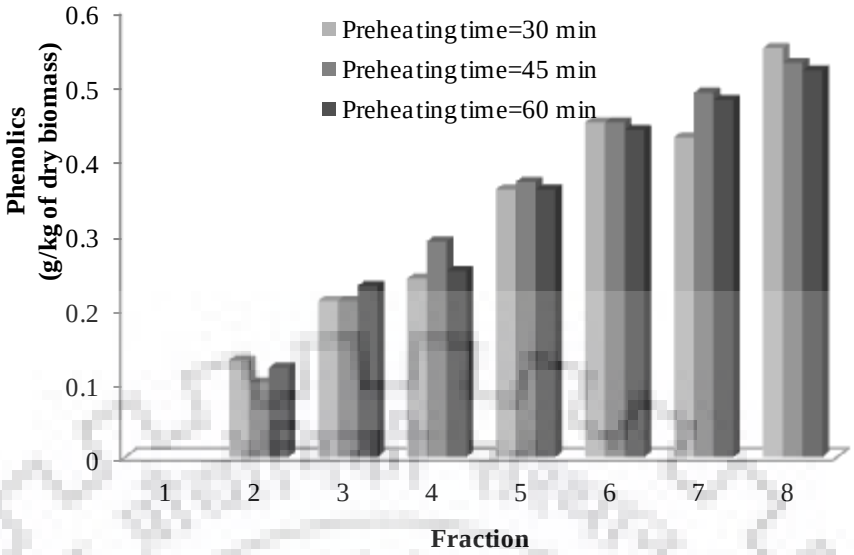
(a)


Xylose (g/10 g of dry biomass)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
Fraction
Preheating time=30 min
Preheating time=45 min
Preheating time=60 min

(b)


Furfural (g/kg of dry biomass)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
Hydrolysate fraction
Preheating time=30 min
Preheating time=45 min
Preheating time=60 min

(c)



(d)

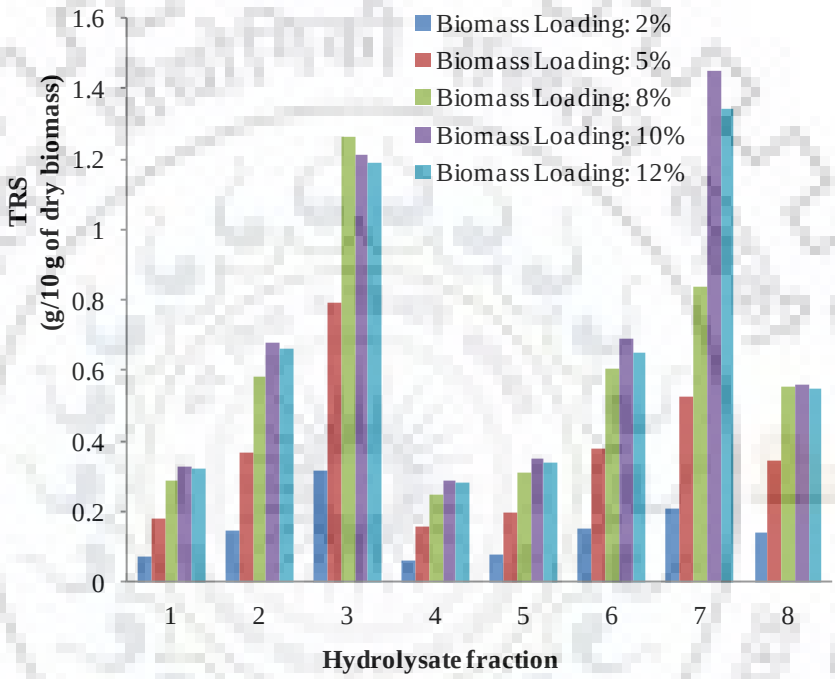
Fig. 3.2. Effect of preheating time on (a) TRS recovery (b) Xylose recovery (c) Furfural formation (d) Phenolic compounds formation in each hydrolysate fraction.

In the first set of experiments, preheating time was kept 30 min resulting in 4.54 g TRS and 1.72 g xylose sugar recovery. Saccharification (%) was calculated as 69.31 with 79.53% xylose recovery. Toxics (2.72×10^{-2} g furfural, 0.94×10^{-2} g phenolics in XRF and 2.1×10^{-2} g in GRF) were formed in minimal amount. In the second set of experiments, after 45 min of preheating, total 5.56 g TRS and 1.89 g xylose sugars were released. Saccharification (%) was 84.88 with 87.9% xylose recovery. Toxics (1.27×10^{-2} g furfural, 0.97×10^{-2} g phenolics in XRF and 2.08×10^{-2} g in GRF) were in negligible concentration. After 60 min of preheating time, 5.37 g TRS and 1.78 g xylose sugar were released. Saccharification (%) was 81.98 with increased toxics concentration.

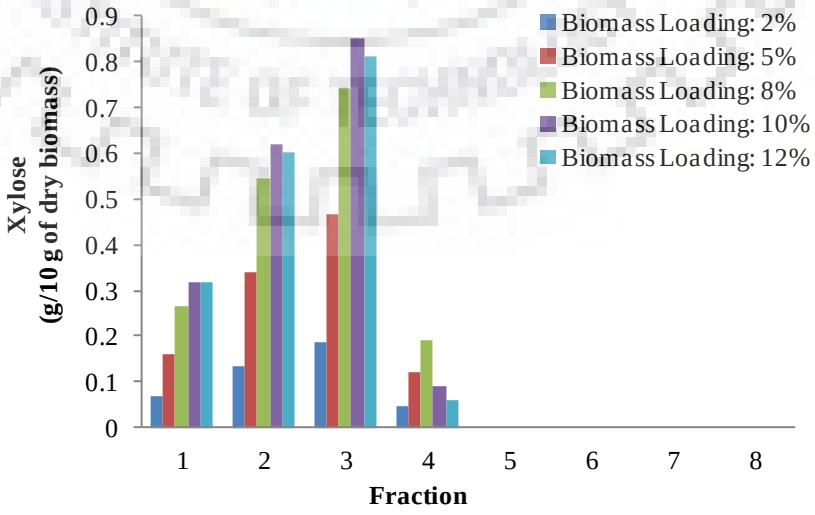
It has been concluded from the results that 30 min of preheating time was not sufficient to break the polymeric backbone of lignocellulosics upon fractional hydrolysis; therefore, TRS recovery was comparatively less. The maximum amount of sugars were recovered after 45 min of preheating, beyond which toxics generation increased significantly due to further conversion of released sugars into furfurals. Therefore 45 min of preheating was considered as the optimum time for maximum sugar recovery with minimum toxics.

3.2.3.2 Effect of biomass loading

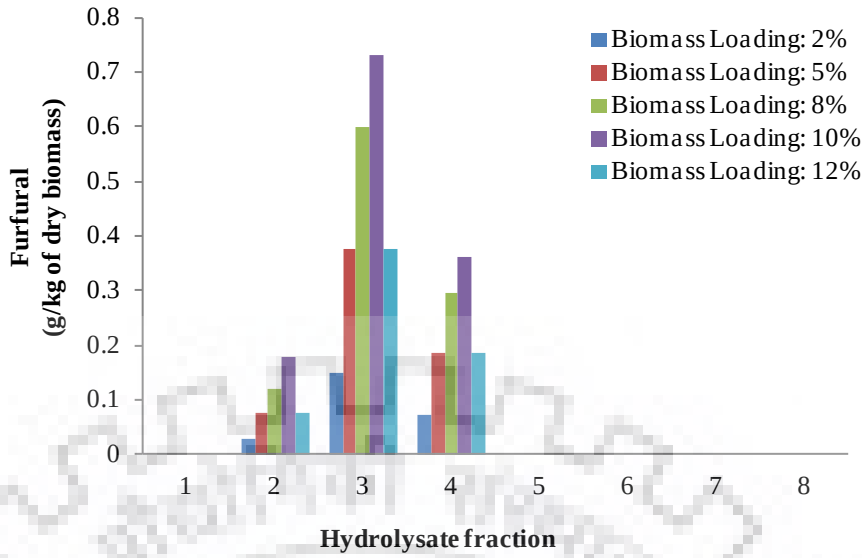
Biomass loading (%) can be expressed as the volume of diluted H_2SO_4 /dry kans grass biomass weight added during the fractional hydrolysis experiment. Different experiments were conducted by varying it from 2-12%, however, during a single experiment; biomass loading was kept same at each stage. Effect of biomass loading on sugar recovery (TRS and xylose) and toxics generation (furfural and phenolics) is shown in Fig. 3.3 (a-d). Sugars recovered and toxics generated are shown at each stage (1-8).



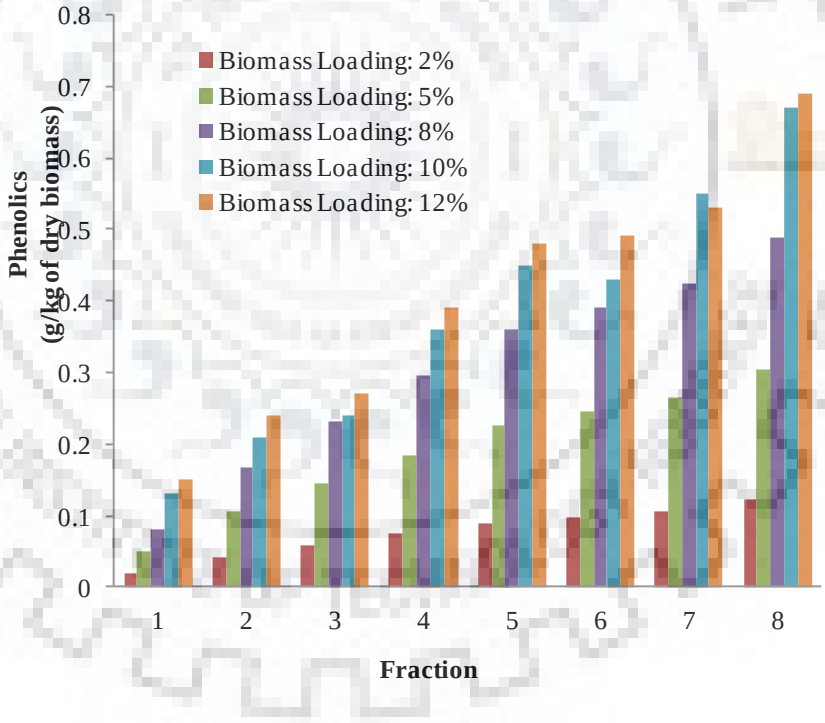
(a)



(b)



(c)



(d)

Fig. 3.3. Effect of Biomass loading on (a) TRS recovery (b) Xylose recovery (c) Furfural formation (d) Phenolic compounds formation in each hydrolysate fraction.

At 2% biomass loading, 1.25 g TRS and 0.44 g xylose sugars were released. Saccharification (%) was 19 with 20.31% xylose recovery. Toxics concentrations were estimated in terms of 0.25×10^{-2} g furfural and 0.76×10^{-2} g total phenolics content. At 5% biomass loading, 3.12 g TRS and 1.09 g xylose sugars were released. Saccharification (%) was 47.25 with 50.46% xylose recovery. Toxics concentrations were estimated in terms of 0.64×10^{-2} g furfural and 1.52×10^{-2} g total phenolics content. Saccharification (%) was observed to be increasing up to 10% biomass loading; beyond it, TRS and xylose recovery was less, and toxics concentration was higher. It was due to further conversion of glucose and xylose sugars into 5-Hydroxy methyl furfuraldehyde and 2-Furfuraldehyde respectively by H_2SO_4. Therefore 10% biomass loading has been considered as an optimum value with maximum sugar (5.56 g TRS and 1.88 g xylose sugar) recovery with minimum toxics. Saccharification (%) was 84.88 with 87.44% xylose recovery. A negligible amount of toxics were formed (1.27×10^{-2} g furfural and 3.75×10^{-2} g total phenolics) whereas, at 12% biomass loading, 5.33 g TRS and 1.79 g xylose sugars were released. Saccharification (%) was 81.37 with 83.25% xylose recovery and toxics compounds formed were in higher concentration (1.35×10^{-2} g furfural and 3.99×10^{-2} g phenolics).

3.2.3.3 Fractional hydrolysis experiments involving the different number of stages

Various strategies up to 30% H_2SO_4 concentration have been tried to reduce the number of stages for acid use minimisation in the process and make it more economic. Acid concentration was varied at each stage to find the minimum number of stages required for maximum TRS recovery with minimum toxics. The study was initiated with 5-stage fractional hydrolysis process (Table 3.3). After 5-stage fractional hydrolysis process, 4.46 g TRS and 1.17 g pentose sugars were released. Saccharification (%) was 68.09 with 54.41% xylose recovery. 1.62×10^{-2} g furfural and 2.83×10^{-2} g phenolics were estimated.

Table 3.3. 5-stage fractional hydrolysis summary.

Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)	Xylose (g/10 g of dry biomass)	Furfural (g/kg of dry biomass)	Phenolics (g/kg of dry biomass)
1	1	0.33	0.32	n.d.	0.13
2	5	1.11	0.73	0.78	0.35
3	12	0.65	0.12	0.84	0.53
4	20	1.29	n.d. (not detected)	n.d.	0.79
5	30	1.08	n.d.	n.d.	1.03

Results of 6-stage fractional hydrolysis process are presented in Table 3.4, where Saccharification (%) was 78.63 and xylose recovery was calculated as 50.7%. 1.05×10^{-2} g furfural and 3.27×10^{-2} g phenolics were generated during the process.

Table 3.4. 6-stage fractional hydrolysis summary.

Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)	Xylose (g/10 g of dry biomass)	Furfural (g/kg of dry biomass)	Phenolics (g/kg of dry biomass)
1	1	0.33	0.32	n.d.	0.14
2	3	0.74	0.69	0.27	0.28
3	10	1.04	0.08	0.78	0.44
4	17	0.96	n.d.	n.d.	0.65
5	24	0.84	n.d.	n.d.	0.86
6	30	1.24	n.d.	n.d.	0.9

Two different strategies were tried to develop for seven step fraction hydrolysis process i.e. 7A and 7B. Saccharification results of both the approaches are summarised in Table 3.5. Saccharification (%) was 79.69 with 66.04% xylose recovery in strategy A whereas in B, it was 81.52 and 85.58% respectively. 8-stage fractional hydrolysis process resulted in maximum TRS recovery (84.88%) with minimum toxics; results are shown in Table 3.6. 9- and 11-stage fractional hydrolysis processes were also carried out which resulted in a high concentration of toxics generation and can be observed from Table 3.7 and Table 3.8 respectively.

Table 3.5. Summary of two different approaches for 7-stage fractional hydrolysis.

S. no.	Acid conc. (%)		TRS (g/10 g of dry biomass)		Xylose (g/10 g of dry biomass)		Furfural (g/kg of dry biomass)		Phenolics (g/kg of dry biomass)	
	Strategy A	Strategy B	StrategyA	Strategy B	Strategy A	Strategy B	Strategy A	Strategy B	Strategy A	Strategy B
1	2	1	0.72	0.33	0.68	0.32	0.14	n.d.	0.2	0.1
2	7	2	1.09	0.68	0.62	0.62	0.69	0.16	0.31	0.22
3	12	5	0.35	1.21	0.12	0.85	0.25	0.78	0.4	0.31
4	17	12	0.85	0.36	n.d.	0.05	n.d.	0.54	0.55	0.63
5	22	19	0.87	0.75	n.d.	n.d.	n.d.	n.d.	0.67	0.85
6	27	26	0.73	0.94	n.d.	n.d.	n.d.	n.d.	0.85	0.91
7	30	30	0.61	1.07	n.d.	n.d.	n.d.	n.d.	0.98	0.97

Table 3.6. 8-stage fractional hydrolysis summary.

Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)	Xylose (g/10 g of dry biomass)	Furfural (g/kg of dry biomass)	Phenolics (g/kg of dry biomass)
1	1	0.33	0.32	n.d.	0.13
2	2	0.68	0.62	0.18	0.21
3	5	1.21	0.85	0.73	0.24
4	10	0.29	0.09	0.36	0.36
5	15	0.35	n.d.	n.d.	0.45
6	20	0.69	n.d.	n.d.	0.43
7	25	1.45	n.d.	n.d.	0.55
8	30	0.56	n.d.	n.d.	0.67

In 9-stage fractional hydrolysis process, 5.33 g TRS and 1.66 g pentose sugars were released. Saccharification (%) was 81.37 with 77.2% xylose recovery. 1.5×10^{-2} g furfural and 4.68×10^{-2} g phenolics were estimated. It has occurred due to further conversion of glucose and xylose sugars into 5-Hydroxy methyl furfuraldehyde and 2-Furfuraldehyde respectively by repeated use of concentrated acid during the entire process.

Table 3.7. 9-stage fractional hydrolysis summary.

Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)	Xylose (g/10 g of dry biomass)	Furfural (g/kg of dry biomass)	Phenolics (g/kg of dry biomass)
1	2	0.68	0.67	0.13	0.19
2	4	1.05	0.82	0.64	0.20
3	8	0.24	0.17	0.73	0.3
4	12	0.38	n.d.	n.d.	0.36
5	16	0.41	n.d.	n.d.	0.41
6	20	0.54	n.d.	n.d.	0.68
7	24	0.76	n.d.	n.d.	0.75
8	28	0.65	n.d.	n.d.	0.88
9	30	0.62	n.d.	n.d.	0.91

11-stage fractional hydrolysis process resulted in 5.36 g TRS and 1.86 g pentose sugar recovery. Saccharification (%) was calculated 81.83 with 1.35×10^{-2} g furfural and 5.4×10^{-2} g phenolic compounds as toxics.

Table 3.8. 11-stage fractional hydrolysis summary.

Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)	Xylose (g/10 g of dry biomass)	Furfural (g/kg of dry biomass)	Phenolics (g/kg of dry biomass)
1.	1	0.32	0.32	n.d.	0.13
2	2	0.68	0.62	0.16	0.26
3	5	1.21	0.85	0.73	0.38
4	8	0.14	0.05	0.46	0.45
5	11	0.16	0.02	n.d.	0.49
6	14	0.37	n.d.	n.d.	0.57
7	17	0.42	n.d.	n.d.	0.65
8	20	0.43	n.d.	n.d.	0.73
9	23	0.45	n.d.	n.d.	0.85
10	26	0.53	n.d.	n.d.	0.89
11	29	0.65	n.d.	n.d.	0.95

From these results, it was observed that 8-stage fractional hydrolysis experiments resulted in maximum TRS recovery although results were almost comparable with the 7-stage hydrolysis. The summarised results for all the hydrolysis experiments performed to find out the optimum number of stages during fractional hydrolysis are presented in Table 3.9. Therefore, for the further fermentation of sugars fractions obtained into ethanol, hydrolysates of 8-stage fractional hydrolysis process (optimum preheating time 45 min and liquid to solid ratio: 10:1) were used as it resulted in maximum sugar recovery from kans grass biomass with minimum toxics (1.27×10^{-2} g furfural and 3.04×10^{-2} g phenolics).

The 8-stage fractional hydrolysis resulted in highest recovery of fermentable sugars with TRS of 4.725±0.3 g/L in XRF and 7.62±0.4 in GRF. Hydrolysate fractions of kans gass biomass were conditioned and concentrated for TRS concentration in XRF up to 60 g/L and GRF up to 200 g/L prior to fermentation.

Table 3.9. Summarised results for different fractional hydrolysis experiments.

	TRS recovery (%)	Xylose recovery (%)	Furfural (g/kg of dry biomass)	Phenolics (g/kg of dry biomass)
5- stage	68.09	54.41	1.62	2.83
6- stage	78.63	50.7	1.05	3.27
7- stage	81.52	85.58	1.48	3.89
8- stage	84.88	87.9	1.27	3.04
9- stage	81.37	77.2	2.1	4.68
11- stage	81.83	86.51	1. 35	5.4

3.3 Conclusion

Recovery of maximum amount of reducing sugars from lignocellulosic feedstocks is the major challenge in 2G ethanol production. In this investigation, we have developed a single step process (pretreatment+hydrolysis+detoxification) of total 6 h duration to convert the hemicellulosic and cellulosic content of lignocellulosic biomass. In previous studies, two or more process steps were involved for the complete fractionation of the lignocellulosic biomass to obtain fermentable sugars. Application of a new popping pretreatment technique was performed on rice straw for enhancing cellulose conversion efficiency, where sugar production under the optimum conditions was 0.567 g/g of straw after 48 h [14]. A novel approach of enzymatic hydrolysis using phosphoric acid impregnated and steam exploded sugarcane bagasse has been developed under high solid (18-22%) and low enzyme loading, resulting in total glucan conversion of 69.2% [15]. Extrusion pretreatment method was developed for pine (softwood), optimum extrusion conditions included screw speed of 150 rpm, barrel temperature 180°C and moisture content 25%. Maximum cellulose, hemicellulose, and total sugar recovery were 65.8, 65.6% and 66.1% respectively [16]. Moreover, in most of the previous studies, due to severe reaction conditions, liberated sugars further were converted into toxics (5-HMF, furfural, and phenolics); thereby reducing the total fermentable sugars recovery which necessitates the requirement of an additional detoxification step.

Fractional hydrolysis process has been developed to recover the maximum amount of soluble pentose and hexose sugars separately; direct from lignocellulosic feedstocks with the negligible concentration of toxic products. Also, separate pretreatment and detoxification steps are not required in this technique. The success of technique can be stated by total 84.88% sugar recovery from the biomass as soluble monomeric sugars with the negligible amount of toxics.

CHAPTER 4

Exploration and evaluation of different inorganic acids and lignocellulosic feedstocks for the fractional hydrolysis process

4.1 Introduction

Bioethanol can be produced from plentiful and domestic cellulosic biomass resources (agricultural and forestry residues, herbaceous and woody plants as well as municipal and industrial solid waste streams). Currently, world ethanol production is about 60% from feedstocks of food and sugar crops, requiring high-quality agricultural land for their growth; thereby giving rise to food-fuel conflict. Bioethanol production overall cost varies widely by feedstock types, conversion processes, production scale, and regions. Feedstock cost (crops) is a significant component in the ethanol production cost [1-3]. Lignocellulosic biomasses are mainly harvested from agricultural wastes materials and forest residues crops. They are easily available in almost every region and different climatic condition [4]. Lignocellulosic biomasses are the most promising alternative for sugar crops because of (a) low cost (b) high yield (c) wide availability throughout the year and (d) ability to grow in marginal lands with almost nill water supply requirement. Major research challenges of 2G ethanol production at commercial level include maximum extraction of fermentable sugars (cellulose and hemicelluloses) from lignocellulosic biomass during saccharification.

Various techniques have been developed during recent years to overcome these challenges for efficient bioethanol production at the commercial level. However, most of the current technologies used for fuel ethanol production are cost ineffective and unable to eliminate process steps significantly. Technological approaches improvements and optimisation of various factors have been prioritised in these studies. Nevertheless, 2G ethanol production still has some challenges that need to be properly addressed in the development of a sustainable bioethanol industry. Therefore, in the present work, a unique approach with just two process steps (fractional hydrolysis and fermentation) was adapted for conversion of lignocellulosic biomass into fuel ethanol with high conversion efficiency and bringing down overall 2G ethanol production cost effectively. Fractional hydrolysis technique has been developed which gives soluble pentose and hexose sugars as separate fractions directly from the lignocellulosic biomass. Obtaining a separate xylose-rich fraction (XRF) and glucose-rich fraction (GRF) of hydrolysates hold a tremendous advantage for further fermentation. Additionally, fractional hydrolysis process merges two different steps (pretreatment and hydrolysis), thereby reducing the total production cost. Furthermore, toxic compounds in hydrolysate were found negligible; hydrolysate can be taken directly for fermentation without undergoing detoxification, thereby reducing overall process cost. Development and effects of various parameters on the fractional

hydrolysis process have been discussed in the previous chapter. In this chapter, four different strong inorganic acids (HCl, H_3PO_4, HNO_3, and H_2SO_4) have been compared for 7- and 8-stage fractional hydrolysis process for maximum sugar recovery with minimum toxics. Also, three different lignocellulosic biomasses were selected (kans grass, sugarcane bagasse, and wheat straw) for the study because of their geographically even distribution and higher polysaccharide content compared to other feedstocks.

4.2 Results and Discussion

4.2.1 Fractional hydrolysis using various inorganic acids

Four strong inorganic acids (HCl, H_3PO_4, HNO_3, and H_2SO_4) were selected to find out the most suitable reagent for maximum soluble sugar recovery with minimum toxics. Also, two different approaches (7 stage and 8-stage fractional hydrolysis) were tried using these acids. All the parameters were kept similar during all the experiments. Results are summarised in Table 4.1 and Table 4.2 for 7-stage and 8-stage fractional hydrolysis respectively using all the four acids. Results were calculated per 10 g of dry kans grass biomass. As hemicelluloses have lower molecular weight compared to cellulose and branches with short lateral chains that can be easily hydrolysed [5], xylose sugar (monomeric unit of hemicelluloses) released before glucose in few initial fractions when acid concentrations were less. Few experiments were conducted at acid conc.>30% and T>100^oC, where liberated glucose sugar further converted into 5-Hydroxymethyl furfural and toxic products generated significantly in the hydrolysate media. Therefore, temperatures up to 100^oC and acid concentration within 30% have been used for all the fractional hydrolysis experiments.

4.2.1.1 7-stage fractional hydrolysis

5.26 g of total reducing sugars (TRS) were recovered using HCl during the 7-stage fractional hydrolysis process. Glucose recovery was calculated as 80.22% whereas xylose recovery was 80.47%. Toxic compounds were present in the form of 1.5×10^{-2} g furfural and 4.03×10^{-2} g total phenolics. Upon treatment with H_3PO_4, TRS recovery was only 3.19 g with 46.36% glucose and 53.49% xylose recovery. HNO_3 resulted in highest sugar recovery with overall 83.52% saccharification giving 82.95% glucose and 84.65% xylose recovery. H_2SO_4 was able to recover total 5.34 g TRS resulting in 81.52% overall saccharification. Glucose and xylose recovery (80.9 and 82.79% respectively) was slightly lower compared to HNO_3. In case of

HNO_3, toxic concentrations (2.1×10^{-2} g furfural and 4.3×10^{-2} g total phenolics) were higher compared to H_2SO_4 (1.48×10^{-2} g furfural and 4.01×10^{-2} g total phenolics).

4.2.1.2 8-stage fractional hydrolysis

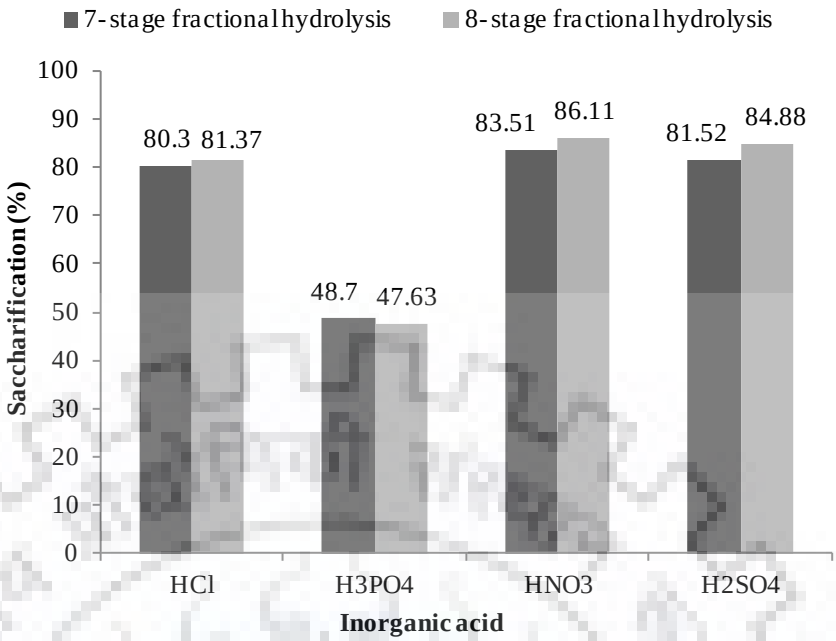
5.33 g TRS was recovered using HCl in the 8-stage fractional hydrolysis process. Glucose recovery was calculated as 81.81% whereas xylose recovery was almost equal to 7-stage with 80.46%. Toxics compounds were present in the form of 1.15×10^{-2} g furfural and 2.96 g total phenolics. Upon treatment with H_3PO_4, TRS recovery was only 3.12 g with 45.45% glucose and 52.09% xylose recovery. Here also, HNO_3 resulted in highest sugar recovery with overall 86.11% saccharification giving 84.31% glucose and 89.69% xylose recovery. H_2SO_4 was able to recover total 5.56 g TRS during 8-stage fractional hydrolysis resulting in 84.88% overall saccharification. Glucose and xylose recovery (83.4 and 87.9% respectively) was slightly lower compared to HNO_3. In case of HNO_3, toxic concentrations (1.52×10^{-2} g furfural and 4.28×10^{-2} g total phenolics) were higher compared to H_2SO_4 (1.27×10^{-2} g furfural and 3.75×10^{-2} g total phenolics). Sugar recovery comparison of 7- and 8-stage fractional hydrolysis process using different inorganic acids has been represented in Fig. 4.1.

Table 4.1. Summary of 7-stage fractional hydrolysis with different inorganic acids using kans grass biomass.

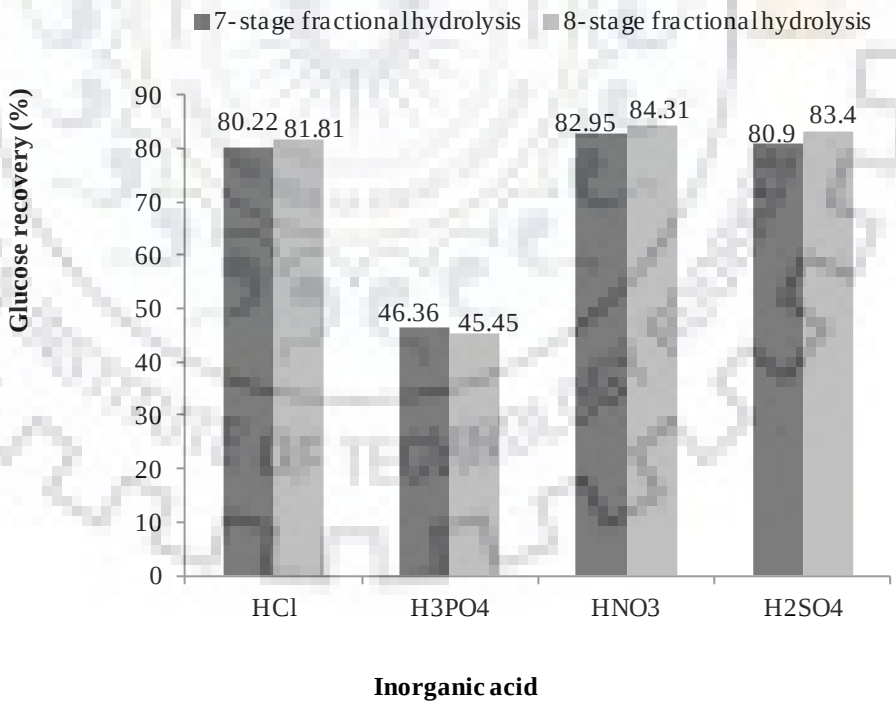
Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)				Xylose (g/10 g of dry biomass)				Furfural (g/kg of dry biomass)				Phenolics (g/kg of dry biomass)			
		HCl	H_3PO_4	HNO_3	H_2SO_4	HCl	H_3PO_4	HNO_3	H_2SO_4	HCl	H_3PO_4	HNO_3	H_2SO_4	HCl	H_3PO_4	HNO_3	H_2SO_4
1	1	0.31	0.17	0.36	0.33	0.29	0.15	0.34	0.32	(n.d.)	n.d.	n.d.	n.d.	0.13	0.07	0.14	0.1
2	2	0.64	0.34	0.75	0.68	0.63	0.33	0.63	0.60	0.18	0.06	0.27	0.16	0.24	0.16	0.27	0.22
3	5	1.51	0.83	1.54	1.21	0.81	0.67	0.85	0.86	0.73	0.23	0.84	0.78	0.30	0.21	0.48	0.31
4	12	0.35	0.18	0.38	0.36	n.d.	n.d.	n.d.	n.d.	0.59	0.39	0.99	0.54	0.62	0.33	0.77	0.63
5	19	0.79	0.56	0.73	0.75	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.87	0.45	0.83	0.85
6	26	1.23	0.84	1.1	0.94	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.92	0.57	0.89	0.91
7	30	0.43	0.27	0.61	1.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.95	0.64	0.92	0.97

Table 4.2. Summary of 8-stage fractional hydrolysis with different inorganic acids using kans grass biomass.

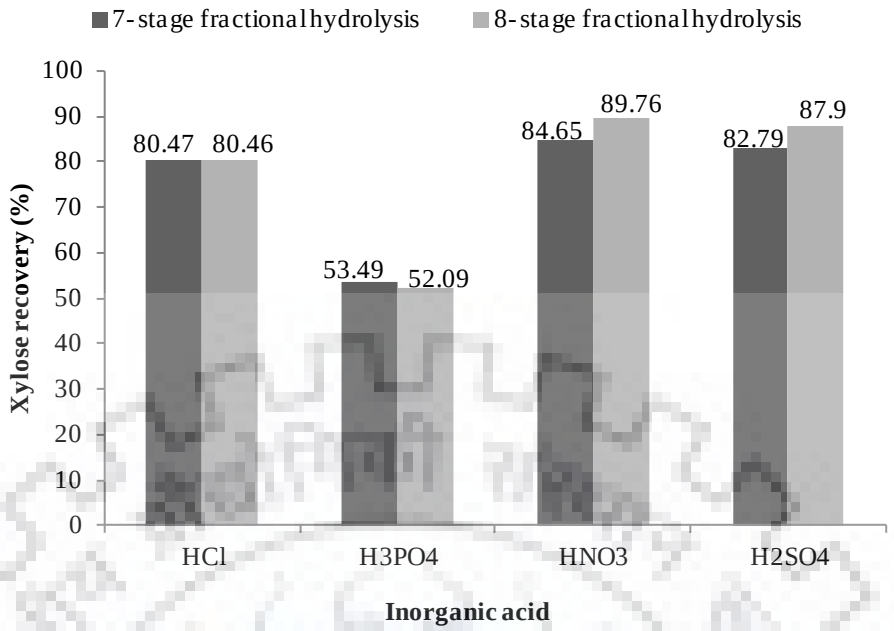
Serial no.	Acid conc. (%)	TRS (g/10 g of dry biomass)				Xylose (g/10 g of dry biomass)				Furfural (g/kg of dry biomass)				Phenolics (g/kg of dry biomass)			
		HCl	H_3PO_4	HNO_3	H_2SO_4	HCl	H_3PO_4	HNO_3	H_2SO_4	HCl	H_3PO_4	HNO_3	H_2SO_4	HCl	H_3PO_4	HNO_3	H_2SO_4
1	1	0.29	0.18	0.32	0.33	0.27	0.16	0.35	0.32	n.d.	n.d.	n.d.	n.d.	0.08	0.05	0.15	0.13
2	2	0.58	0.37	0.66	0.68	0.54	0.34	0.64	0.63	0.12	n.d.	0.29	0.18	0.17	0.11	0.19	0.21
3	5	1.27	0.79	1.11	1.21	0.73	0.47	0.89	0.85	0.6	0.19	0.78	0.73	0.23	0.14	0.25	0.24
4	10	0.34	0.16	0.28	0.29	0.14	0.12	0.05	0.09	0.3	0.23	0.45	0.36	0.3	0.18	0.39	0.36
5	15	0.46	0.2	0.39	0.35	0.05	0.03	n.d.	n.d.	0.13	0.28	n.d.	n.d.	0.36	0.22	0.48	0.45
6	20	0.61	0.38	0.73	0.69	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.39	0.24	0.56	0.43
7	25	0.84	0.53	1.54	1.45	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.42	0.26	0.63	0.55
8	30	0.55	0.35	0.61	0.56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.49	0.30	0.74	0.67
9	35	0.39 (Discarded)	0.16	n.d. (Discarded)	0.1 (Discarded)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.52	0.33	0.89	0.71


7-stage fractional hydrolysis
8-stage fractional hydrolysis
Saccharification (%)
100
90
80
70
60
50
40
30
20
10
0
80.3
81.37
48.7
47.63
83.51
86.11
81.52
84.88
HCl
H3PO4
HNO3
H2SO4
Inorganic acid

(a)


7-stage fractional hydrolysis
8-stage fractional hydrolysis
Glucose recovery (%)
90
80
70
60
50
40
30
20
10
0
80.22
81.81
46.36
45.45
82.95
84.31
80.9
83.4
HCl
H3PO4
HNO3
H2SO4
Inorganic acid

(b)



(c)

Fig. 4.1. Comparison of sugar recovery during 7- and 8-stage fractional hydrolysis process using different inorganic acids (a) Overall saccharification (b) Glucose recovery (c) Xylose recovery.

From these results, it can be stated that 8-stage hydrolysis experiment resulted in maximum TRS recovery with negligible toxics compared to 7-stage hydrolysis. Among all the acids used, TRS recovery was very less using phosphoric acid whereas nitric acid resulted in maximum sugar recovery, but the high cost makes it economically non-feasible. Results of both HCl and H_2SO_4 are the comparable but comparatively lower price of H_2SO_4 makes it most suitable reagent for fractional hydrolysis process resulting in maximum sugar recovery with minimum toxics. Based on these results, further studies have been carried out using 8-stage fractional hydrolysis process with H_2SO_4.

To emphasise the advantages of fractional hydrolysis results, one-step acid hydrolysis experiment was conducted as a control using kans grass biomass. At 12% acid conc., where maximum xylose was recovered during 8-stage fractional hydrolysis process; only 54.23% xylose sugar was recovered after 2.5 h whereas toxics concentration increased gradually upon increasing reaction time. At 23% acid concentration, where maximum glucose was recovered; 40.03% TRS was recovered with a high amount of toxics generation upon increasing reaction time. These results again verify novelty of fractional hydrolysis technique.

4.2.2 Fractional hydrolysis using different lignocellulosic feedstocks

The 8-stage fractional hydrolysis process using H_2SO_4 resulted in maximum sugar recovery with minimum toxics, therefore, to validate fractional hydrolysis results of kans grass, some other widely and easily available feedstocks were explored. Results were almost comparable in all three biomasses (saccharification%: kans grass 84.88, sugarcane bagasse 82.55, and wheat straw 81.66) which strengthens the fact that this novel "fractional hydrolysis" technique is independent of lignocellulosic biomass type and form. Therefore, feedstock type should not be a matter of worry for implementation of this technique during 2G ethanol production at commercial scale. Results of 8-stage fractional hydrolysis process using three different lignocellulosic feedstocks with H_2SO_4 treatment has been summarised in Table 4.3.

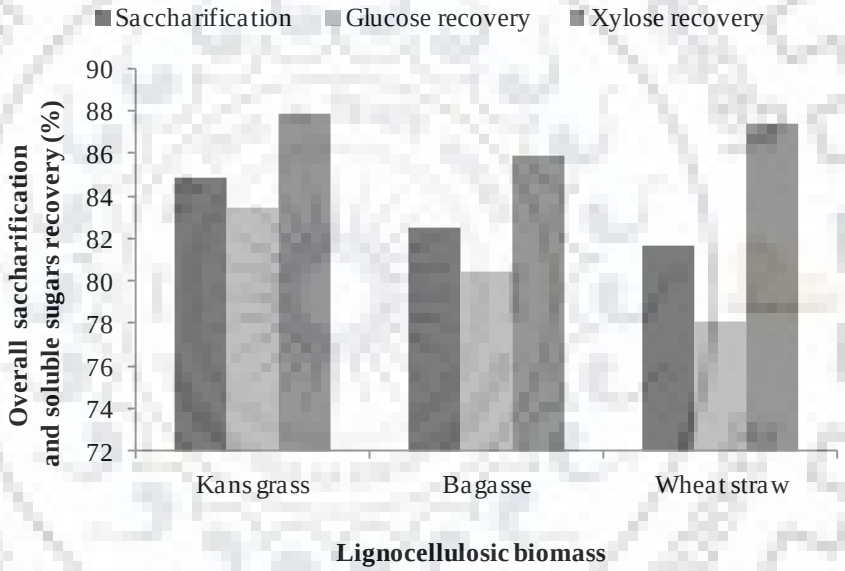


Fig. 4.2 Comparison of sugar recovery during 8-stage fractional hydrolysis process using different lignocellulosic feedstocks.

Bagasse recovered 5.3 g TRS with 2.26 g xylose recovery whereas wheat straw resulted in 5.39 g TRS with 2.22 g xylose recovery. A comparative representation of sugar recovery in three different wide and easily available lignocellulosic feedstocks using 8-stage fractional hydrolysis processes with H_2SO_4 treatment has been shown in Fig. 4.2.

Fractional hydrolysis is a single stage process, carried out in a single fractional hydrolysis column. Initially, fractional hydrolysis column is packed with lignocellulosic biomass; after 45 min preheating, only acid concentration is changed at 30 min time interval during the entire saccharification. There is no requirement of separate pretreatment step also for hemicellulosic

sugars release. Additionally, as toxics concentrations were very low and below the tolerance limits of microbes during ethanol fermentation, no additional detoxification was required prior to fermentation. Therefore, fractional hydrolysis is a replacement for three major steps involved in ethanol production (pretreatment, hydrolysis, and detoxification) from lignocellulosic feedstocks and may help in reducing the total production cost.

Entire process is performed in a single column, initially xylose sugars are obtained followed by glucose, therefore, no need of many reactors with many sets of controllers and pumps, mixers etc. 7- and 8- stage fractional hydrolysis terms are used to denote the total number of sugar fractions collected at 30 min time interval during entire process.

4.2.3 Structural analysis of raw and treated biomass

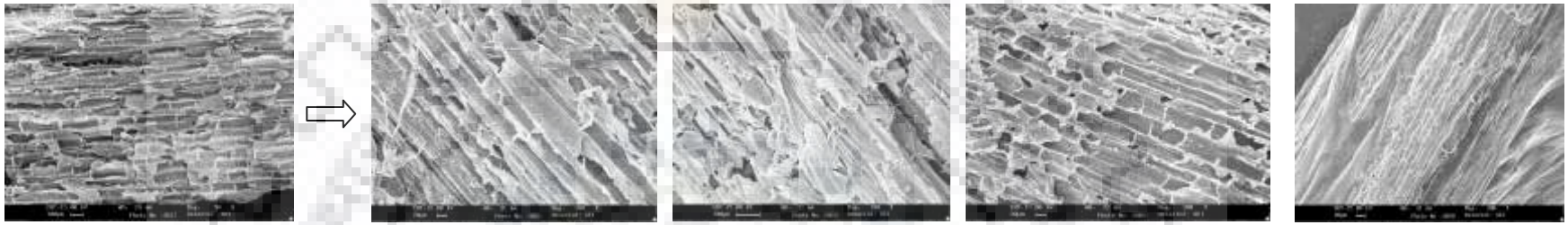
4.2.3.1 SEM analysis

The SEM images [(Fig. 4.3 (a-c)] of untreated biomass and acid treated biomass at every stage shows the effect of increasing acid concentrations on all the three lignocellulosic feedstocks. These images represent total 8 fractional hydrolysis stages. From the images, it can be seen that surface architecture was intact in untreated biomass while upon sequential hydrolysis, it starts to disrupt and completely destroyed at stage 8. In the case of untreated biomass, texture was compact and covered with a thin wax film found in herbaceous materials [6] which was disappeared upon hydrolysis.

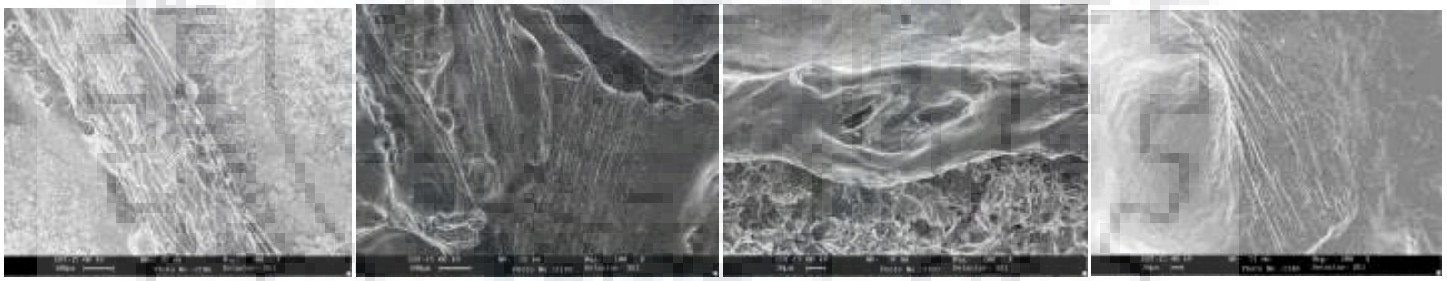
Table 4.3. Summary of 8-stage fractional hydrolysis using different lignocellulosic feedstocks.

S. no.	H_2SO_4 conc.	Kans grass				Bagasse				Wheat straw			
		TRS (g/10g of dry biomass)	Xylose (g/10g of dry biomass)	Furfural (g/10g of dry biomass $\times10^{-2}$)	Phenolics (g/10g of dry biomass $\times10^{-2}$)	TRS (g/10g of dry biomass)	Xylose (g/10g of dry biomass)	Furfural (g/10g of dry biomass $\times10^{-2}$)	Phenolics (g/10g of dry biomass $\times10^{-2}$)	TRS (g/10g of dry biomass)	Xylose (g/10g of dry biomass)	Furfural (g/10g of dry biomass $\times10^{-2}$)	Phenolics (g/10g of dry biomass $\times10^{-2}$)
1	1	0.33	0.32	n.d.	0.13	0.35	0.34	n.d.	0.19	0.34	0.33	n.d.	0.17
2	2	0.68	0.63	0.18	0.21	0.73	0.7	0.05	0.27	0.71	0.69	0.08	0.26
3	5	1.21	0.85	0.73	0.24	1.25	0.93	0.33	0.31	1.21	0.95	0.37	0.34
4	10	0.29	0.09	0.36	0.36	0.31	0.29	0.35	0.38	0.34	0.25	0.31	0.39
5	15	0.35	n.d.	n.d.	0.45	0.27	n.d.	n.d.	0.42	0.29	n.d.	n.d.	0.45
6	20	0.69	n.d.	n.d.	0.43	0.59	n.d.	n.d.	0.49	0.63	n.d.	n.d.	0.51
7	25	1.45	n.d.	n.d.	0.55	1.31	n.d.	n.d.	0.56	1.34	n.d.	n.d.	0.58
8	30	0.56	n.d.	n.d.	0.67	0.49	n.d.	n.d.	0.64	0.53	n.d.	n.d.	0.69

(a)

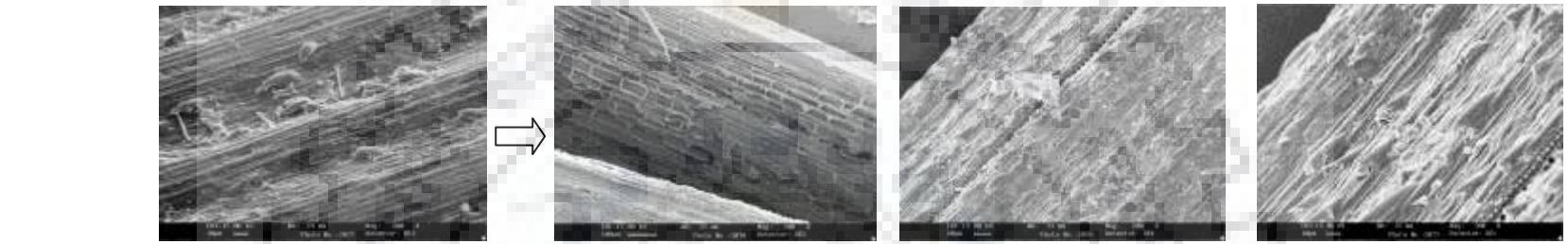


Untreated Bagasse **1** **2** **3** **4**

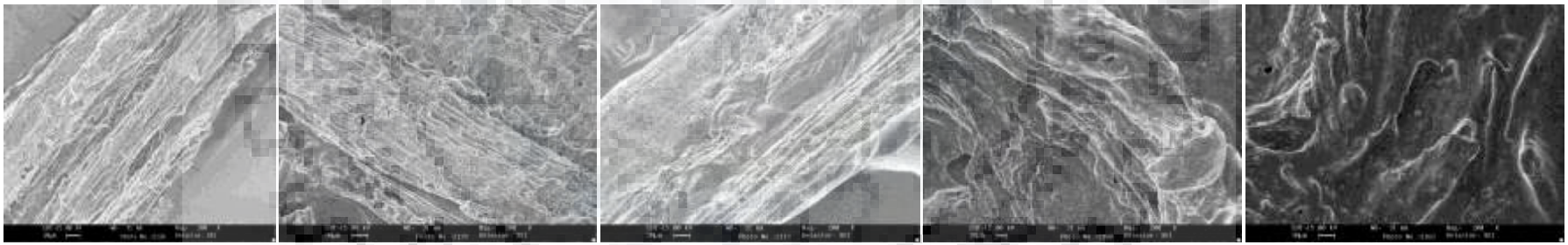


5 **6** **7** **8**

(b)



Untreated kans grass **1** **2** **3**



4 **5** **6** **7** **8**

(c)

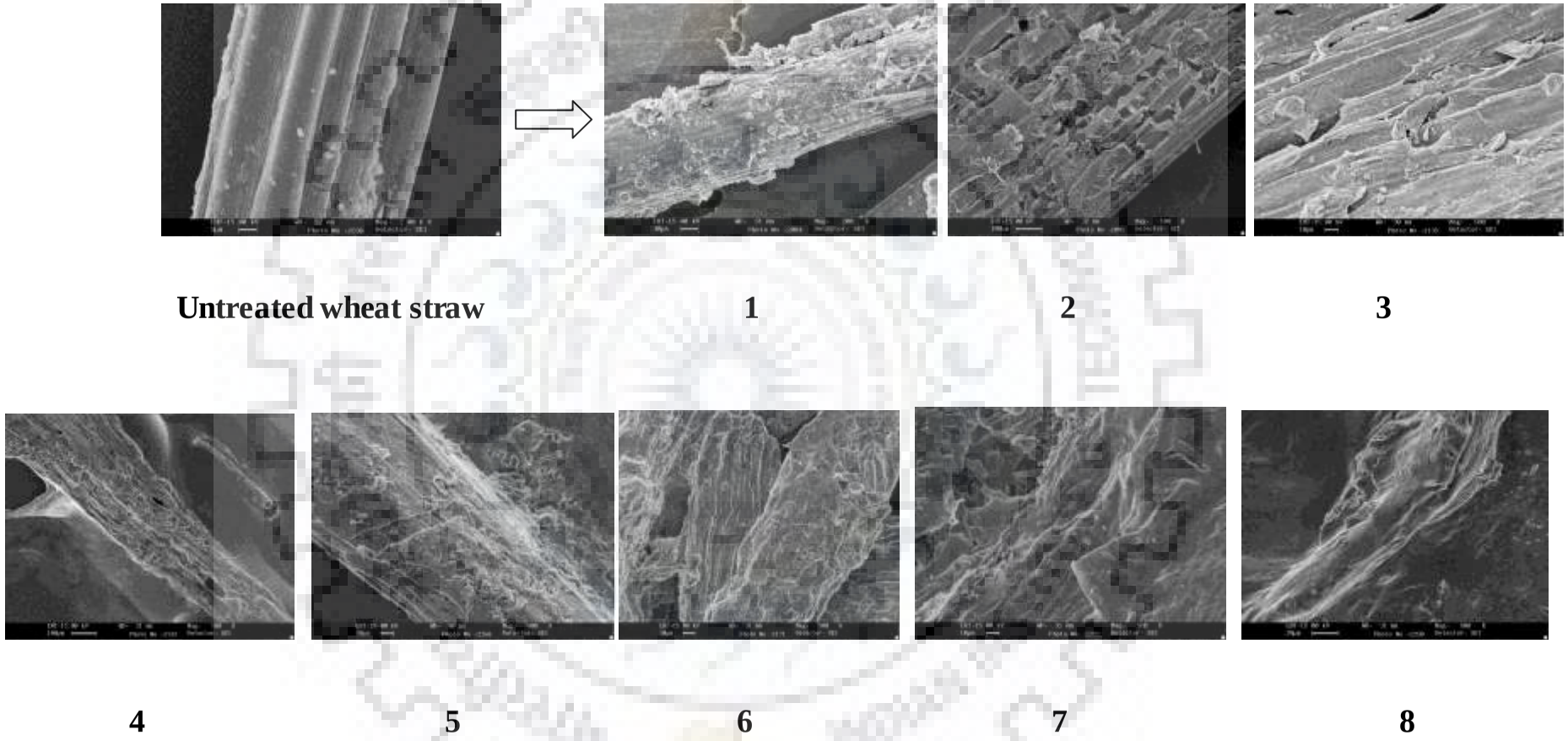
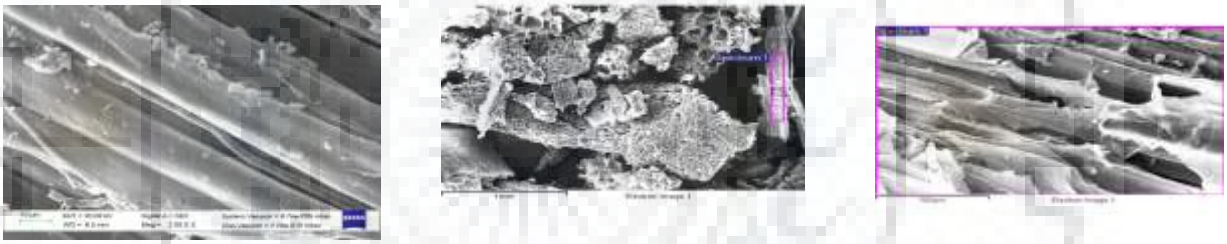


Fig. 4.3. Scanning electron microscopic images of lignocellulosic biomass upon fractional hydrolysis (a) Bagasse (b) Kans grass (c) Wheat straw.

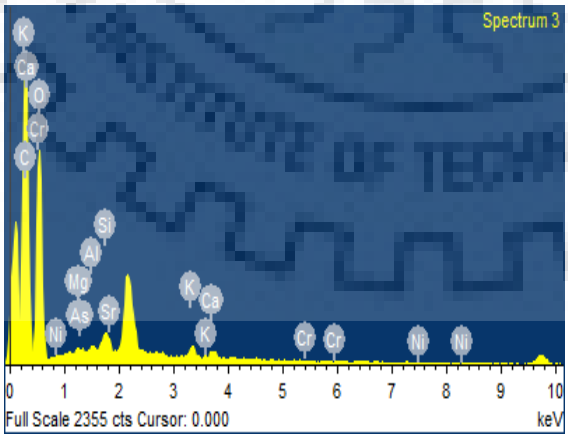
4.2.3.2 FE-SEM analysis

The surface texture of the treated biomass was observed to be distorted after sequential addition of acid (Fig. 4.4-4.6). It is believed to expose the carbohydrate moieties during initial stages of hydrolysis and increase the biomass porosity subsequently, facilitating easy accessibility to cellulolytic agents [7-8]. The extensive surface distortions of the hydrolysed biomass compared to the raw might be due to the effective action of the acid. FE-SEM images provide higher resolution in the whole range of accelerating voltages compared to SEM. Therefore, the surface architecture of raw biomass, biomass after XRF removal (stage 5), and treated biomass were examined through FE-SEM study and shown in Fig. 4.4, Fig. 4.5, and Fig. 4.6 for bagasse, kans grass, and wheat straw respectively. FE-SEM along with EDX provided elemental compositions of the biomass though results are comprehensive, not absolute as hydrogen was not analysed in it.

(a)



Untreated Bagasse (L+H+C)



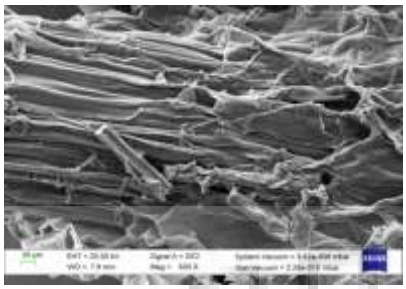
EDX graph of Untreated Bagasse (L+H+C)

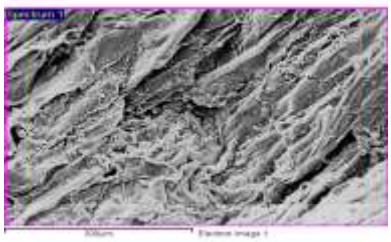
Element	Weight%	Atomic%
C K	42.71	50.67
O K	53.91	48.01
Mg K	0.27	0.16
Al K	0.16	0.09
Si K	0.99	0.50
K K	0.73	0.27
Ca K	0.48	0.17
Cr K	0.07	0.02
Ni K	0.04	0.01
As L	0.03	0.01
Sr L	0.59	0.10
Totals	100.00	

EDX elemental analysis of Untreated Bagasse (L+H+C)

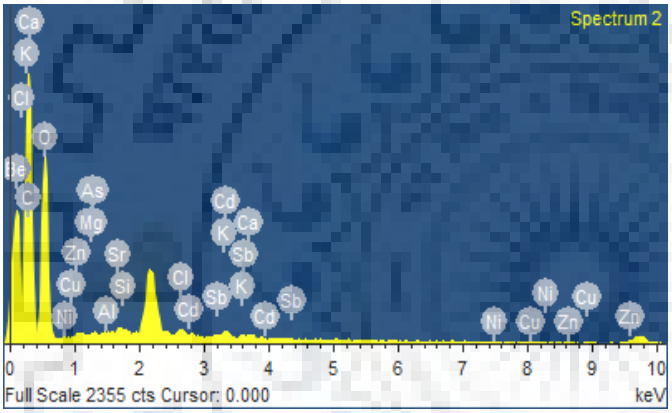
(L: Lignin, H: Hemicellulose, C: Cellulose; K, L in EDX graph and Table represent shell of an atom).

(b)





Bagasse after XRF removal (L+C)

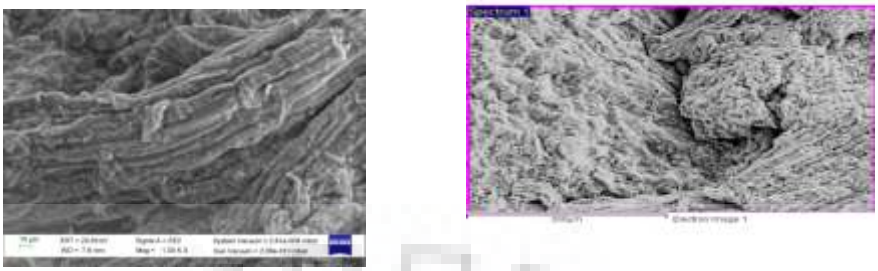


EDX graph after XRF removal (L+C)

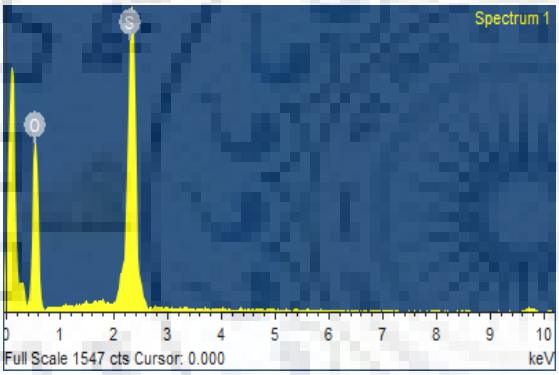
Element	Weight%	Atomic%
C K	30.32	37.27
O K	56.21	52.0
S K	11.04	10.06
Mg K	0.02	0.01
Al K	0.03	0.02
Si K	0.18	0.09
Cl K	0.37	0.15
K K	0.38	0.14
Ca K	0.16	0.06
Ni K	0.06	0.02
Cu K	0.10	0.02
Zn K	0.21	0.04
As L	0.32	0.06
Sr L	0.22	0.03
Cd L	0.22	0.03
Sb L	0.16	0.02
Totals	100.00	

EDX elemental compositions after XRF removal

(c)



Treated Bagasse (L)



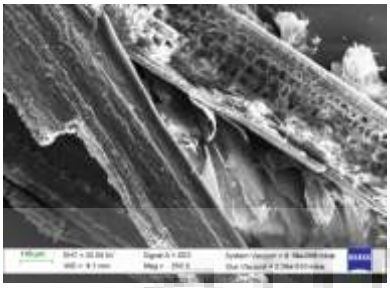
EDX graph of treated bagasse (L)

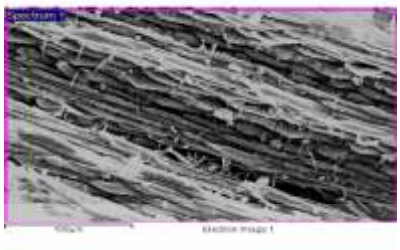
Element	Weight%	Atomic%
O K	67.96	80.96
S K	32.04	19.04
Totals	100.00	

EDX elemental compositions of treated bagasse (L)

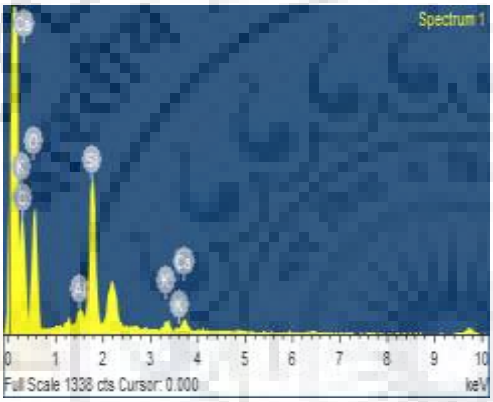
Fig. 4.4. FE-SEM with EDX analysis of bagassse (a) Untreated biomass (b) After XRF removal (c) Treated biomass.

(a)





Untreated kans grass (L+H+C)

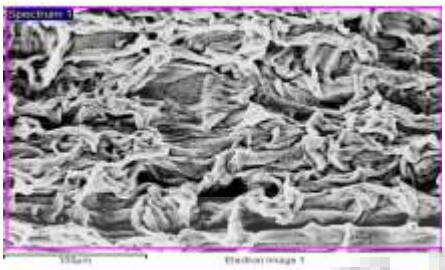


EDX graph of untreated kans grass (L+H+C)

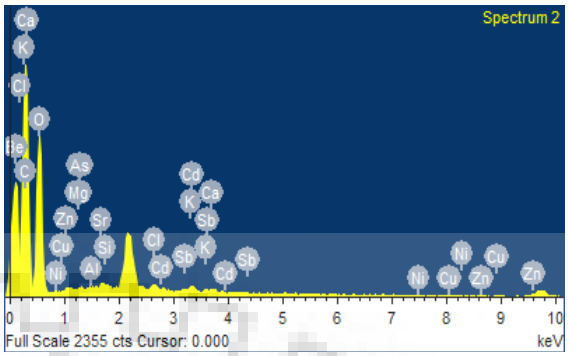
Element	Weight%	Atomic%
C K	47.51	54.25
O K	50.10	44.52
Al K	0.14	0.07
Si K	1.58	0.74
K K	0.51	0.17
Ca K	0.16	0.05
Totals	100.00	

EDX elemental compositions of untreated kans grass (L+H+C)

(b)



FE-SEM image after XRF removal (L+C)

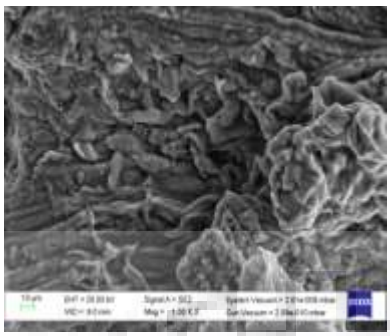


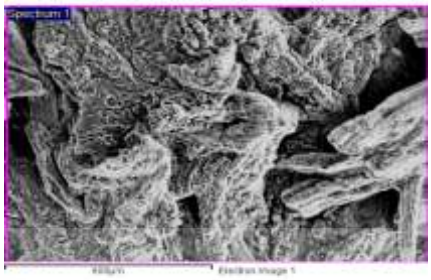
EDX graph after XRF removal (L+C)

Element	Weight%	Atomic%
C K	29.32	36.27
O K	58.25	50.79
S K	10.0	11.34
Mg K	0.02	0.01
Al K	0.03	0.02
Si K	0.18	0.09
Cl K	0.37	0.15
K K	0.38	0.14
Ca K	0.16	0.06
Ni K	0.06	0.02
Cu K	0.10	0.02
Zn K	0.21	0.04
As L	0.32	0.06
Sr L	0.22	0.03
Cd L	0.22	0.03
Sb L	0.16	0.02
Totals	100.00	

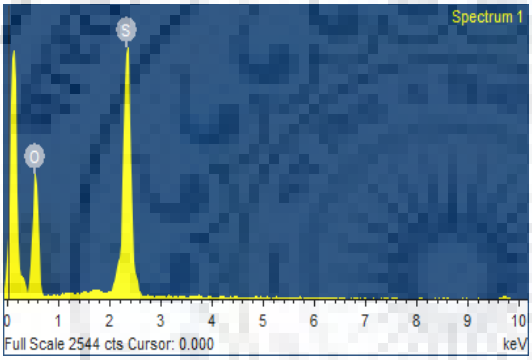
EDX elemental compostions after XRF removal from kans grass (L+C)

(c)





Treated kans grass (L)



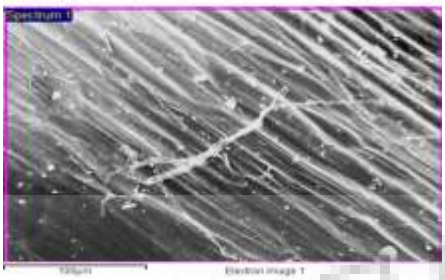
EDX graph of treated kans grass (L)

Element	Weight%	Atomic%
O K	67.76	80.82
S K	32.24	19.18
Totals	100.00	

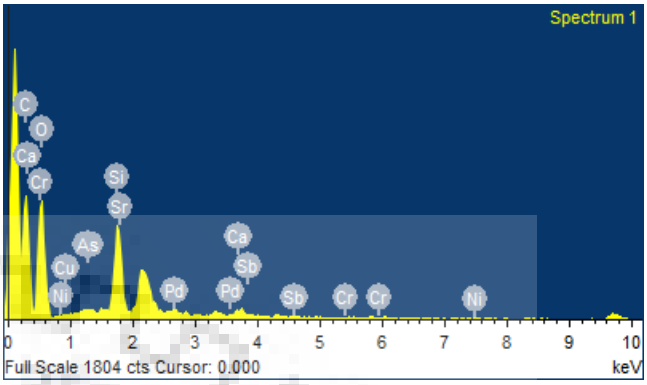
EDX elemental compostions of treated kans grass (L)

Fig. 4.5. FE-SEM with EDX analysis of kans grass (a) Untreated biomass (b) After XRF removal (c) Treated biomass.

(a)



Untreated wheat straw (L+H+C)

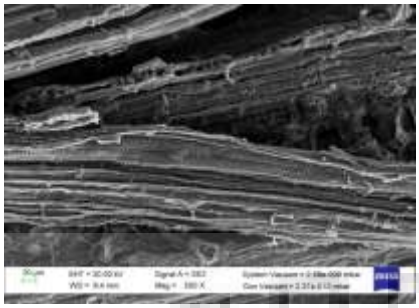


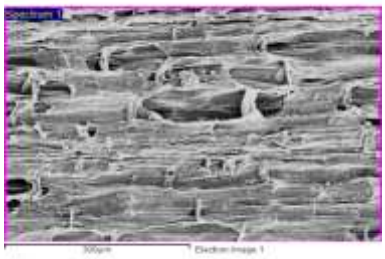
EDX graph of untreated wheat straw (L+H+C)

Element	Weight %	Atomic %
C K	42.43	61.43
O K	50.82	35.91
Si K	4.29	2.15
Ca K	0.50	0.18
Cr K	0.09	0.02
Ni K	0.16	0.04
Cu K	0.09	0.02
As L	0.23	0.04
Sr L	0.99	0.16
Pd L	0.21	0.03
Sb L	0.20	0.02
Totals	100.00	

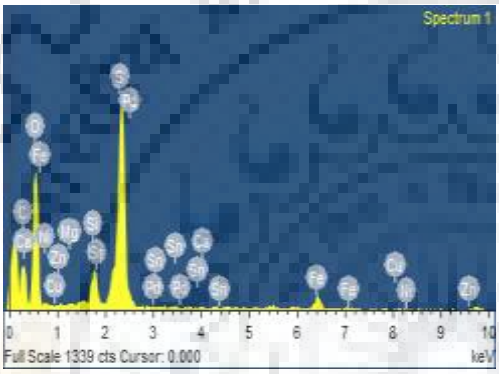
EDX elemental compositions of untreated wheat straw (L+H+C)

(b)





FE-SEM images of wheat straw after XRF removal (L+C)

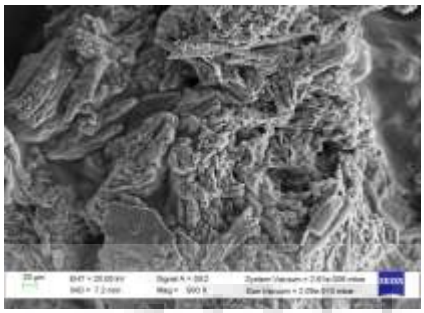


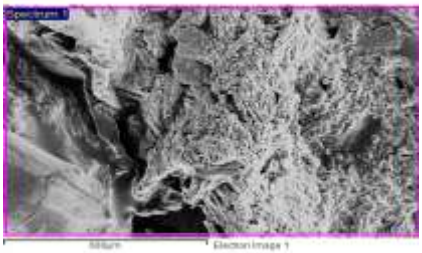
EDX graph of wheat straw after XRF removal (L+C)

Element	Weight%	Atomic%
C K	33.82	44.50
O K	52.55	49.73
Mg K	0.07	0.04
Si K	1.47	0.78
S K	9.09	4.23
Ca K	0.03	0.01
Fe K	1.98	0.53
Ni K	0.05	0.01
Cu K	0.19	0.04
Zn K	0.08	0.02
Sr L	0.31	0.05
Pd L	0.01	0.00
Sn L	0.35	0.04
Totals	100.00	

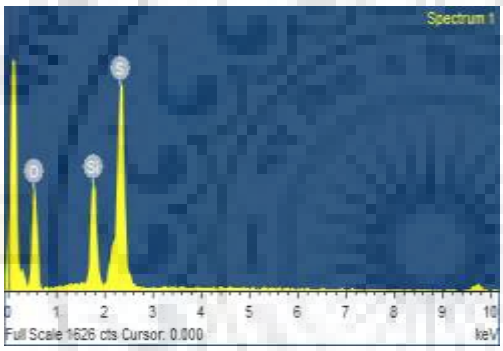
EDX elemental compositions of wheat straw after XRF removal (L+C)

(c)





FE-SEM images of treated wheat straw (L)



EDX graph of treated wheat straw (L)

Element	Weight%	Atomic%
O K	59.73	73.99
Si K	12.81	9.04
S K	27.46	16.97
Totals	100.00	

EDX elemental compositions of treated wheat straw (L)

Fig. 4.6. FE-SEM with EDX analysis of wheat straw (a) Untreated biomass (b) After XRF removal (c) Treated biomass.

4.2.3.3 SPM analysis

An increase in surface roughness was observed in terms of cracks and pores which is an indication of the removal of cell wall materials that result into exposure of buried cellulose microfibrils. To measure these changes, SPM analysis was performed using 3D-imaging. Therefore, the surface roughness of raw biomass, biomass after XRF removal (stage 5), and treated biomass were examined through SPM study and shown in Fig. 4.7, Fig. 4.8, and Fig. 4.9 (for bagasse, kans grass, and wheat straw respectively).

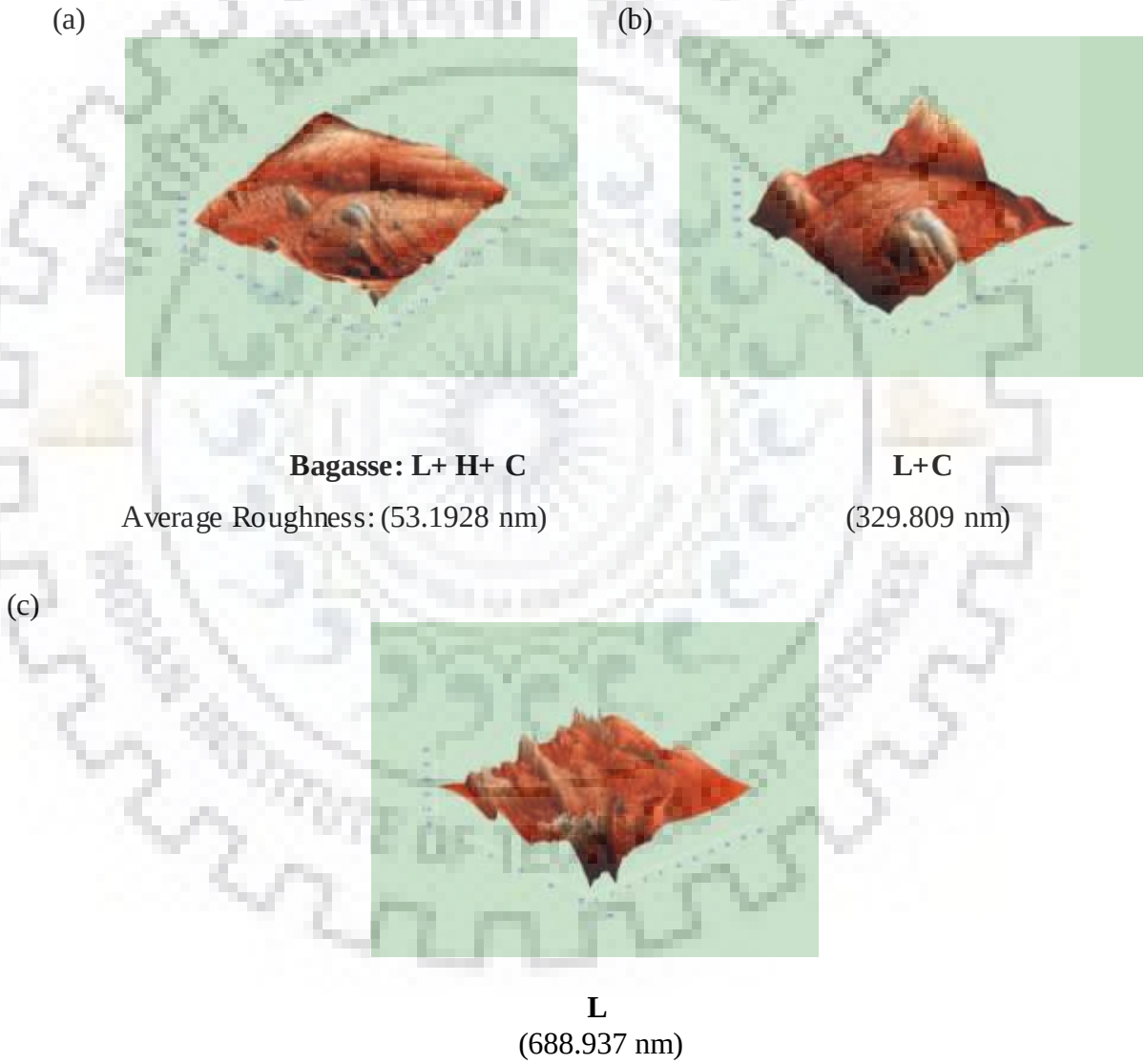


Fig. 4.7. SPM analysis of bagasse (a) Untreated biomass (b) After XRF removal (c) Treated biomass.

(a) (b)

Kans grass: L+ H+ C **L+C**

Average Roughness: (108.697 nm) (519.743 nm)

(c)

L

(248.801 nm)

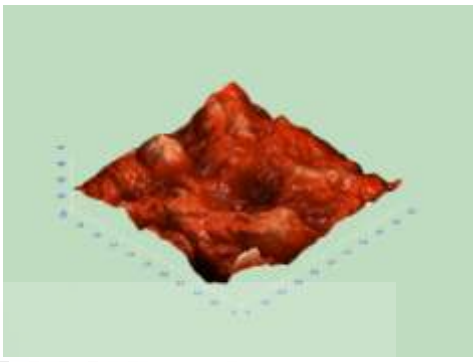
Fig. 4.8. SPM analysis of kans grass (a) Untreated biomass (b) After XRF removal (c) Treated biomass.

(a)

Wheat straw: L+ H+ C

Average Roughness: (184.124 nm)

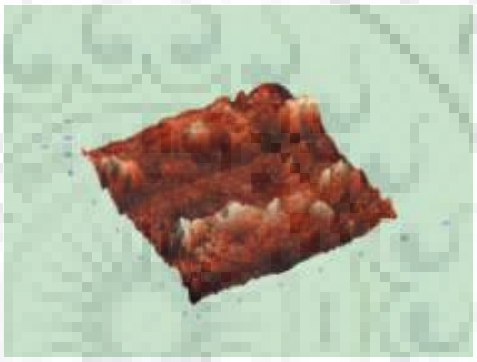
(b)



L+C

(432.253 nm)

(c)



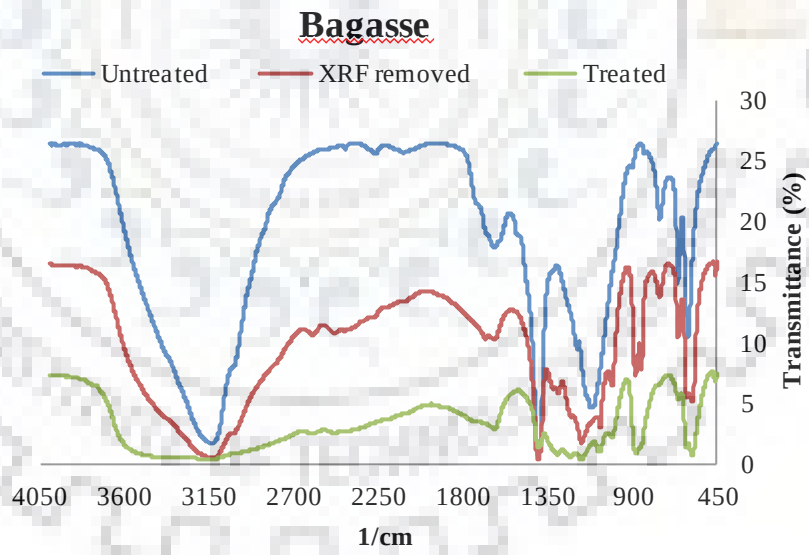
L

(204.814 nm)

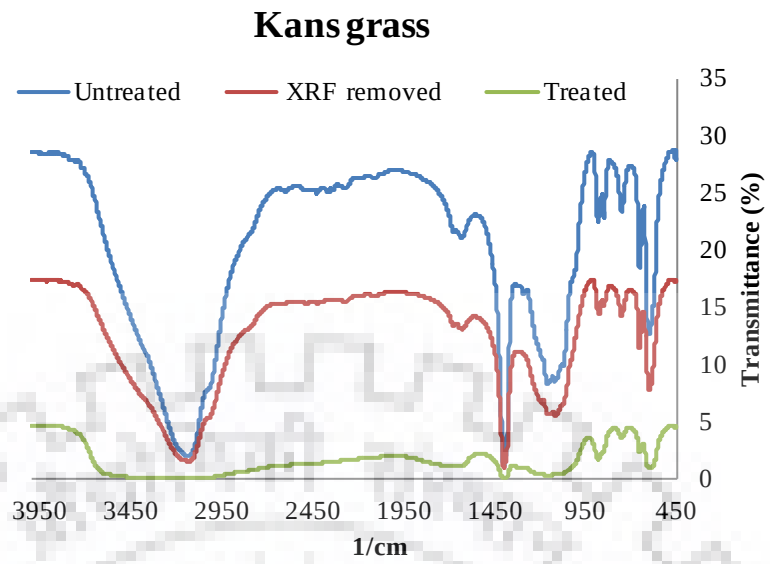
Fig. 4.9. SPM analysis of wheat straw (a) Untreated biomass (b) After XRF removal (c) Treated biomass.

4.2.3.4 FTIR analysis

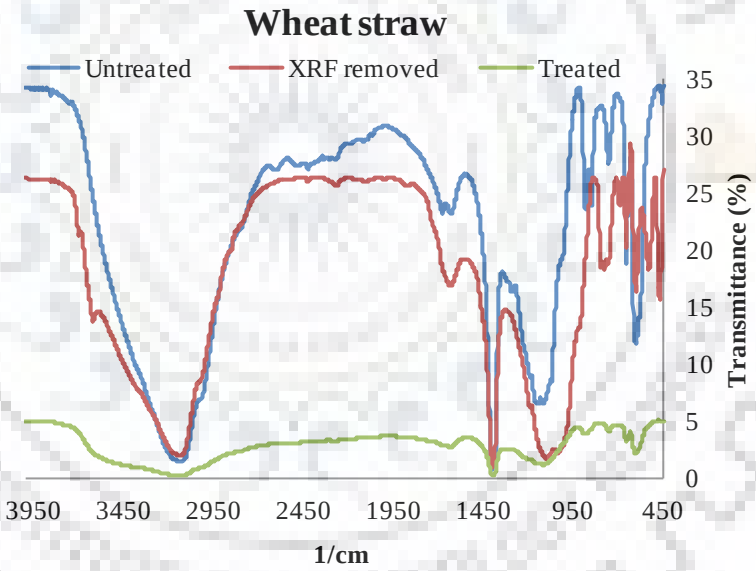
FTIR analysis is commonly used to study the compositional modifications, changes and/or stretching in functional groups, vibrations of different molecules during the course of biomass treatment. Fig. 4.10 shows the FTIR spectra of raw biomass, biomass after XRF removal, and solid residue left after the fractional hydrolysis treatment. The spectrum region from 1000 cm^{-1}-2000 cm^{-1} is usually assigned to the major biological components (cellulose, hemicelluloses and lignin). Marked differences with a reduction in transmittances were observed in the FTIR spectra of untreated and treated biomass. The transmittance at 2852 cm^{-1}, 2930-2910 cm^{-1}, and 3450-3300 cm^{-1} corresponds to stretching of $–CH_2$, $–CH_3$ and –OH groups, respectively [9], however, slight variations were observed in different studies in the correlation of transmittance and functional group modifications assignment [10-11]. Therefore, a generalised detected peak summary is provided along with functional groups and polymeric molecules assignment (Table 4.4). The reason behind the decrease in transmittance of the hydrolysed biomass is due to the cleavage of cellulose and hemicellulose moieties by acid [12].



(a)



(b)



(c)

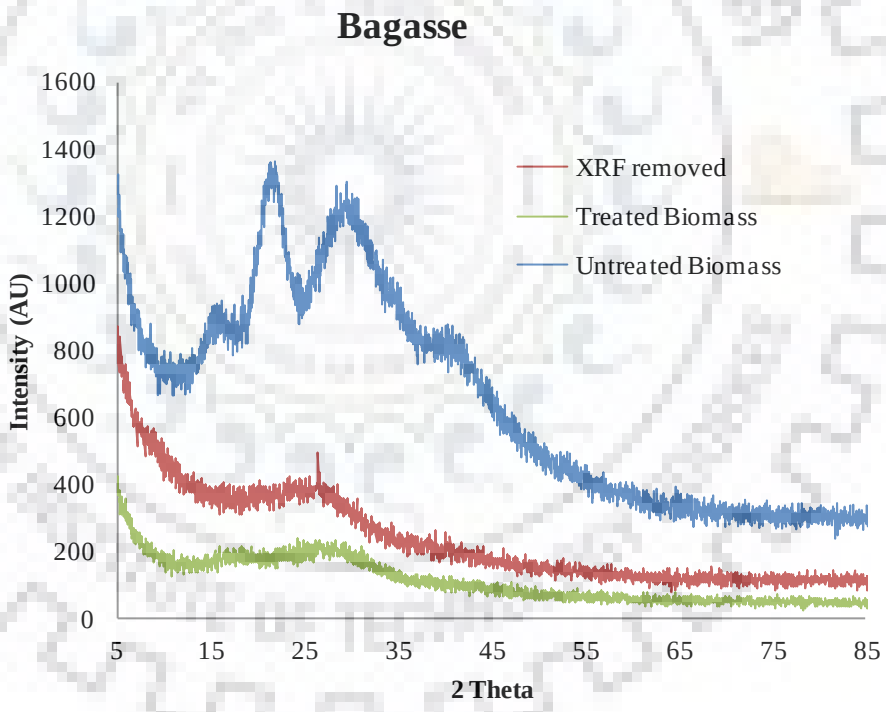
Fig. 4.10. FTIR spectra of biomass (a) Bagasse (b) Kans grass (c) Wheat straw.

Table 4.4. Peaks summary of lignocellulosic biomass obtained during FTIR analysis [13].

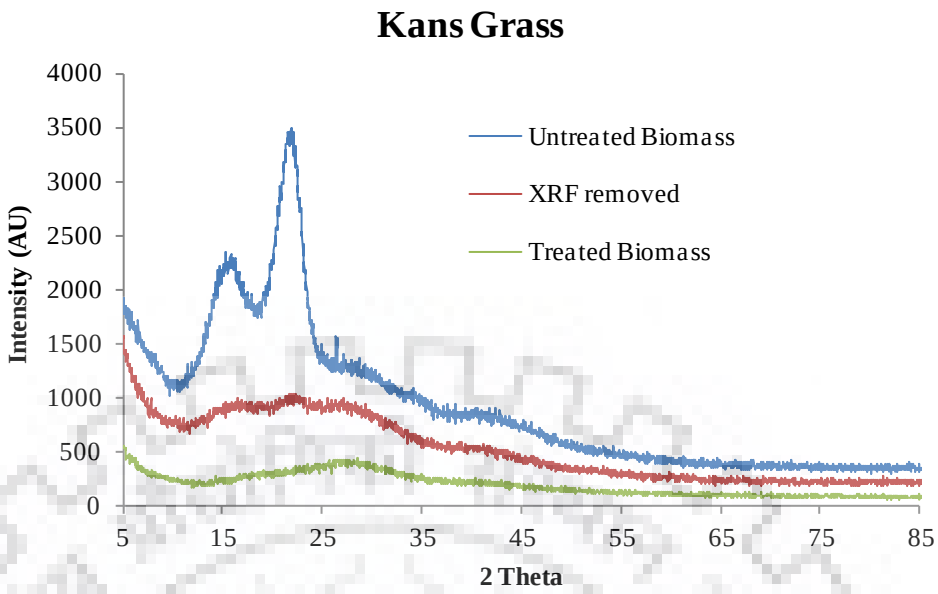
Wavenumber (cm^{-1})	**Functional group**	**Polymer**
875	Glycosidic linkage	Hemicellulose
930	Glycosidic linkage	Cellulose, hemicellulose
990	C-O valence vibration	Cellulose
1035	C-O, C=C, and C-C-O stretching	Cellulose, hemicellulose, lignin
1160	C-O-C asymmetrical stretching	Cellulose, hemicellulose
1200	O-H bending	Cellulose, hemicellulose
1215	C-C + C-O stretch	Lignin (wood)
1270	Aromatic ring vibration	Guaicyl lignin
1280	C-H bending	Crystalline cellulose
1310	CH_2 wagging	Cellulose, hemicellulose
1327	C-O of syringyl ring	Lignin (wood)
1335	C-H vibration, O-H in-plane bending	Cellulose, hemicellulose, lignin
1380	C-H bending	Cellulose, hemicellulose, lignin
1425	C-H in-plane deformation	Lignin (wood)
1440	O-H in-plane bending	Cellulose, hemicellulose, lignin
1465	C-H deformation	Lignin
1500	Aromatic ring vibration	Lignin
1595	Aromatic ring vibration +C=O stretch	Lignin
1682	C=O stretching (unconjugated)	Lignin (wood)
1730	Ketone/aldehyde C=O stretch	Hemicellulose
1750	Free ester	Hemicellulose
2840, 2937	C-H stretching Lignin	Lignin (wood)
3421	O-H stretching Lignin	Lignin (wood)

4.2.3.5 XRD analysis

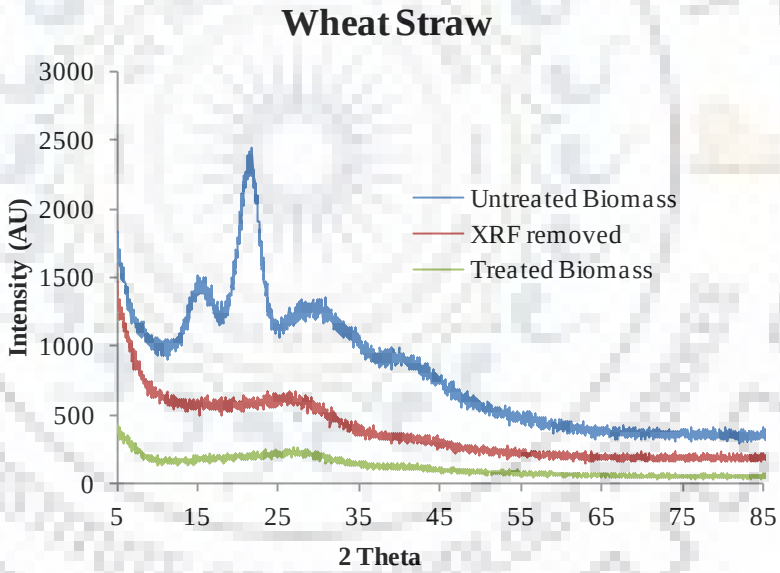
Cellulose crystallinity is one of the critical issues in second generation bioethanol production. Cellulose can be present in amorphous, paracrystalline (between amorphous and crystalline phases), or crystalline form in the biomass. The amorphous form is defined by the presence of more disordered cellulosic chains; therefore, it can be easily hydrolysed compared to the compact structure of crystalline cellulose. Crystallinity occurs due to the presence of crystalline cellulose in the biomass and is one of the major governing factors for its conversion to corresponding reducing sugars. The digestibility of lignocellulosic biomass with acidic/alkaline or enzymatic treatment mainly depends upon crystalline behaviour of cellulose. After the solubilisation of amorphous cellulose, crystalline portion was utilised which leads to an increased reducing sugars production and decrease in the relative % cellulose crystallinity (Table 4.5). Crystallinity Index was calculated by the formula given in chapter 2. Fig. 4.11 shows the XRD spectra of raw biomass, after XRF removal, and treated biomass.



(a)



(b)



(c)

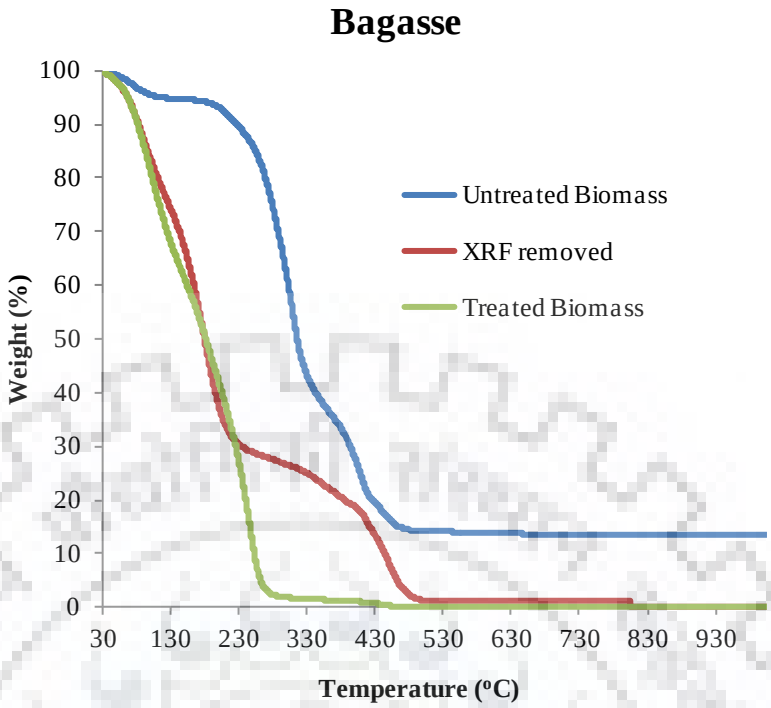
Fig. 4.11. XRD spectra of biomass (a) Bagasse (b) Kans grass (c) Wheat straw.

Table 4.5. Crystallinity indices of lignocellulosic biomass after fractional hydrolysis.

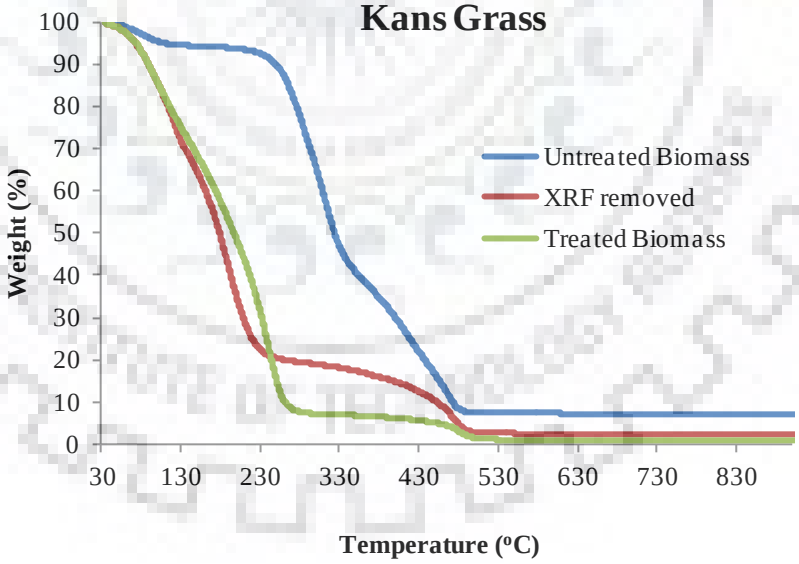
	Bagasse	Kans Grass	Wheat Straw
Untreated Biomass	38.72	50.0	49.05
XRF Removed Biomass	22.63	38.6	38.42
Treated Biomass	20.93	17.8	20.96

4.2.3.6 TGA analysis

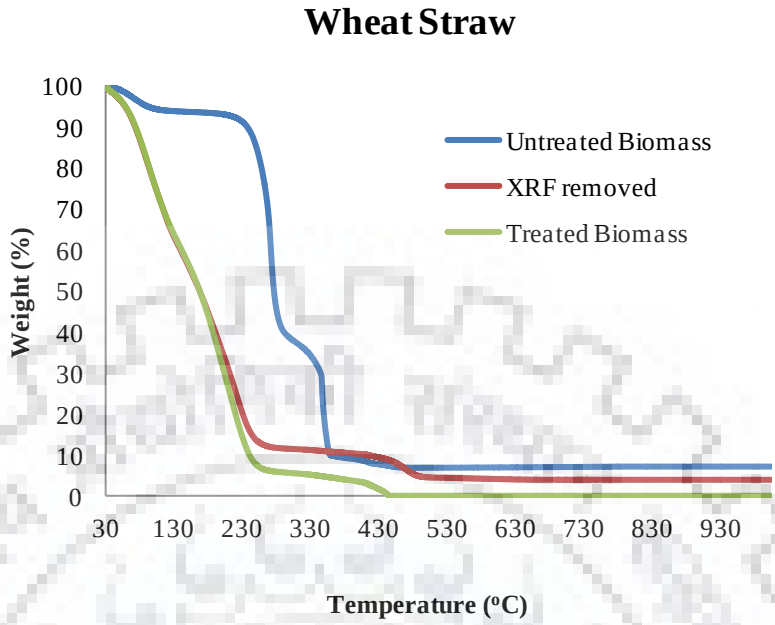
Fig. 4.12 shows the TGA curve of all three lignocellulosic feedstocks upon fractional hydrolysis. The loss of weight between 30-200^{o}C is associated with dehydration in case of untreated biomass i.e. moisture adsorbed on the sample surface and volatile compounds present within the inner cells is released [14-15]. This temperature reduced to 100^{o}C upon fractional hydrolysis. The second stage of thermal decomposition of feedstocks occurred in the temperature range of 200-500^{o}C, three peaks were detected for the maximum weight loss in this range for untreated biomass (Table 4.6). After XRF removal, two peaks detected for maximum weight loss whereas after complete hydrolysis it reduced to one (up to 250^{o}C). Hemicellulose degrades between 200-350^{o}C, cellulose (305-375^{o}C), and lignin (150-600^{o}C) in untreated biomass [16]. The cellulose decomposition peak probably overlaps with that of hemicelluloses [17]. Burn-out temperature (temperature at which complete pyrolysis of all the major components present in the biomass occurred, apart from fixed residues) also reduces after fractional hydrolysis. Some materials present in the biomass remain unburned, termed as fixed residues (highest in case of untreated biomass).


Bagasse
Weight (%)
100
90
80
70
60
50
40
30
20
10
0
30
130
230
330
430
530
630
730
830
930
Temperature (°C)
Untreated Biomass
XRF removed
Treated Biomass

(a)


Kans Grass
Weight (%)
100
90
80
70
60
50
40
30
20
10
0
30
130
230
330
430
530
630
730
830
Temperature (°C)
Untreated Biomass
XRF removed
Treated Biomass

(b)



(c)

Fig. 4.12. TGA curves of biomass (a) Bagasse (b) Kans grass (c) Wheat straw.

Table 4.6. TGA summary (a) Bagasse (b) Kans grass (c) Wheat straw.

Lignocellul-osic biomass		Moisture & Volatile compounds (%)	Ignition temperature (°C)	Peak temperatures (°C)	Burn-out temperature (°C)	Weight loss (%)	Fixed residues (%)
Bagasse	Untreated Biomass	6.78	201	310, 414, 444	500	79.03	13.64
	XRF removed	13.93	100	97, 187, 462	500	84.34	1.09
	Treated Biomass	16.82	100	102, 252	400	82.11	0.0
Kans grass	Untreated Biomass	6.34	200	280, 308, 468	490	85.96	7.24
	XRF removed	13.45	100	126, 190, 473	490	83.45	2.42
	Treated Biomass	11.54	100	103, 249	274	79.66	0.968
Wheat straw	Untreated Biomass	7.2	220	290, 316, 383	463	85.9	6.9
	XRF removed	20.53	100	97, 230	400	78.35	4.16
	Treated Biomass	20.51	100	218	400	76.01	0.0

4.2.3.7 ICP-MS analysis

Plants grown in uncultivable and barren lands might uptake heavy metals and accumulate them. When such feedstocks are used for biofuel production, these toxic elements dispensed through the process and inhibit fermentation process. Wastes from agricultural sector and industries also contribute to it. Therefore, heavy metal concentration analysis is a prerequisite for ethanol production. Kans grass grows in a barren sandy alluvial soil whereas other two feedstocks are agricultural residues. Therefore, the concentration of heavy metal in hydrolysates was detected through ICP-MS (Table 4.7-4.9) for all three biomasses. The content of chromium, strontium, and zinc was in significant quantities, but within the prescribed standard limit of heavy metals for fermentation [18].

Table 4.7. Heavy metal analysis in hydrolysates (XRF and GRF) of bagasse.

Analytes	XRF (ppb)	GRF (ppb)	Standard Limit
Pb	0.620	0.623	0.075×10^{-6}
As	1.673	1.139	-
Be	0.177	0.094	-
Cr	34.500	33.854	1×10^{-6}
Cu	1.655	1.779	0.1×10^{-6}
Sr	5.839	4.153	-
Ni	10.214	10.437	-
Zn	8.532	14.560	5×10^{-6}
Cd	0.296	0.123	5×10^{-6}

Table 4.8. Heavy metal analysis in hydrolysates (XRF and GRF) of kans grass.

Analytes	XRF (ppb)	GRF (ppb)	Std. Limit (ppb)
Pb	0.322	0.202	0.075×10^{-6}
As	0.497	0.717	-
Be	0.035	0.027	-
Cr	48.683	35.152	1×10^{-6}
Cu	1.201	1.161	0.1×10^{-6}
Sr	5.340	22.673	-
Ni	10.267	10.21	-
Zn	13.947	8.031	5×10^{-6}
Cd	0.056	0.053	5×10^{-6}

Table 4.9. Heavy metal analysis in hydrolysates (XRF and GRF) of wheat straw.

Analytes	XRF (ppb)	GRF (ppb)	Std. Limit (ppb)
Pb	1.774	0.160	0.075×10^{-6}
As	0.403	0.425	-
Be	0.016	0.021	-
Cr	67.350	34.560	1×10^{-6}
Cu	1.556	0.787	0.1×10^{-6}
Sr	8.103	3.359	-
Ni	11.293	9.875	-
Zn	11.885	10.552	5×10^{-6}
Cd	0.040	0.039	5×10^{-6}

4.2.3.8 Calorific values of feedstocks

The energy density or calorific value of the lignocellulosics is one of the major concerns because of its key role in energy and cost balance during the production process. The heating value is 21.13, 17.0, and 16.63 MJ/kg for lignin, cellulose, and hemicellulose respectively in the lignocellulosic biomass [19]. It was estimated using bomb calorimeter and was found highest in kans grass (24.19). The calorific values of all the three feedstocks, before and after fractional hydrolysis are presented in Table 4.10.

Table 4.10. Calorific value analysis.

	Bagasse	Kans Grass	Wheat Straw
Untreated Biomass	22.97	24.19	21.7
XRF Removed Biomass	17.23	18.69	16.23
Treated Biomass	12.52	11.29	10.45

4.2.3.9 Surface area analysis

Several factors play important roles during the saccharification of lignocellulosic biomass (degree of polymerisation, biomass porosity, cellulose crystallinity, and behaviour of hydrolytic agents) [20]. The texture of the biomass gives a primary indication about the biomass digestibility which depends upon its surface area. The surface area, pore size and pore volume before and after fractional hydrolysis were estimated through BET and BJH method and represented in Table 4.11. Generally, small pores are not easily accessible for hydrolysis

whereas large pores increase the hydrolysis rate [21]. In the present study, biomass porosity after hydrolysis was studied and compared with the raw biomass. The results showed a reduction in surface area, pore volume and size of the hydrolysed biomass compared to the raw biomass.

Table 4.11. Surface area analysis.

	Bagasse		**Kans Grass**		**Wheat Straw**	
	Raw	Treated	Raw	Treated	Raw	Treated
Surface area (m^2/g)	2.26	0.82	2.26	0.97	2.14	0.73
Pore volume (mL/g)	0.004	0.0016	0.005	0.0013	0.0053	0.0014
Pore radius (nm)	11.24	7.26	12.25	8.53	11.97	6.23

4.3 Conclusion

8-stage hydrolysis experiments resulted in maximum sugar recovery with negligible toxics compared to 7-stage hydrolysis. Among all the acids used, TRS recovery was very less using phosphoric acid whereas nitric acid resulted in maximum sugar recovery, but the high cost makes it economically non-feasible. Results of both HCl and H_2SO_4 were comparable, but cheap price of H_2SO_4 makes it as the most suitable reagent for fractional hydrolysis process resulting in maximum sugar recovery with minimum toxics. As 8-stage fractional hydrolysis process with H_2SO_4 resulted in maximum sugar recovery with minimum toxics, to validate fractional hydrolysis results of kans grass, some other wide and easily available feedstocks were explored. Results were almost in the same range for all the three biomasses (saccharification%: kans grass 84.88, sugarcane bagasse 82.55, and wheat straw 81.66) which strengthened the statement that fractional hydrolysis technique is independent of the lignocellulosic biomass type and form. Therefore, feedstock type should not be a matter of worry for implementation of this technique during 2G ethanol production at commercial scale.

The structural characterisation of raw biomass, biomass after XRF removal, and fully treated biomass showed marked differences during all the analyses. SEM images of biomass showed surface distortions in the form of cracks and pores on the surface compared to the intact surface of raw feedstocks (Fig. 4.3). Such types of structural distortions were observed in various lignocellulosic biomass after pretreatment and hydrolysis [8, 22-23]. FESEM gave elemental composition at each stage with high resolution images. SPM was used to measure the

roughness analyses (Fig. 4.7-4.9). FTIR spectroscopic analysis of the biomass showed decrease in the absorption peaks indicating the loss of cellulose and hemicelluloses (Fig.4.10). Biomass crystallinity is believed to play a critical role in lignocellulosics digestibility in terms of cellulose conversion to reducing sugars [22]. XRD analysis was done to measure the changes in crystallinity indices of feedstocks during the course of fractional hydrolosis (Fig. 4.11). The fractional hydrolysis resulted in a decrease of % crystallinity. TGA provided information about the pyrolysis temperatures of cellulose, hemicelluloses, and lignin present in the feedstocks along with weight loss %. These results validate the efficiency of 8-stage fractional hydrolysis technique for the maximum sugar recovery with negligible toxics.

CHAPTER 5

Development of co-culture fermentation system using Zymomonas mobilis and Scheffersomyces shehatae

5.1 Introduction

In recent years, to cope with the fossil fuel's depletion, bioethanol industry has developed rapidly. The utilisation of edible raw material (corn) for bioethanol production has genarated criticism for creating undue pressure on the global food supply [1,2]. Therefore, the lignocellulosic materials which are unsuitable for human consumption have been considered as ideal feedstocks for ethanol production. The efficient conversion of two dominant sugars (pentose and hexose) present in the lignocellulosic hydrolysates to ethanol is a prerequisite for maximisation of the profitability of an industrial bioethanol production process [3,4]. Since no wild-type microorganism has the capability of efficiently accomplishing this process, two common approaches have been followed; the simultaneous utilisation of two microorganisms and the construction of genetically modified organisms. Providing optimal environmental conditions for the two microorganisms simultaneously is the main difficulty of using two strains for the fermentation of both sugars [5]. As reported by the majority of previous co-cultures studies, glucose fermentation in the sugar mixture proceeded efficiently while the xylose fermentation was of low efficiency and slow. Reasons might be the different oxygen requirements of two strains and/or the catabolite repression of glucose on the xylose assimilation [6–9]. To circumvent these difficulties and improve efficiency of the system, approaches in both strain and process engineering have been carried out. Process engineering modifications include immobilization of one or both the strains [7,8,12], continuous culture [8,10,11], two stage fermentation in a single bioreactor or sequential culture [13], and separate fermentation in two different bioreactors [14-15]. For the strain engineering, respiratory deficient mutants of *Saccharomyces cerevisiae and Saccharomyces diastaticus* [6,9,16,10,11,17], genetically modified ethanologenic *Escherichia coli* [18], and *Pichia stipitis* mutant showing restricted glucose catabolite repression have been developed [6].

Another improved process engineering approach has been proposed in the present study utilising *Z. mobilis* (for XRF fermentation) and *S. shehatae* (for GRF fermentation) co-culture system. The proposed system is believed to overcome the research challenge of maximum utilisation of pentoses and hexoses during fermentation, and enhances the ethanol production efficiency. *Z. mobilis* has many desirable characteristics as natural ethanologen:

- High specific productivity
- High ethanol tolerance,
- a broad pH range (3.5-7.5) for production, and

- Generally regarded as safe status [19-24]

For glycolysis, *Z. mobilis* uses the Entner-Doudoroff (ED) pathway instead of classical Embden-Meyerhof-Parnas (EMP). The EMP pathway is found in classical model ethanologens whereas ED pathway conducts fermentation with <50% ATP generation leading to improved ethanol yield. Moreover, it has a high-specific cell surface area that allows consuming sugars faster than model ethanologens (*S. cerevisiae*), thereby higher ethanol productivity is achieved by *Z. mobilis* [25]. Moreover, during the fermentation process scale-up, facultative anaerobic nature of *Z. mobilis* reduces the production cost for advanced aeration control.

Among xylose fermenting organisms, *Scheffersomyces shehatae* has been found to ferment ethanol faster compared to other organisms [26]; also the specific rate of ethanol production was highest [27]. It can utilise all the major sugars present in lignocellulosic hydrolysates, and no hydrolysate pretreatment is required as other yeast strains [28-29].

5.2 Results snd Discussion

5.2.1 Effect of toxics on the microbial growth

5.2.1.1 Effect of furfurals on *S. shehatae* growth

During the hemicellulose hydrolysis, xylose and other pentose sugars are liberated which may further degrade into furfural. Furfural is inhibitory to the growth of fermentative microorganisms. Therefore, the experiment was conducted to examine the effect of furfural on *S. shehatae* growth. In these experiments, different concentrations of 2-Furfuraldehyde (up to 0.5 g/L) was added to the xylose fermentation media along with the control (with no added furfural). The specific growth rate was calculated from the slope of $\ln(x/x_o)$ vs. Time during exponential growth phase. The % reduction in specific growth rate was calculated. 13% reduction in specific growth rate was found when the furfural concentration was 0.4 g/L, whereas it was more than 20% in 0.5 g/L of furfural. Therefore, furfural concentration up to 0.3 g/L was considered tolerable for *S. shehatae* growth in the xylose fermentation media. Boyyer et al. 1992 and Navarro 1994 have also reported that furfural reduces microbial growth rate significantly [30-31]. The reduction (%) in specific growth rate by increasing furfural concentration is shown in Fig. 5.1.

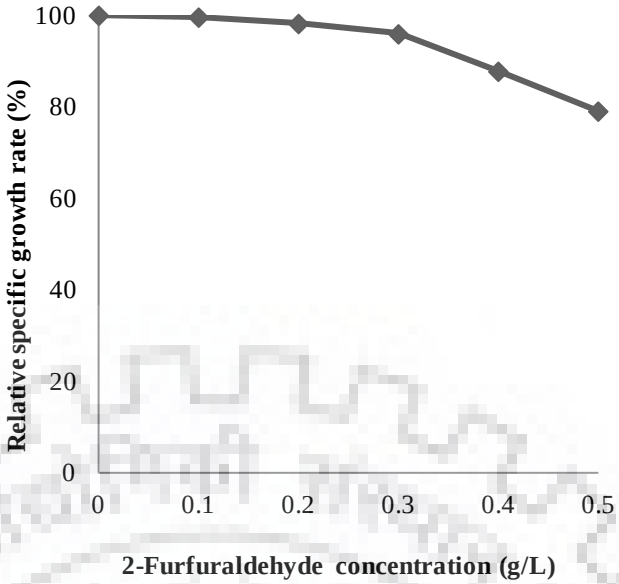


Fig. 5.1. Effect of furfurals on the specific growth rate of *S. shehatae*.

5.2.1.2 Effect of phenolics on *S. shehatae* growth

During the lignocellulosic biomass hydrolysis, phenolics and other aromatic compounds are liberated in large quantities from degradation of lignin. Phenolics are inhibitory to the growth of microorganisms during fermentation. Therefore, the experiment was conducted to examine the effect of vanillin (4-Hydroxy-3-methoxy benzaldehyde), a model phenolic compound on *S. shehatae* growth. In these experiments, different concentrations of vanillin (up to 2 g/L) was added to the xylose fermentation media along with the control (with no added vanillin). The specific growth rate was calculated from the slope of $\ln(x/x_o)$ vs. Time during exponential growth phase. The reduction in specific growth rates (%) were calculated. 7-8% reduction in specific growth rate was found when the vanillin concentration was 0.5 g/L, whereas it was more than 20% on increasing the concentration beyond it. Therefore, phenolics concentration up to 0.5 g/L in the fermentation media was considered tolerable for *S. shehatae* growth. Delgenes et al. 1996 have reported that vanillin and syringaldehyde reduce microbial growth rate significantly during fermentation [11]. The reduction (%) in specific growth rate by increasing vanillin concentration is shown in Fig. 5.2.

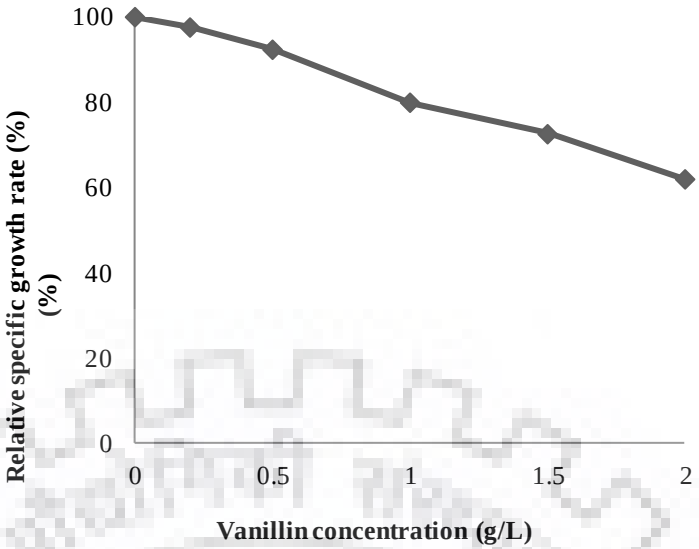


Fig. 5.2. Effect of phenolics on specific growth rate of *S. shehatae*.

5.2.1.3 Effect of 5-HMF on the growth of *Z. mobilis*

During the cellulose hydrolysis of lignocellulosic biomass, glucose and other hexose sugars are liberated which further degrade into 5-Hydroxymethyl furfuraldehyde (5-HMF). 5-HMF is inhibitory to the growth of glucose-fermenting organisms. Therefore, the experiment was conducted to examine the effect of 5-HMF on *Z. mobilis*. In these experiments, different concentration of 5-HMF (up to 2 g/L) was added to the glucose fermentation media along with the control (with no 5-HMF addition). The specific growth rate was calculated from the slope of ln (x/x_o) vs. time during exponential growth phase. The % reduction in specific growth rate was calculated. 11-12% reduction in the specific growth rate was found in presence of 1g/L 5-HMF, beyond it reduction was intense. Therefore, 1 g/L was considered tolerable for *Z. mobilis* growth in glucose-fermentation media. Fein et al. 1981 reported that 1.47% (w/v) 5-HMF concentration is inhibitory for *Z. mobilis* during glucose fermentation [32]. The reduction (%) in specific growth rate by increasing 5-HMF concentration is shown in Fig. 5.3.

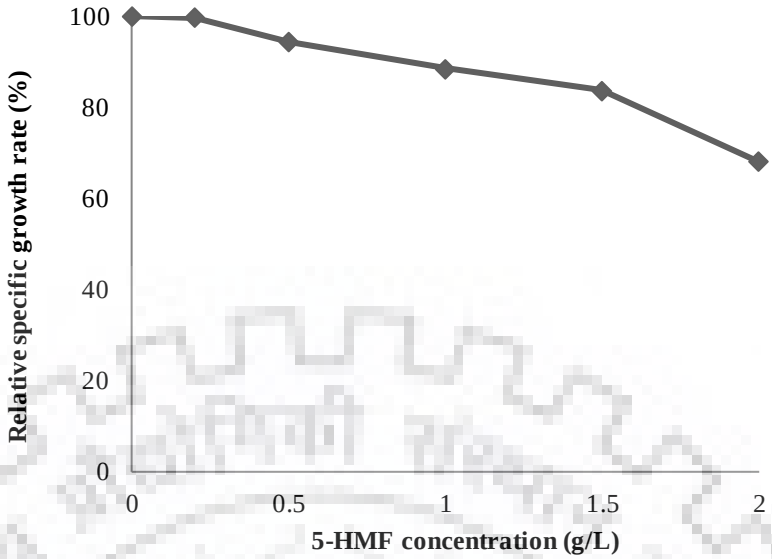


Fig. 5.3 Effect of 5-HMF on specific growth rate of *Z. mobilis*.

5.2.1.4 Effect of phenolics on the growth of *Z. mobilis*

As discussed previously, phenolics and other aromatic compounds are liberated in large quantities from degradation of lignin during the lignocellulosics hydrolysis. Phenolics are inhibitory to the growth of glucose-fermenting microorganisms also during fermentation. Therefore, the experiment was conducted to examine the effect of vanillin on the *Z. mobilis* growth. In these experiments, different concentrations of vanillin (up to 2 g/L) was added to the glucose fermentation media along with the control. The specific growth rate was calculated from the slope of $\ln(x/x_o)$ vs. Time during exponential growth phase. The % reduction in specific growth rate was calculated. 7-8% reduction in specific growth rate was found when the vanillin concentration was 0.5 g/L, whereas it was more than 17% on increasing phenolics concentration up to 1 g/L. Therefore, 0.5 g/L phenolics concentration in the glucose fermentation media was considered tolerable for *Z. mobilis* growth. Delgenes et al. 1996 observed that 0.5 g/L concentration of hydroxybenzaldehyde reduces *Z. mobilis* growth rate by 16% [11]. The reduction (%) in specific growth rate of *Z. mobilis* by increasing vanillin concentration is shown in Fig. 5.4.

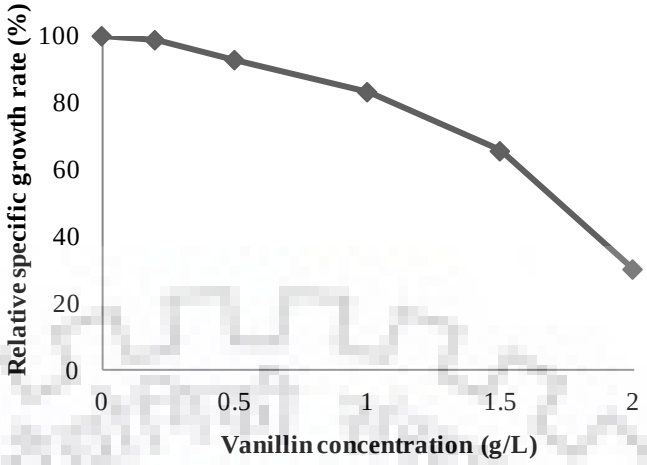


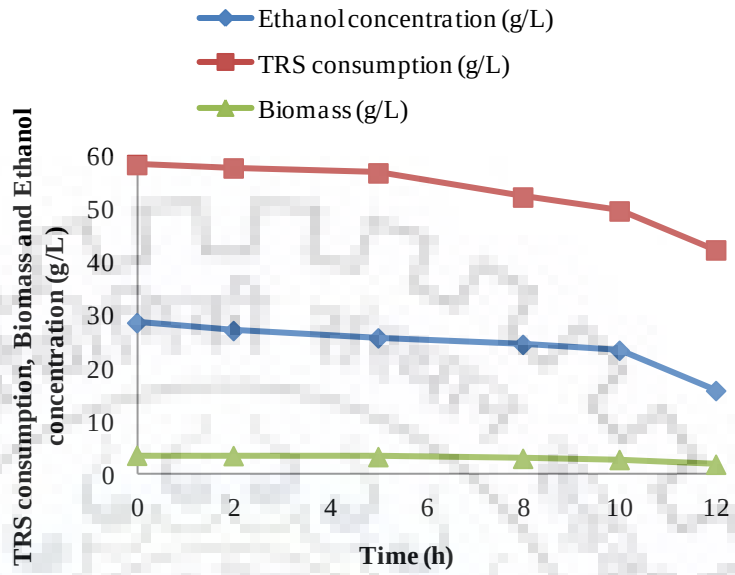
Fig. 5.4. Effect of Phenolics on the specific growth rate of *Z. mobilis*.

5.2.2 Ethanol tolerance of microorganisms used in co-culture fermentation

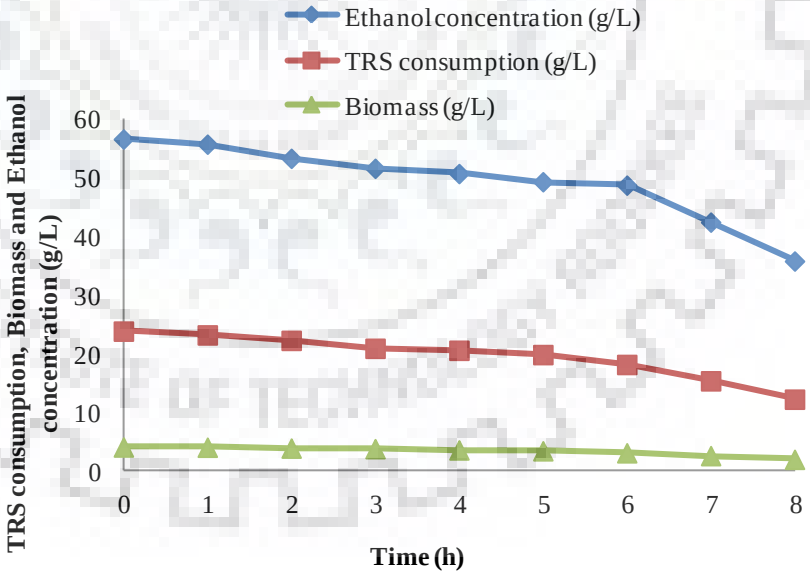
In the case of *Z. mobilis* MTCC 91, a gradual decrease in the biomass growth and glucose consumption has been observed upon increasing exogenous ethanol, thereby reducing ethanol production. Biomass growth and ethanol production were significantly inhibited after 10% exogenous ethanol, clearly seen from Fig. 5.5a. In the figure, ethanol concentration represents only the ethanol produced during the fermentation i.e. exogenous ethanol concentration was substituted from the final ethanol concentration. At 2% initial ethanol and 60 g/L sugar, 26.14 g/L of ethanol was produced after utilising 96.3% glucose whereas it was reduced to 23.42 g/L after 10% initial ethanol addition utilising glucose only up to 82.8%. Ethanol production was reduced significantly (15.83 g/L) by adding 12% exogenous alcohol. Therefore, it was concluded that *Z. mobilis* MTCC 91 had shown ethanol tolerance up to 10%. Panesar et al. 2006 reported 8% (v/v) ethanol tolerance of *Z. mobilis* MTCC 2428 in glucose medium at 30^{o}C [33].

S. shehatae NCIM 3501 has shown ethanol tolerance only up to 6%. Here also, biomass growth and xylose consumption were observed decreasing gradually upon increasing exogenous ethanol concentration; hence ethanol production was minimised. Biomass growth and ethanol production were significantly inhibited at 7% exogenous ethanol which can be observed clearly from the Fig. 5.5b. Ethanol concentration was 23.19 g/L after 1% initial ethanol addition utilising TRS up to 92.73% while it was reduced to 18.16 g/L after 6% initial ethanol addition utilising 81.11% TRS. Beyond 6% exogenous ethanol, a significant reduction in ethanol production was exhibited by the yeast. Wayman and Parekh 1985 reported that *Candida*

shehatae ATCC 22984 growth was ceased after 100 g/L ethanol production from 260 g/L sugar (glucose to xylose ratio was 70:30) [34].



(a)



(b)

Fig 5.5 Effect of exogenous ethanol addition on (a) *Z. mobilis* MTCC 91 (b) *S. shehatae* NCIM 3501.

Therefore, it is quite evident that glucose fermenting bacterial strain *Z. mobilis* MTCC 91 (up to 10%) shows better ethanol tolerance level compared to xylose-fermenting yeast *S. shehatae* NCIM 3501 (up to 6%).

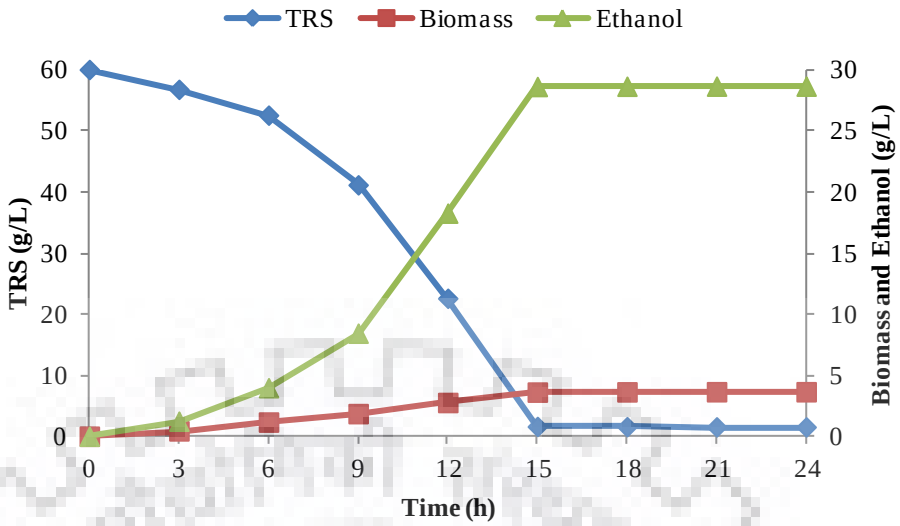
5.2.3 Monoculture fermentation of the kans grass hydrolysate

As discussed earlier in chapter 3, 8-stage fractional hydrolysis resulted in the highest recovery of fermentable sugars with TRS of 4.725±0.3 g/L in XRF and 7.62±0.4 in GRF. Therefore, for all the fermentation processes, acid-hydrolysate of kans gass biomass was conditioned and concentrated for desired sugar level in XRF and GRF after conditioning. Batch fermentation of XRF and GRF were conducted using *S. shehatae* and *Z. mobilis* strain respectively. Although the concentrations of toxics were quite low, few experiments were performed after detoxifying XRF and GRF using 5 g/L activated charcoal powder and results were compared with the un-detoxified and synthetic media (Table 5.1). The results were comparable in un-detoxified and detoxified media with insignificant improvement. Therefore, further co-culture experiments were conducted without any detoxification step prior to fermentation. In *Z. mobilis* fermentation medium, majorly glucose sugar contributes to TRS whereas, in case of *S. shehatae* fermentation medium, TRS represents xylose sugar.

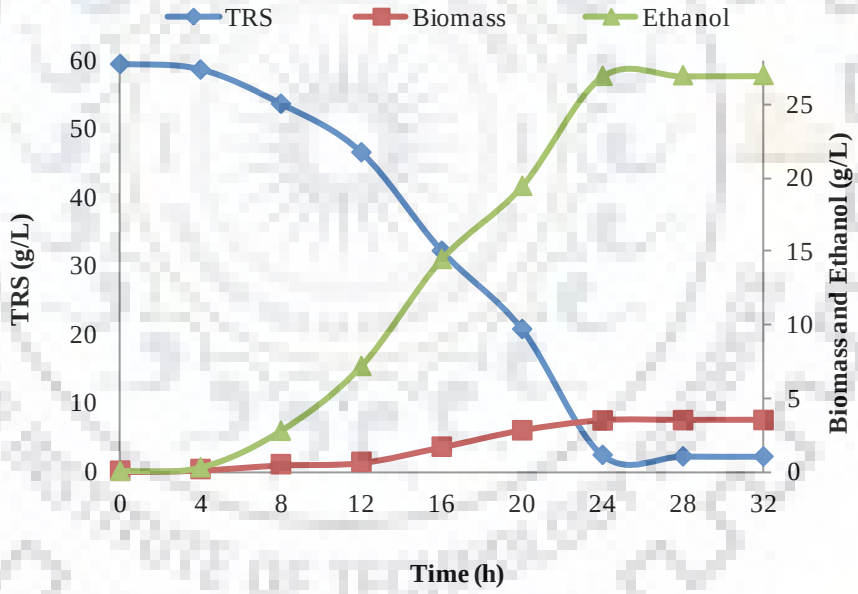
5.2.3.1 Monoculture fermentation using *Z. mobilis* in GRF obtained from kans grass hydrolysis

Conditioned GRF with an initial TRS of 59.36 g/L was fermented by *Z. mobilis*, whereas in the synthetic medium it was 60 g/L. Fermentation profiles of TRS, biomass, and ethanol during *Z. mobilis* monoculture system using synthetic and hydrolysate media are shown in Fig. 5.6a and 5.6b respectively. 97.33% glucose was utilised by the microbe in synthetic medium within 15 h and 95.06% sugar was utilised in hydrolysate medium after 24 h of fermentation. Comparison of kinetic parameters of GRF fermentation and synthetic media is shown in Table 5.1.

Almost three times longer lag phase was observed compared to synthetic medium. It can be reduced by propagating cells in the same hydrolysate medium up to 5-6 generations. Ethanol yield coefficient ($Y_{P/S}$) was observed as 0.47 and 0.49 in hydrolysate and synthetic medium respectively. Values of fermentation kinetic parameters were lower in hydrolysate medium compared to synthetic medium.



(a)



(b)

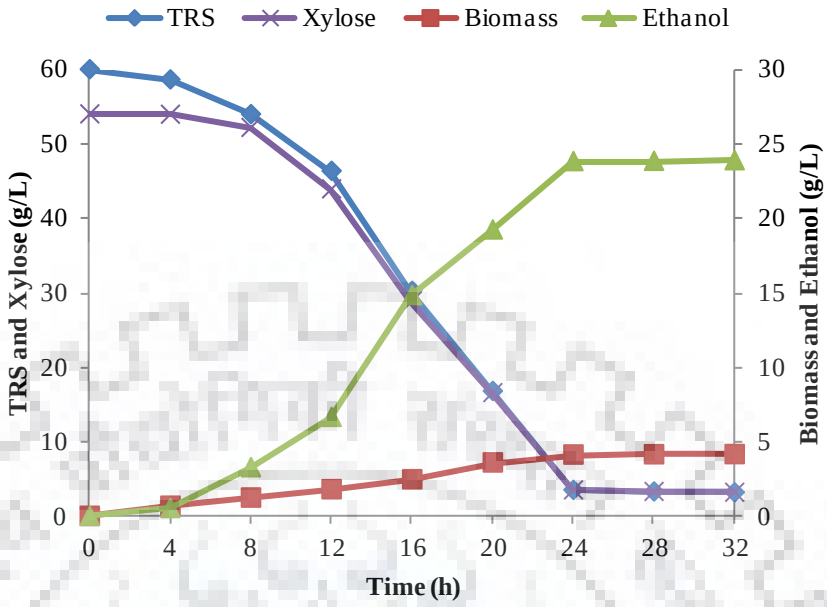
Fig. 5.6. Fermentation profile of *Z. mobilis* in (a) Synthetic medium (Initial sugar: 60 g/L) (b) Kans grass hydrolysate medium (Initial sugar: 59.36 g/L).

5.2.3.2 Monoculture fermentation using *S. Shehatae* in XRF obtained from kans grass hydrolysis

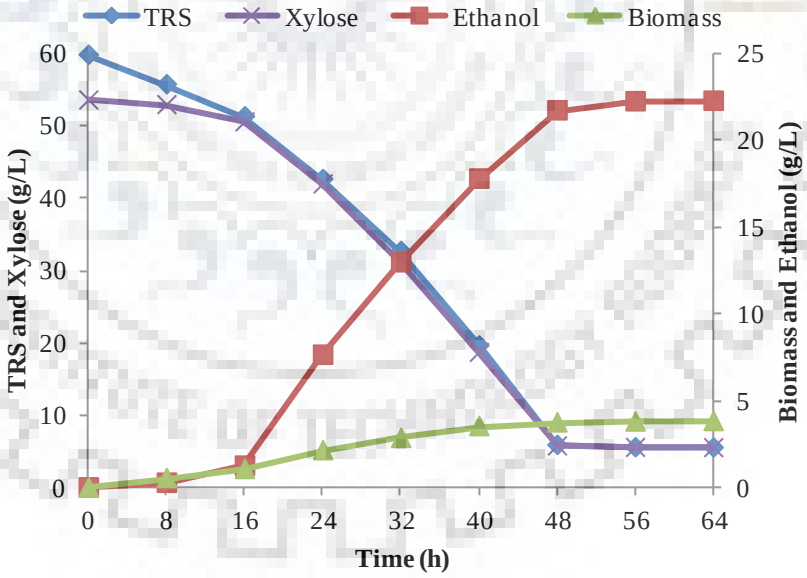
Conditioned XRF with an initial TRS of 59.72 g/L was fermented by *S. shehatae* while in the synthetic medium TRS was kept 60 g/L. Fermentation profiles of TRS, biomass, and ethanol during *S. shehatae* monoculture fermentation using synthetic and hydrolysate media is shown in Fig. 5.7a and 5.7b respectively. In synthetic medium, 94.18% xylose was utilised by the microbe within 24 h of fermentation and 90.18% sugar was utilised in hydrolysate medium after 48 h of fermentation. Comparison of kinetic parameters of XRF fermentation and synthetic media is shown in Table 5.1.

Duration of lag phase was double in case of hydrolysate medium, which can be further reduced by propagating cells in the same hydrolysate medium up to 3-4 more generations. Ethanol yield coefficient ($Y_{P/S,}$ g/g) was observed as 0.40 and 0.42 in the hydrolysate and synthetic medium respectively.

It should be noted down that no detoxification was required after saccharification to remove or reduce toxic compounds in the XRF and GRF; still, results are comparable with previously reported works on lignocellulolosic biomass hydrolysate fermentation system. Therefore, fractional hydrolysis technique can be considered as an efficient technique for soluble sugar production from lignocellulosic feedstocks, with minimal production of toxic compounds (for fermentation of sugars into ethanol).



(a)



(b)

Fig. 5.7 Fermentation profile of *S. shehatae* in (a) Synthetic medium (Initial sugar: 60 g/L) (b) Kans grass hydrolysate medium (Initial sugar: 59.72 g/L).

Table 5.1. Kinetic performances of *Z. mobilis* and *S. shehatae* in different media.

	Un-detoxified XRF	Detoxified XRF	Synthetic medium	Un-detoxified GRF	Detoxified GRF	Synthetic medium
Initial TRS (g/L)	59.72	59.13	60	59.36	59.17	60
Fermentation time (h)	48	45	24	24	22	15
Maximum cell conc. (g/L)	3.74	3.81	4.12	3.43	3.48	3.63
Biomass yield coefficient(g/g)	0.069	0.07	0.072	0.06	0.061	0.062
Maximum ethanol conc. (g/L)	21.69	22.48	23.83	26.9	26.94	28.63
Sugar consumption (%)	90.18	91.05	94.18	96.09	96.56	97.4
Ethanol yield coefficient (g/g)	0.402	0.417	0.42	0.47	0.471	0.49
Ethanol productivity (g/(L*h)	0.45	0.5	1.0	1.12	1.22	1.9
Residual sugar (g/L)	5.86	5.29	3.49	2.32	2.03	1.56
Sugar consumption rate (g/(L*h)	1.12	1.19	2.35	2.37	2.59	3.9
% theoretical yield	78.82	81.76	82.35	92.15	92.35	96.07

5.2.4 Co-culture fermentation of the kans grass hydrolysate using *S. shehatae* and *Z. mobilis*

Various strategies have been developed using *S. shehatae* and *Z. mobilis* for the ethanol production. Simultaneous additions of both the strains were done in the hydrolysate medium initially which resulted in very poor ethanol production due to their different aeration requirements and most of the xylose remained unutilised. Therefore, it was concluded that both the organisms must function separately either in one reactor by adding them sequentially or in two different reactors. Two-reactor strategy would require additional control and monitoring during the course of fermentation, therefore, the co-culture system was developed in a single reactor with sequential addition of microbes and their fermentation media.

5.2.4.1 Two- step sequential co-culture system

Prior addition of *S. shehatae* strain and accordingly the medium was based on the facts that 1. Glucose (as a carbon source) is assimilated by *S. shehatae* preferentially over xylose for growth and ethanol production 2. It has low ethanol tolerance (<7%, w/v) compared to *Z. mobilis* (>10%, w/v) 3. High concentration of glucose can suppress xylose fermentation by the yeast due to catabolite repression at initial stage [35]. Xylose bioconversion is completely inhibited at a glucose concentration of 2.3 g/L or higher [8], resulting in poor ethanol yield and/or delay of fermentation [36]. Limited co-culture work has been done for efficient 2G ethanol production in the past at a bioreactor level. Therefore, the present approach was developed, and results of hydrolysate medium were compared with the synthetic medium.

5.2.4.1.1 Two-step sequential co-culture fermentation of the kans grass hydrolysate (at low sugar concentration)

The process was initiated using synthetic medium with 20 g/L xylose using *S. shehatae* culture. After 20 h of fermentation, around 93% of xylose was utilised to produce 7.84 g/L ethanol. Glucose sugar along with other medium components was added after 20 h of fermentation. After *Z. mobilis* addition, the process can be considered as co-culture system. Fermentation profile during the whole process is shown in Fig. 5.8. The process was converted into strict anaerobic after *Z. mobilis* addition. *Z. mobilis* uses Entner-Doudoroff pathway instead of Entner-Meyerhof-Parnas pathway for glucose consumption in such case. It results in less biomass formation and more carbon utilisation for ethanol fermentation [37]. Sugar

assimilation rate was slower in case of *S. shehatae*. Kinetic parameters of the process are presented in Table 5.2. Average ethanol yield coefficient ($Y_{P/S,}$ g/g) was observed as 0.45.

For kans grass hydrolysate fermentation, xylose and glucose sugars were replaced with XRF (21.87 g/L TRS) and GRF (40.32 g/L TRS) respectively. XRF was assimilated after 42 h of fermentation whereas GRF was utilised within 44-68 h to produce ethanol. More extended lag phase was observed with respect to synthetic medium. Fermentation profile of both the organisms is shown in Fig. 5.9. 87.33% sugar of XRF and 92.08% of GRF was utilised to produce 25.0 g/L ethanol. Xylose was assimilated even after GRF addition to the fermentation medium but at a very slow rate. 6-7% less ethanol concentration was observed compared to synthetic medium. Kinetic parameters were compared to the co-culture fermentation using synthetic media (Table 5.2). Values of most of the fermentation parameters were lower in hydrolysate media compared to synthetic.

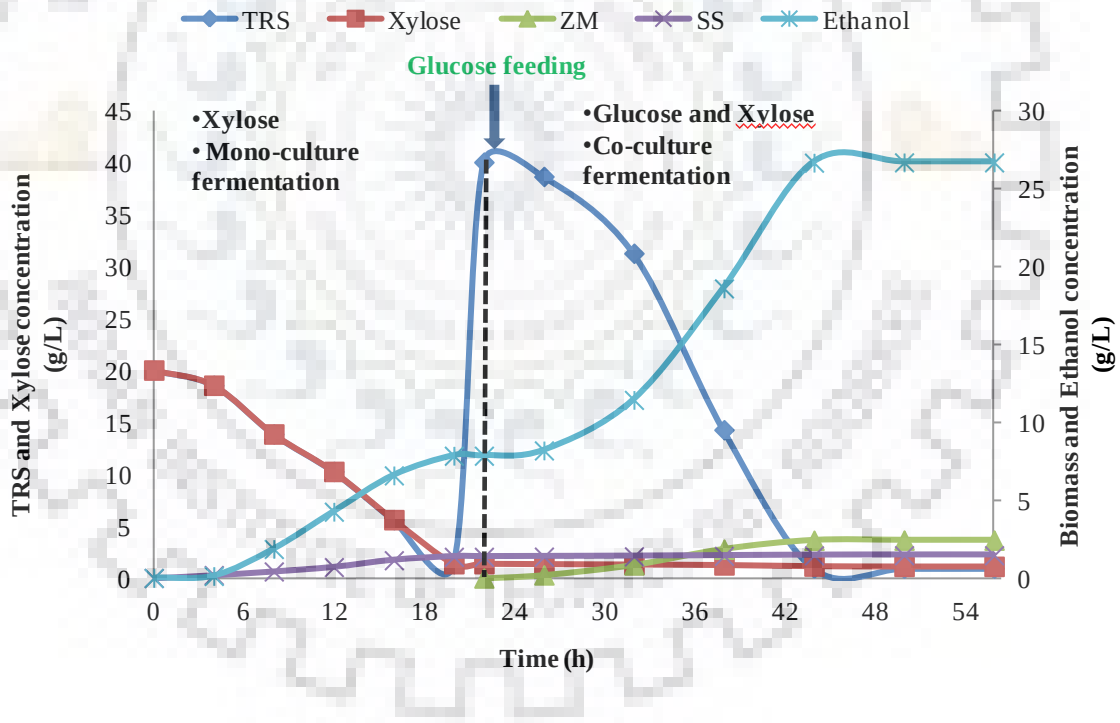


Fig. 5.8 Fermentation profile of *Z. mobilis* and *S. shehatae* co-culture system for ethanol production in synthetic medium with 60 g/L initial sugar (40 g/L glucose+ 20 g/L xylose). (ZM: *Zymomonas mobilis*; SS: *Scheffersomyces shehatae*)

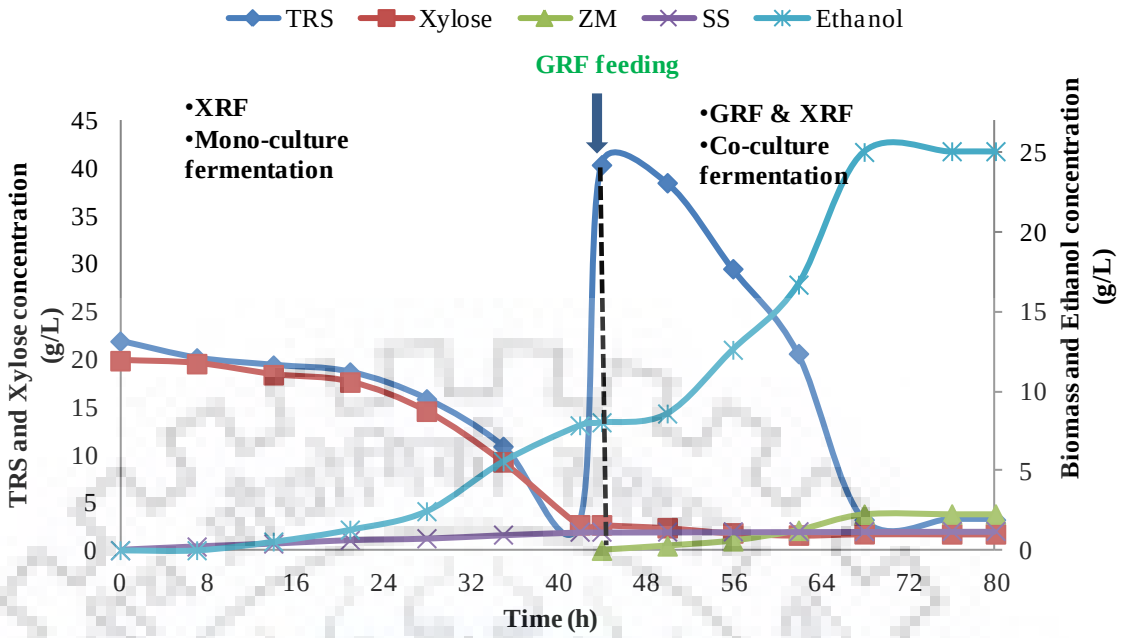


Fig. 5.9. Fermentation profile of *Z. mobilis* and *S. shehatae* co-culture fermentation system for ethanol production in kans grass hydrolysate medium with 62.19 g/L initial TRS (40.32 g/L in GRF+ 21.87 g/L XRF).

Table 5.2. Kinetic parameters comparison of co-culture fermentation using *Z.mobilis* and *S. shehatae* in synthetic and kans grass hydrolysate media (at low sugar concentration).

	Synthetic	**Hydrolysate**
Initial TRS (g/L)	20(X)+40(G)	21.87(XRF)+40.32(GRF)
Fermentation time (h)	44	68
Maximum cell conc. (g/L)	1.46 (SS) +2.43 (ZM)	1.29(SS) +2.26 (ZM)
Biomass yield coefficient(g/g)	0.077(SS) +0.062 (ZM)	0.071 (SS) +0.059 (ZM)
Maximum ethanol conc. (g/L)	26.63	25.0
Ethanol yield coefficient (g/g)	0.42 (Stage 1) + 0.48 (Stage 2)	0.41 (Stage 1) + 0.46 (Stage 2)
Ethanol productivity (g/(L*h)	0.61	0.37
Residual sugar (g/L)	1.15 (X) + 0.96(G)	1.72 (X) + 1.48(G)
Sugar consumption rate (g/(L*h)	0.931(Stage 1) + 1.95 (Stage 2)	0.45(Stage 1) + 1.55 (Stage 2)
Average % theoretical yield	88.23	85.3

5.2.4.1.2 Two-step sequential co-culture fermentation of the kans grass hydrolysate (At high sugar concentration)

The co-culture strategy described in previous section was repeated using high initial TRS concentration. In the synthetic medium, the process was initiated with 60 g/L xylose using 10% *S. shehatae* inoculum. After 24 h of fermentation, 91.95% of xylose was utilised to produce ethanol concentration of 23.26 g/L. Glucose sugar along with other media supplements was added after 24 h of fermentation, after which N_2 gas was sparged for 2 h to create the strict anaerobic environment for GRF fermentation by *Z. mobilis*. After *Z. mobilis* addition, the process can be considered as co-culture. Fermentation profile during the whole process has been shown in Fig. 5.10. Sugar assimilation rate was slower in case of *S. shehatae*. Kinetic parameters of the process have been presented in Table 5.3. Average ethanol yield coefficient ($Y_{P/S}$, g/g) has been observed as 0.435.

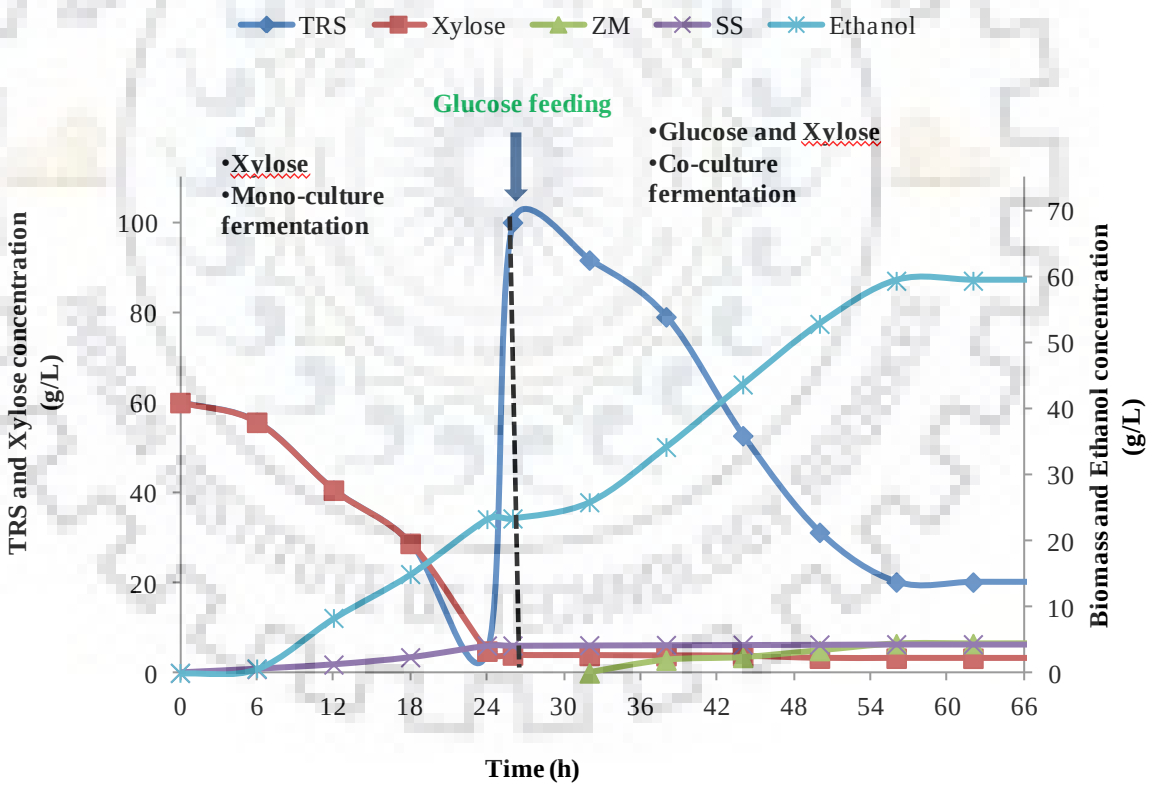


Fig. 5.10. Fermentation profile of *Z. mobilis* and *S. shehate* co-culture system for ethanol production in synthetic media with 100 g/L glucose and 60 g/L xylose concentration.

For the hydrolysate fermentation, xylose and glucose sugar in synthetic media were replaced with XRF and GRF containing 63.79 g/L and 100.25 g/L TRS respectively. XRF was assimilated within first 48 h of fermentation whereas GRF was utilised in 50-86 h to produce ethanol. Longer lag phase has been observed with respect to synthetic media. Fermentation profile is shown in Fig. 5.11. 90.31% sugar of XRF and 76.43% of GRF was utilised to produce 55.95 g/L of ethanol. Xylose was assimilated even after GRF addition to the fermentation media. 6-7% less ethanol concentration was observed compared to synthetic media. All kinetic parameters have been compared to synthetic media and presented in Table 5.3. Ethanol production rate was very high in synthetic media (1.05) compared to kans grass hydrolysate media (0.65). Values of most of the fermentation parameters were lower in hydrolysate media compared to synthetic media. Overall ethanol yield coefficient ($Y_{P/S,}$ g/g) has been observed as 0.41.

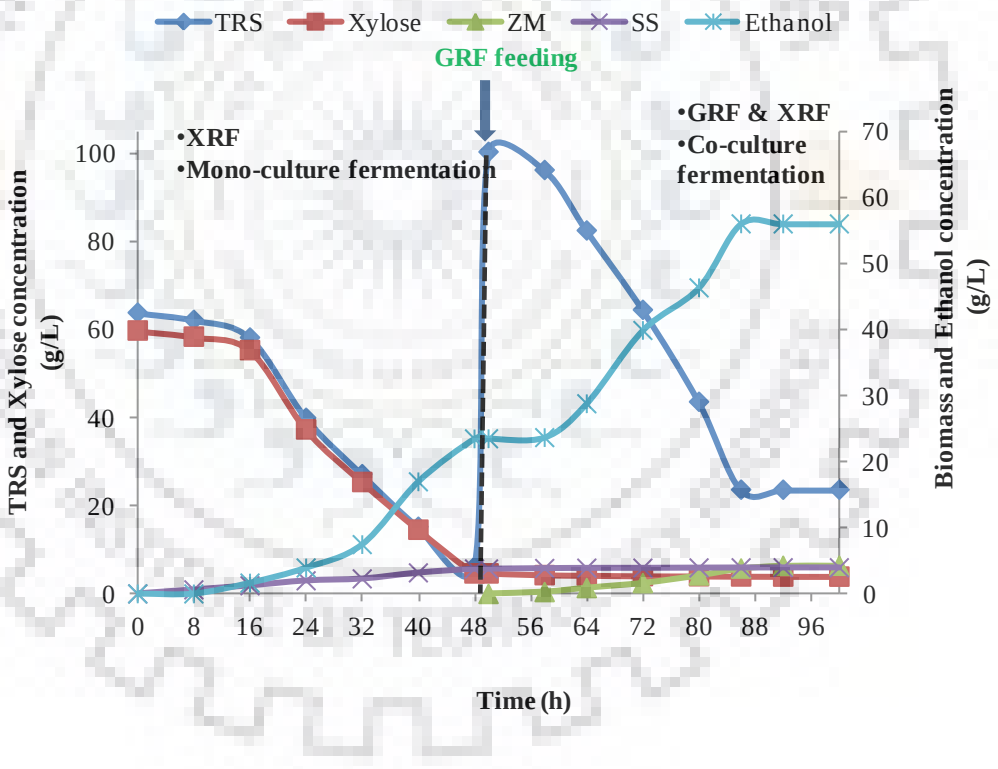


Fig. 5.11. Fermentation profile of *Z. mobilis* and *S. shehate* co-culture system for ethanol production in hydrolysate media with initial sugar of 100.25 g/L in GRF and 59.74 g/L in XRF.

Table 5.3. Kinetic parameters comparison of co-culture fermentation using *Z.mobilis* and *S. shehatae* in synthetic and kans grass hydrolysate media (At high sugar level).

	Synthetic media	**Hydrolysate media**
Initial TRS (g/L)	100 (G)+ 60 (X)	100.25 (GRF)+ 63.79 (XRF)
Fermentation time (h)	56	86
Maximum cell conc. (g/L)	4.42 (ZM)+ 4.15 (SS)	4.26 (ZM) +3.77 (SS)
Biomass yield coefficient(g/g)	0.056 (ZM)+ 0.075(SS)	0.051 (ZM)+ 0.064 (SS)
Maximum ethanol conc. (g/L)	59.34	55.95
Ethanol yield coefficient (g/g)	0.42 (Stage 1) + 0.45 (Stage 2)	0.4 (Stage 1) + 0.42 (Stage 2)
Ethanol productivity (g/L/h)	1.05	0.65
Residual sugar (g/L)	20.14 (G)+ 3.25 (X)	23.62 (G)+ 3.93 (X)
Sugar consumption rate (g/L/h)	2.29 (Stage 1) + 2.66 (Stage 2)	1.22(Stage 1) + 2.13 (Stage 2)
Average % theoretical yield	85.29	80.39

5.2.4.2 Multi-step successive glucose feeding co-culture system

It was observed from the previous co-culture strategy that upon increasing sugar concentration, sugar consumption rate decreases to a large extent. Moreover, ethanol concentration >55 g/L is very difficult to obtain even on increasing glucose up to 200 g/L (when 60g/L TRS in XRF and 200 g/L in GRF were used). Therefore, multi-step successive glucose feeding co-culture system was developed to overcome these problems.

5.2.4.2.1 Effect of initial glucose level

To determine the effect of the initial TRS concentration on ethanol production by *Z. mobilis*, experiments were conducted using glucose concentrations range of 50-300 g/L in the fermentation medium. An increase in the initial glucose concentration resulted in prolonged fermentation and reduced biomass and ethanol yield coefficients. The ethanol concentrations achieved by using 200 g/L and 250 g/L glucose were in the range of 77-80 g/litre (Table 5.4). Lag phase was increased and ethanol yield was decreased with an increase in the initial glucose concentration (Table 5.4). Highest ethanol yield was obtained by using 50 g/L glucose. Sugar consumption (%) did not vary significantly up to 200 g/L glucose, whereas, at higher glucose concentrations, it was reduced. An inoculums level of 10% (v/v) was used in all experiments.

Table 5.4. Effect of initial glucose concentration on the ethanol production by *Z. mobilis*.

Initial glucose (g/L)	50	100	150	200	250	300
Fermentation time (h)	15	28	42	54	75	84
Maximum cell conc. (g/L)	2.95	4.47	5.53	5.97	6.49	6.83
Biomass yield coefficient(g/g)	0.06	0.047	0.039	0.032	0.029	0.026
Maximum ethanol conc. (g/L)	23.94	46.34	65.25	77.18	79.48	80.34
Sugar consumption (%)	97.5	95.75	93.55	92.07	89.3	87.21
Ethanol yield coefficient (g/g)	0.491	0.484	0.465	0.397	0.356	0.307
Ethanol productivity (g/(L*h)	1.33	1.66	1.56	1.43	1.06	0.96
Residual sugar (g/L)	1.25	4.24	9.67	15.85	26.73	38.37
Sugar consumption rate (g/(L*h)	3.25	3.42	3.34	3.41	2.97	3.11
% theoretical yield	96.27	94.9	91.17	77.84	69.8	60.19

5.2.4.2.2 Step-feeding of glucose

To enhance the ethanol production and reduce possible substrate inhibition, glucose addition was done in a stepwise manner. The experiments were conducted using glucose concentrations of 50-250 g/L, which were divided into 1-5 equal fractions. As concluded from the earlier experiments, an initial glucose concentration of 50 g/L was consumed after 15 h of fermentation; therefore, sugar was added as 50 g/L increments. Second increment was made after 15 h interval (Table 5.5). The next additions were made at 10 h intervals, substituting the growth lag time from total fermentation time. The maximum ethanol concentration in the step-feeding system for a total glucose concentration of 100 g/L was 45.6 g/L, which was achieved at 25 h. For a total glucose of 150 g/L, the ethanol concentration was found to be 68.39 g/L by using a step-feeding system, during 35 h of fermentation. Table 5.5 shows the kinetic parameters at different times of glucose addition for a total glucose concentration up to 250 g/L. Bacterial growth coefficient decreased with different steps of glucose addition, whereas ethanol yield coefficient was in the range of 0.4-0.5 up to 200 g/L glucose. The step-feeding system reduced the fermentation time by about 9 h compared with the batch process with an initial glucose concentration of 200 g/L. The maximum ethanol concentration of 89.35 g/L was obtained with this sugar addition. For 250 g/L of total glucose addition, no substantial increase in ethanol level was observed, while the residual glucose concentration was 30.75 g/L. It was

concluded that the stepwise addition of glucose did not allow the inhibition exercised by product and/or substrate or by the formation of metabolically inactive cells.

Table 5.5. Step feeding of glucose for ethanol production by *Z. mobilis*.

Fermentation time (h)	0	15	25	35	45	55
Added Glucose (g/L)	50	100	150	200	250	-
Initial Glucose (g/L)	-	50	51.23	53.77	52.85	53.59
Maximum cell conc. (g/L)	-	2.95	4.19	5.27	6.25	6.46
Biomass yield coefficient(g/g)	-	0.06	0.027	0.023	0.02	0.009
Maximum ethanol conc. (g/L)	-	23.94	45.6	68.39	89.35	90.69
Sugar consumption (%)	-	97.5	90.92	87.8	92.77	44.68
Ethanol yield coefficient (g/g)	-	0.49	0.47	0.48	0.43	0.06
Ethanol productivity (g/(L*h)	-	1.33	1.78	1.93	1.96	1.64
Residual sugar (g/L)	-	1.25	4.65	6.69	4.18	30.75
Sugar consumption rate (g/(L*h)	-	3.25	4.6	4.8	5.36	2.48
% theoretical yield	-	96.27	92.15	94.11	84.31	11.76

5.2.4.2.3 Multi-step successive glucose feeding co-culture fermentation using synthetic media (At flask level)

The above strategy of glucose addition in a stepwise manner to enhance the ethanol production and reduce the possible substrate inhibition was tried in a co-culture system. Initially xylose sugar with concentration of 50 g/L was fermented. After the exhaustion of xylose, glucose along with other media supplements was added for *Z. mobilis* fermentation. The experiments were conducted using TRS concentrations of 50-250 g/L, which were divided into 1-5 equal fractions. The important parameters and process conditions favourable for better fermentation performance were examined at flask level. The prior addition *S. shehatae* and fermentation medium were done due to glucose assimilation by the yeast preferentially over xylose as main carbon source for their growth and ethanol production. Furthermore, it has lower ethanol tolerance (<7%, w/v) than *Z. mobilis* (>10%, w/v). Moreover, high glucose concentration can suppress xylose fermentation due to catabolite repression, resulting in poor ethanol yield and/or productivity [36]. Therefore, *S. shehate* fermentation using xylose sugar as carbon source was conducted first, followed by *Z. mobilis* fermentation using glucose. Occurrence of any inhibition of xylose fermentation due to rapid ethanol production from glucose (using *Z. mobilis*) was also

taken care and efficient utilisation of both sugars was achieved for their conversion to ethanol. N_2 sparging at the onset of stage 2 under static condition removed the dissolved oxygen completely from the fermentation medium and provided a strict anaerobic environment for glucose fermentation by the bacterium. The results are presented in Table 5.6. The fermentation parameters (ethanol yield coefficient, substrate utilisation rate, and volumetric ethanol productivity) were calculated to evaluate performance of the co-culture system. The kinetic parameters were calculated for each stage as well as for the whole system. The fermentation profile for this co-culture system is shown in Fig. 5.12. The xylose consumption by *S. shehatae* was observed to be slower compared to the *Z. mobilis*-mediated glucose fermentation system. The actual co-culture system exists only during 2nd–5th stages. Stage 1 represents the mono-culture batch fermentation by *S. shehatae*, whereas stage 2-5 can be termed as the co-culture system of *S. shehatae* and *Z. mobilis*. Average ethanol yield and productivity have been shown only up to stage 4; beyond it, ethanol production rate reduced significantly. Hence, the process can be carried out only up to 4th stage.

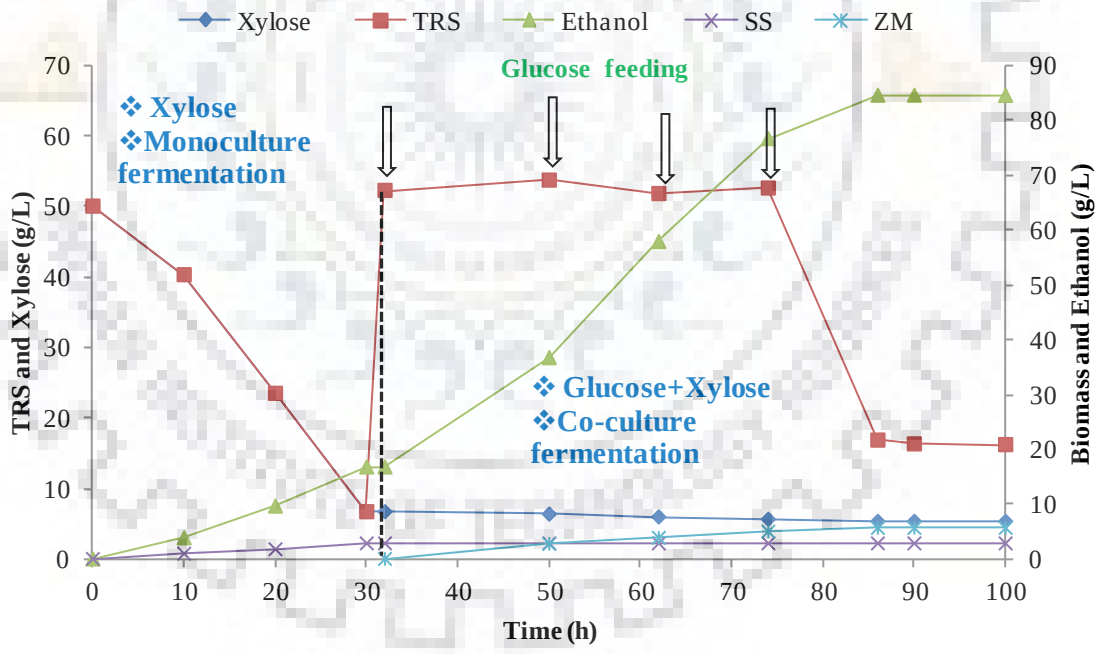


Fig. 5.12. Fermentation profile of multi-step glucose feeding co-culture system (At flask level).

Table 5.6. Kinetic performance of multi-step glucose feeding co-culture system (At flask level).

Stage	1	2	3	4	5
Type of sugar	***Xylose***			***Glucose***	
Initial TRS (g/L)	50.0	52.23	53.69	51.78	52.57
Fermentation time (h)	30	50	62	74	86
Maximum cell conc. (g/L)	2.83 (SS)	2.74 (ZM)	3.85 (ZM)	4.98 (ZM)	5.65 (ZM)
Maximum ethanol conc. (g/L)	16.76	36.67	57.83	76.59	84.48
Residual sugar (g/L)	6.79	8.26	7.83	6.75	16.84 [5.32 (X)+11.52 (G)]
Biomass yield coefficient(g/g)	0.065	0.062	0.024	0.024	0.019
Ethanol yield coefficient (g/g)	0.39	0.45	0.46	0.42	0.22
Sugar consumption rate (g/(L*h)	1.44	2.44	3.82	3.75	2.97
Sugar consumption (%)	86.42	84.18	85.41	87.0	67.96
% theoretical yield	76.47	88.23	90.2	82.35	76.47
Overall ethanol productivity (g/(L*h)	1.03 (Up to 4th stage)				
Average ethanol yield coefficient (g/g)	0.43 (Up to 4th stage)				

5.2.4.2.4 Multi-step successive glucose feeding co-culture fermentation using synthetic media (At bioreactor level)

The selected strategy at flask level for multi-step feeding-co-culture system was further applied at bioreactor level for ethanol production to validate the results and provide more controlled environment. The working volume was increased up to 2.75 L (>10x). Total 4 stages were used to evaluate the overall fermentation system performance. 1st stage represents mono-culture fermentation of xylose by *S. shehatae* and 2nd-4th stage were the co-culture system using *Z. mobilis* and *S. shehatae*. The fermentation profile of this co-culture system is shown in Fig. 5.13.

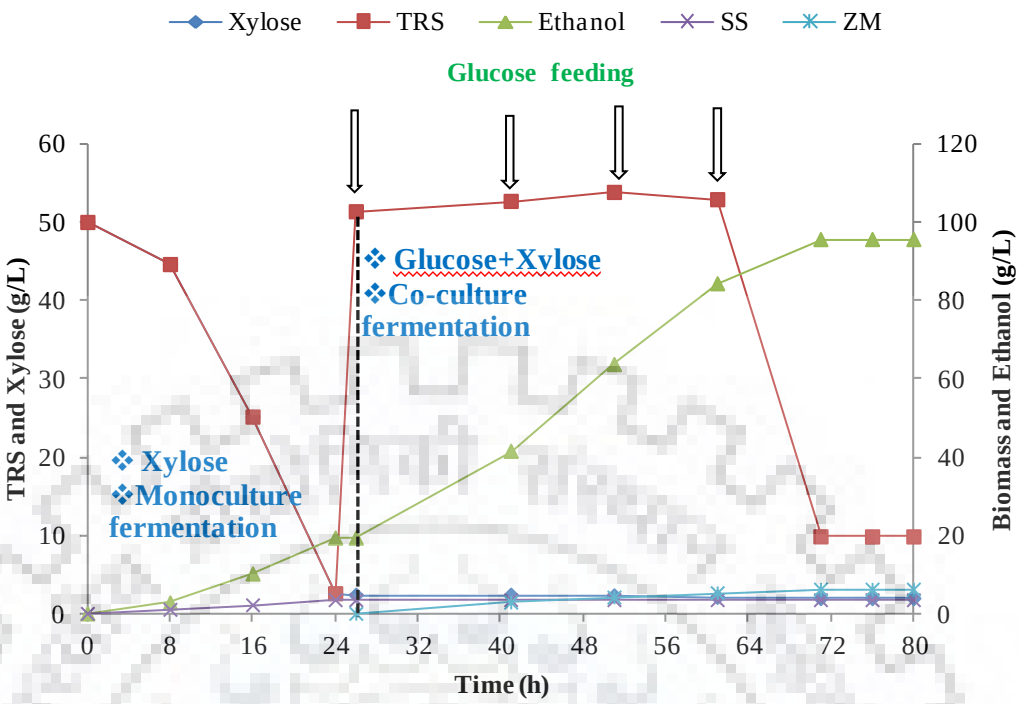


Fig. 5.13. Fermentation profile of multi-step glucose feeding co-culture system (At bioreactor level).

The patterns of sugar consumption, biomass, and ethanol production were same as flask level fermentation. Microaerobic condition at stage 1 and strict anaerobic condition at later stage (by sparging N_2), and controlled pH resulted in better fermentation. The results are given in Table 5.7. The average ethanol yield coefficient and overall volumetric ethanol productivity were calculated as 0.46 g/g and 1.38 g/L/h respectively. The average ethanol yield was 1.07 times and overall volumetric ethanol productivity was about 1.34 times higher than flask level system. The results were compared with the previously reported co-culture studies, although few have been conducted using glucose-xylose mixture at bioreactor level. In this case also, average ethanol yield and productivity have been shown only up to stage 4 as ethanol production rate reduced significantly beyond it. Hence, the process can be carried out only up to 4^{th} stage.

Table 5.7. Kinetic performance of multi-step glucose feeding co-culture system (At bioreactor level).

Stage	1	2	3	4	5
Type of sugar	Xylose			Glucose	
Initial TRS (g/L)	50	51.37	52.69	53.84	52.91
Fermentation time (h)	24	41	51	61	71
Maximum cell conc. (g/L)	3.48 (SS)	2.87 (ZM)	4.16 (ZM)	5.28 (ZM)	6.19 (ZM)
Maximum ethanol conc. (g/L)	19.36	41.54	63.69	84.34	95.63
Residual sugar (g/L)	2.57	4.74	6.83	8.77	9.84 [1.95 (X)+7.89(G)]
Biomass yield coefficient(g/g)	0.073	0.061	0.028	0.025	0.021
Ethanol yield coefficient (g/g)	0.41	0.48	0.48	0.46	0.26
Sugar consumption rate (g/(L*h)	1.98	3.1	4.58	4.5	4.3
Sugar consumption (%)	94.86	90.77	87.04	83.71	81.4
% theoretical yield	80.39	94.11	94.11	90.19	64.7
Overall ethanol productivity (g/(L*h)	1.38 (Up to 4th stage)				
Average ethanol yield coefficient (g/g)	0.46 (Up to 4th stage)				

5.2.4.2.5 Multi-step successive glucose feeding co-culture fermentation of kans grass hydrolysate

The aforementioned co-culture strategy was extended for kans grass hydrolysate fermentation media (using XRF and GRF) for ethanol production. In the multistep glucose feeding co-culture fermentation system, the XRF along with other medium components were first fermented by *S. shehatae* to suppress the catabolite repression of pentose by hexose sugar and avoid the possible ethanol inhibition. After XRF fermentation, GRF was fermented by *Z. mobilis* under anaerobic conditions. The fermentation profile during the present co-culture system is shown in Fig. 5.14. Total fermentation time was 101 h (0-45 h for XRF and 45-101 h for GRF), whereas, with synthetic sugars, it was only 61 h (0-24 h for xylose and 24–61 h for glucose). Longer adaptation period of *S. shehatae* fermentation during stage 1 majorly

contributed to it, which may be reduced by using adaptive cells. Average sugar consumed during stage 1 and stages 2nd-4th were 91.19 and 83.94% respectively. Less than 1% xylose was utilised during stage 2-4. The average ethanol yield coefficient and overall volumetric ethanol productivity were found as 0.44 and 0.79 g/L/h respectively up to stage 4 (Table 5.8). 79.59 g/L of ethanol concentration was achieved up to the 4th stage which was about 5-6% less than synthetic sugar fermentation medium. Biomass yield coefficients were also lower for both the organisms after utilising kans grass acid hydrolysate medium. Presence of some toxics might be the possible reason for it [38]. Comparison of multi-step successive co-culture and 2-step sequential co-culture system has been presented in Table 5.9 which proves the advancement of multi-step co-culture system over the 2-step in terms of average ethanol yield, maximum ethanol concentration and productivity.

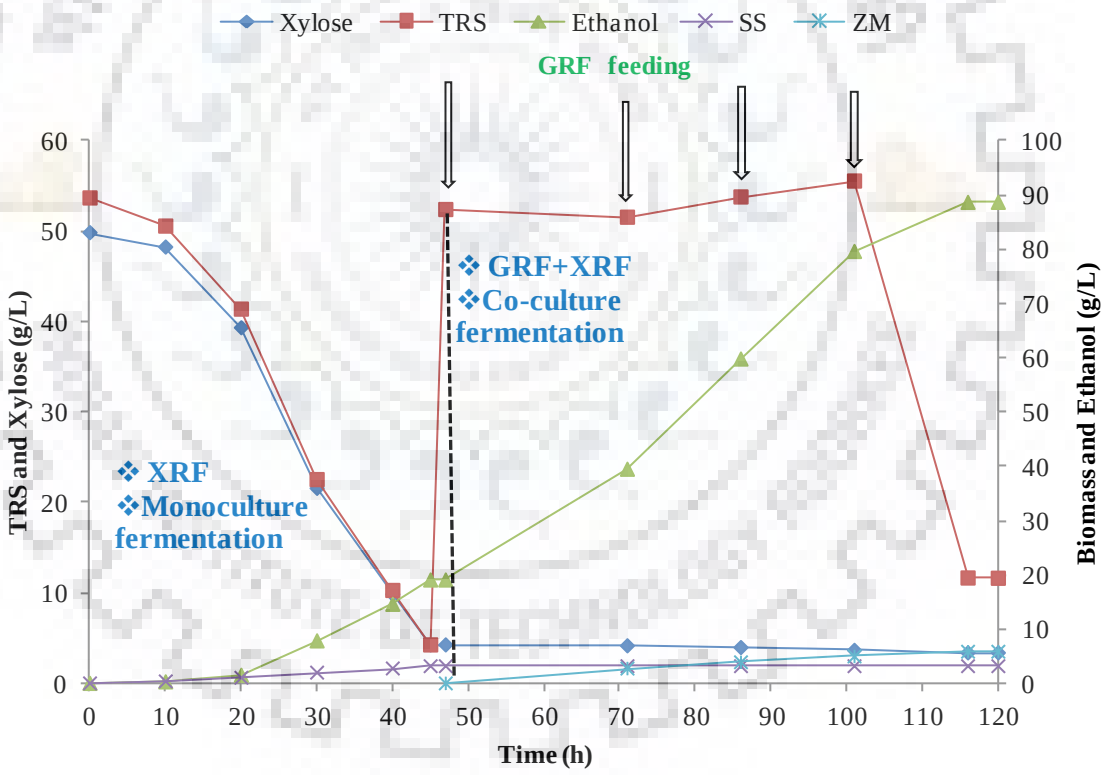


Fig. 5.14. Fermentation profile of multi-step glucose feeding co-culture system in kans grass acid hydrolysate media.

Table 5.8. Kinetic performance of multi-step glucose feeding co-culture system in kans grass acid hydrolysate media.

Type of sugar	XRF	GRF			
Stage	1	2	3	4	5
Initial TRS (g/L)	53.67 [49.83 (X)+ 3.84 (G)]	52.39	51.54	53.78	55.54
Fermentation time (h)	45	71	86	101	116
Maximum cell conc. (g/L)	3.16 (SS)	2.73 (ZM)	3.88 (ZM)	4.97 (ZM)	5.84 (ZM)
Maximum ethanol conc. (g/L)	19.05	39.46	59.77	79.59	88.63
Residual sugar (g/L)	4.73 [4.25 (X)+0.48(G)]	6.68	8.59	10.07	11.65 [3.37 (X)+5.28(G)]
Biomass yield coefficient(g/g)	0.065	0.06	0.027	0.025	0.02
Ethanol yield coefficient (g/g)	0.39	0.45	0.47	0.45	0.21
Sugar consumption rate (g/(L*h)	1.09	1.9	2.86	2.91	2.92
Sugar consumption (%)	91.19	87.24	83.33	81.27	79.02
% theoretical yield	76.47	88.23	92.16	88.23	41.17
Overall ethanol productivity (g/(L*h)	0.79 (Up to 4th stage)				
Average ethanol yield coefficient (g/g)	0.44 (Up to 4th stage)				

Table 5.9. Comparison of multi-step successive co-culture and 2-step sequential co-culture system.

	2-step sequential co-culture system	**Multi-step successive glucose feeding co-culture system**
Initial TRS (g/L)	Up to 200 g/L	Up to 200 g/L
Fermentation time (h)	86	101
Maximum ethanol conc. (g/L)	55.95	79.59
Average ethanol yield coefficient (g/g)	0.41	0.44
Ethanol productivity (g/L/h)	0.65	0.79
Total residual sugar (g/L)	25.62	10.07
Average % theoretical yield	80.39	86.27

5.3 Conclusion

Therefore, using multi-step co-culture system containing *Z. mobilis* and *S. shehatae*, a high ethanol yield coefficient (0.44) was achieved with a concentration of 79.59 g/L using concentrated kans grass hydrolysate. The difficulties in controlling different oxygen requirements, pH, and temperature of two organisms in a co-culture system were also removed with maximum sugar utilisation. The lower ethanol tolerance of pentose fermenting organisms and catabolite repression of xylose metabolism (at high glucose concentration) was successfully addressed with a % theoretical yield of 86.27. Moreover, the single reactor operation definitely reduces the process costs. The number of research articles describing the lignocellulosic biomass hydrolysate fermentation for ethanol production using co-culture technique in bioreactor is few, although all the batch co-fermentation studies of glucose and xylose sugars (synthetic/hydrolysate and/or flask level/bioreactor level) are presented and compared with the present co-culture studies in Table (5.10). Overall advantage in terms of ethanol yield, maximum ethanol concentration, and productivity of the multi-step co-culture system can be clearly seen from the Table 5.10.

Table 5.10. Co-culture studies for glucose and xylose sugars in batch mode for bioethanol production.

S.no	Organisms used in co-fermentation	Feedstocks used	Operating conditions	Key findings	Reference
1.	*Z. mobilis* MTCC 91- *S. sthehatae* NCIM 3501	Hydrolysate obtained from kans grass biomass (50 g/L XRF media and 200 g/L GRF media)	1. Temperature: 30°C 2. Agitation speed: 150 rpm for *S. stipitis* 3. Aeration: 0.5 vvm	1. Ethanol productivity: 0.79 g/L*h 2. Ethanol yield: 0.44 3. Maximum ethanol concentration (g/L): 79.59	Present study
2.	*Z. mobilis* MTCC 91- *S. sthehatae* NCIM 3501	Synthetic media (xylose 50 g/L and glucose up to 200g/L) in bioreactor	1. Temperature: 30°C 2. Agitation speed: 150 rpm for *S. stipitis* 3. Aeration: 0.5 vvm	1. Ethanol productivity: 1.38 g/L*h 2. Ethanol yield: 0.46 3. Maximum ethanol concentration (g/L): 84.34	Present study
3.	*Z. mobilis* MTCC 91- *S. sthehatae* NCIM 3501	Syntheic xylose 50 g/L and glucose up to 200g/L (At flask level)	1. Temperature: 30°C 2. Agitation speed: 150 rpm for *S. stipitis*	1. Ethanol productivity: 1.03 g/L*h 2. Ethanol yield: 0.43 3. Maximum ethanol concentration (g/L): 76.6	Present study
4.	*Z. mobilis* MTCC 91- *S. sthehatae* NCIM 3501	Hydrolysate obtained from kans grass biomass (XRF 60 g/L and GRF media up to 200g/L) in bioreactor	1. Temperature: 30°C 2. Agitation speed: 150 rpm for *S. stipitis* 3. Aeration: 0.5 vvm	1. Ethanol productivity: 0.65 g/L*h 2. Ethanol yield: 0.41 3. Maximum ethanol concentration (g/L): 55.95	Present study
5.	*Z. mobilis* MTCC 91- *S. sthehatae* NCIM 3501	Hydrolysate obtained from kans grass biomass (XRF 20 g/L and GRF media up to 40g/L) in bioreactor	1. Temperature: 30°C 2. Agitation speed: 150 rpm for *S. stipitis* 3. Aeration: 0.5 vvm	1. Ethanol productivity: 0.37 g/L*h 2. Ethanol yield: 0.435 3. Maximum ethanol concentration (g/L): 25.0	Present study
6.	*Z. mobilis* MTCC 91- *P. stipitis* NCIM 3498	Hydrolysate obtained from kans grass biomass (XRFM 60 g/L and GRFM 200g/L)	1. Temperature: 30°C 2. Agitation speed: 150 rpm for *S. stipitis*	1. Ethanol productivity: 1.58 g/L*h 2. Ethanol yield: 0.453 3. Maximum ethanol concentration (g/L): 57.8	[35]
7.	*Clostridium thermocellum* CT2–Clostridium *thermosaccharolyticum* HG8	Alkali treated banana waste (100 g/L)	1. Inoculum size: 5% (v/v) 2. Temperature: 60°C 3. pH: 7.5 4. Fermentation time: 5 days	1. Ethanol yield: 0.41 2. Maximum ethanol concentration (g/L): 22	[39]
8.	Immobilised *Z. mobilis*+ *P. stipitis*	30 g/L glucose and 20 g/L Xylose mixture	1. Temperature: 30°C 2. Stirring speed: 150 rpm 3. Working volume: 800 mL 4. Air flow rate level: 80 cm^3/min	1. Ethanol yield: 0.49-0.50 2. Volumetric ethanol productivity (g/L*h): 1.277	[40]
9.	*E. coli* KO11–*S. cerevisiae* TJ1	Waste house wood hydrolysate medium (27.0 g/L glucose and 17.0 g/L xylose) with 1% (v/v) corn steep liquor	1. Inoculum size: *E. coli* (0.2 g-dry cell weight/L) and *S. cerevisiae* (0.02 g-dry cell weight/L) 2. Temperature: 35°C 3. pH: 6.0 (controlled by 10 N KOH) 4. Working volume: 200 mL 5. Shaking rate: 80 rpm 6. Oxygen transfer rate: 5-7 mmol/(L* h)	1. Xylose utilisation efficiency: 46% 2. Ethanol yield: 0.43 3. Maximum ethanol concentration (g/L): 30.3	[41]

10.	*Z. mobilis-P. tannophilus*	60 g/L glucose and 40 g/L xylose)	1. Temperature: 30°C 2. Working volume: 900 mL 3. No aeration at glucose fermentation stage 4. Aeration Level<1 mmol/L/h at xylose fermentation stage	1. Ethanol yield: 0.33 2. Maximum biomass yield (CFU/mL) *Z. mobilis*: 5.1×10^7 *P. tannophilus*: 5.7×10^7 3.Volumetric ethanol productivity (g/L*h): 2.32	[13]
11.	*Z. mobilis* MTCC 92–*C. tropicalis* TERI SH110	Hydrolyzed fruit and vegetable residues	1. Inoculum size: 10% (v/v) 2.Working volume: 50 mL 3. Temperature: 30°C	97.7% of the theoretical yield of ethanol	[42]
12.	*P. stipitis* CCUG18492-*K. Marxianus*	30 g/L glucose, 30 g/L xylose, 12 g/L mannose, 8 g/L galactose mixture	1. pH: 4.5 2. Working volume:100 mL 3.Shaking rate:100 rpm	1.Ethanol yield: 0.36 2. Biomass yield: 0.08 3. Maximum volumetric ethanol productivity (g/L*h): 1.08 4. Maximum ethanol concentration (g/L): 31.87 5. Substrate utilisation efficiency: 99%	[43]
13.	*P. stipitis* CCUG18492–*S. cerevisiae*	30 g/L glucose, 30 g/L xylose,12 g/L mannose, 8 g/L galactose mixture	1. pH: 4.5 2. Working volume: 100 mL 3. Shaking rate: 100 rpm	1. Ethanol yield: 0.41 2. Biomass yield: 0.08 3. Maximum volumetric ethanol productivity (g/L*h): 0.77 4. Maximum ethanol (g/L): 29.45 5. Substrate utilisation efficiency: 94%	[44]
14.	*S. cerevisiae* 2.535-*P. tannophilis* ATCC 2.1662	Softwood hydrolysate	1.Working volume: 150 mL hydrolysate 2. Temperature: 30°C 3. pH: 5.5	1. Ethanol yield: 0.49 2. Volumetric ethanol productivity (g/L*h): 0.38 3. Sugar consumption: 99% 4. Maximum ethanol (g/L): 18.2	[18]
15.	*S. cerevisiae*–Recombinant *E. coli*	Softwood hydrolysate	1. Working volume: 150 mL hydrolysate 2.Temperature: 30°C 3. pH: 7.0	1. Ethanol yield: 0.45 2. Volumetric ethanol productivity (g/L*h): 0.71 3. Sugar consumption: 99% 4. Maximum Ethanol (g/L): 17.1	[12]
16.	*P. stipitis* NRRL Y-11544)-*S. cerevisiae* (Baker Yeast Type II) coimmobilised in Calcium alginate	45 g/L glucose and 12 g/L xylose) mix sugar syrups and enzymatic hydrolysates of stream exploded aspen chips (40 g/L glucose and 10 g/L xylose)	1. Temperature: 30°C 2. pH: 5.5	1. Ethanol yield: 0.396 2. Conversion: 0.995 g consumed/g initial	[12]
17.	*Z. mobilis* 3881-*K. fragilis*	Hydrolyzed mashed tubers	1. Working volume: 200 g medium 2. Temperature: 30°C	1. Substrate utilisation efficiency: 99 2. Ethanol yield: 0.48	[45]
18.	Restricted catabolite repressed mutant *P. stipitis* CCY39501(P5-200-16)-respiratory deficient mutant *S. cerevisiae*	35 g/L glucose and 15 g/L xylose mixture	1.Temperature: 28°C 2. pH: 5.5 3. Fermentation time: 120 h 4. 150 mL F3 medium	1. Glucose utilisation efficiency: 100% 2. Xylose utilisation efficiency: 68% 3. Maximum ethanol (g/L): 20.30 4. Ethanol yield: 0.45 5. Biomass yield: 0.20 6. Volumetric ethanol productivity (g/L*h): 0.169	[6]
19.	*S. cerevisiae-C. tropicalis*	Alkali hydrolyzed corn cobs	1. Temperature: 37°C 2. Shaking rate: 150 rpm	1. Maximum ethanol (g/L): 27 2. Volumetric ethanol productivity (g/L*h): 0.28	[46]

20.	*P. stipitis* CCY39501-*S. cerevisiae* V30	35 g/L glucose and 15 g/L xylose sugar mixture	1. pH: 5.5 2.Fermentation time: 96 h	1. Glucose utilisation efficiency: 99.71% 2. Xylose utilisation efficiency: 26.67% 3. Maximum ethanol (g/L): 15 4. Ethanol yield: 0.39 5. Biomass yield: 0.14 6. Volumetric ethanol productivity (g/L*h): 0.318	[47]
21.	*P. stipitis* CCY39501–respiratory deficient mutant *S. cerevisiae* V30	35 g/L glucose and 15 g/L xylose mixture	1. Temperature: 28^{o}C 2. pH: 5.5 3. Fermentation time: 96 h	1. Glucose utilisation efficiency: 100% 2. Xylose utilisation efficiency: 99.67% 3. Maximum ethanol (g/L): 18.8 4. Ethanol yield: 0.38 5. Biomass yield: 0.12 6. Volumetric ethanol productivity (g/L*h): 0.264	[47]
22.	*S. cerevisae* CBS 1200-*C. shehatae* ATCC 22984 co-immobilised	35 g/L glucose and 15 g/L xylose mixture	1. Initial cell loading: 0.65 mg dry wt/mL (*C. shehatae*); 5.00 mg dry wt/mL (*S. cerevisiae*) 2. Temperature:30^{o}C 3. pH: 5.0	1. Ethanol yield: 0.47 2. Biomass yield: 0.088 3. Volumetric ethanol productivity (g/L*h): 7.5	[7]
23.	*P. stipitis* CBS5773-*S. cerevisiae* no. 7	50 g/L glucose and 25 g/L xylose mixture	1. Initial concentration (g/L): 7.1 (*P. stipitis*); 1.5 (*S. cerevisiae*) 2. pH: 5.0 3. Working volume: 1 L 4. Fermentation time: 40 h 5. Specific oxygen uptake rate (mg/g cell*h): 66.7 for glucose consumption; 14.3 for xylose consumption	1. Ethanol yield: 0.39 2. Volumetric ethanol productivity (g/L*h): 0.74 3. Maximum ethanol (g/L): 29.4	[16]
24.	*P. stipitis* CBS5773-respiratory deficient mutant *S. cerevisiae* no. 7	50 g/L glucose and 25 g/L xylose mixture	1. Initial concentration (g/L): 7.1 (*P. stipitis*); 1.5 (*S. cerevisiae*) 2. pH: 5.0 3. Working volume: 1 L 4. Fermentation time: 40 h	1. Ethanol yield: 0.5 2. Volumetric ethanol productivity (g/L*h): 0.94 3. Maximum ethanol (g/L): 37.5	[16]
25.	*P. stipitis* CBS5773-*S. cerevisiae* no. 7	50 g/L glucose and 25 g/L xylose mixture	1. Initial concentration (g/L): 3.5 (*P. stipitis*); 0.75 (*S. cerevisiae*) 2. Fermentation time: 68 h 3. Specific oxygen uptake rate (mg/g cell*h): 66.7 for glucose fermentation stage and 14.3 for xylose fermentation stage	1. Ethanol yield: 0.35 2. Volumetric ethanol productivity (g/L*h): 0.39 3. Maximum ethanol (g/L): 26.2	[14]
26.	*P. stipitis* CBS5773 (fermentor A) - *S. cerevisiae* no. 7 (fermentor B)	50 g/L glucose and 25 g/L xylose mixture	1. Initial concentration (g/L): 7 (*P. stipitis*); 0.75 (*S. cerevisiae*) 2. Air flow rate: 0.2 vvm into fermentor A. 3.Nitrogen gas flow rate: 0.2 vvm into fermentor B. 4. Fermentation time: 56 h 5. Total working volume:2 L	For *P. stipitis* 1. Maximum ethanol (g/L): 33.1 2. Ethanol yield: 0.44 3. Volumetric ethanol productivity (g/L*h): 0.59 For *S. cerevisiae:* 1. Maximum ethanol (g/L): 33.7 2. Ethanol yield: 0.45 3. Volumetric ethanol productivity (g/L*h): 0.6	[14,48]
27.	*Z. mobilis-Saccharomyces sp*.	200 g/L Reducing sugars	Working volume: 0.5 L	1. Ethanol yield: 0.5 2. Volumetric ethanol productivity (g/L*h): 1.5	[49]

28.	*C. shehatae* ATCC 22984-*S. cerevisiae* CBS 1200	14 g/L glucose and 6 g/L xylose mixture	1. Inoculum: 1.5%. 2. Temperature: 30°C 3. pH: 5.0 4. Working volume: 1.5 L 5. Stirring speed: 800 rpm 6. Aeration rate: 0.005 vvw 7. Oxygen transfer rate: 1.75 mmol/L*h	1. Maximum ethanol (g/L): 14.5 2. Ethanol yield: 0.39 3. Glucose utilisation efficiency: 100% 4. Xylose utilisation efficiency: 8%	[9]
29.	*C. shehatae* ATCC 22984-Respiratory deficient mutant *S. cerevisiae* CBS1200	14 g/L glucose and 6 g/L xylose mixture	1. Inoculum: 1.5%. 2. Temperature: 30°C 3. pH: 5.0 4. Working volume: 1.5 L 5. Stirring speed: 800 rpm 6. Aeration rate: 0.005 vvm 7. Oxygen transfer rate: 1.75 mmol/L*h	1. Maximum ethanol (g/L): 14.7 2. Ethanol yield: 0.4 3. Glucose utilisation efficiency: 100% 4. Xylose utilisation efficiency: 6%	[9]
30.	*P. tannophilus m-S. cerevisiae*	46% glucose, 44% xylose, 4% mannose, 2% arabinose, 4% galactose mixed sugar media	1. Temperature: 30°C 2. Working volume: 100 mL 3. Shaking rate:100 rpm 4. Initial sugar concentration: 125 g/L	1. Ethanol yield: 0.46 2. Maximum ethanol (g/L): 27	[50]
31.	*P. tannophilus s -S. cerevisiae*	46% glucose, 44% xylose, 4% mannose, 2% arabinose, 4% galactose mixed sugar media	1. Temperature: 30°C 2. Working volume: 100 mL 3. Shaking rate:100 rpm 4. Initial sugar concentration: 200 g/L	1. Ethanol yield: 0.44 2. Maximum ethanol (g/L): 42.7	[50]
32.	*E. coli-S. cerevisiae*	46% glucose, 44% xylose, 4% mannose, 2% arabinose, 4% galactose galactose mixed sugar media	1. Temperature: 30°C 2. Working volume: 100 mL 3. Shaking rate: 100 rpm 4. Initial sugar concentration: 200 g/L	1. Ethanol yield: 0.43 2. Maximum ethanol (g/L): 41.2	[50-51]

CHAPTER 6

Concluding Remarks and Future Perspectives

6.1 Concluding Remarks

Recovery of maximum amount of reducing sugars from lignocellulosic feedstocks is the major challenge in second generation ethanol production. Fractional hydrolysis process has been developed to deal with this issue, by recovering the maximum amount of soluble pentose and hexose sugars separately with the negligible toxics. No separate pretreatment and detoxification steps are required using this technique for the saccharification of biomass. Additionally, single vessel operation using a co-culture system of *Z. mobilis* (for GRF fermentation) and *S. shehatae* (for XRF fermentation) efficiently utilised maximum sugars present in the lignocellulosic biomass hydrolysate. This may be able to reduce the 2G ethanol production cost further and simultaneously resulted in high ethanol yield and concentration. The important outcomes of the present study have been represented below:

- 8-stage fractional hydrolysis process resulted in maximum TRS (84.88%) and Xylose recovery (87.9%) with minimum toxics (4.31 g/kg of dry biomass) with comparable 7-stage fractional hydrolysis process results.
- Nitric acid resulted in maximum TRS recovery but, the lower cost of H_2SO_4 makes it as the most suitable and economic reagent for fractional hydrolysis process.
- Fractional hydrolysis resulted in comparable results in all the feedstocks with saccharification (%): Kans grass 84.88, sugarcane bagasse 82.55, and wheat straw 81.66), therefore, can be termed independent of lignocellulosic feedstocks type and form.
- Multi-step glucose feeding co-culture system containing *Z. mobilis* and *S. shehate* resulted in high ethanol concentration (79.59 g/L) and yield (0.44) from the concentrated kans grass hydrolysate media (sugar concentration up to 200 g/L).

A comparative study with some previous significant studies on the lignocellulosic ethanol production clearly shows new contributions from the present work (Table 6.1).

Table 6.1. Some recent significant findings in 2G ethanol production.

Number of major steps	Feedstock	Major steps involved	Main results	Reference
2	Kans grass +Wheat straw + Sugarcane bagasse	Fractional hydrolysis+ co-culture fermentation	-84.88% saccharification - Yp/s 0.44 g/g -86.27% of theoretical ethanol yield -Maximum ethanol conc. is 79.59 g/L.	Present study
2	Pretreated oil palm fronds	Ultrasonic-assisted Pretreatment+simult-aneous saccharification and fermentation (SSF)	-Maximum bioethanol concentration (18.2 g/L) and yield (57.0%).	[1]
3	Pretreated wheat straw	Pretreatment+ enhanced saccharification process+fermentation	- Sugar yield increased from 33 to 54% and enzymatic mixture quantity reduced by 40%.	[2]
3	Sugarcane bagasse	Sono-assisted pretreatment+enzym-atic saccharification (it must be followed by fermentation for ethanol production; not shown in the study)	-91.28% glucose yield obtained -38.4 g/L glucose obtained. -Hemicellulose and lignin removed.	[3]
3	Switchgrass	Pretreatment+ enzymatic hydrolysis(fermentati-on study not shown)	-Glucose yields: 70%-90% - Xylose yields: 70%-100% - Ethanol yields 72- 92% of the theoretical maximum.	[4]
4	Lantana camara	Pretreatment+ saccharification+fer-mentation	-87.2% lignin removal -80.0% saccharification; -17.7 g/L of ethanol with corresponding yields of 0.48 g/g (*Saccharomyces cerevisiae*).	[5]
3	Sugarcane bagasse	Steam explosion pretreatment+ SSF+ Dehydration	- Highest exergy efficiency (Steam Explosion Pretreatment+ SSF+ Dehydration) reaching 79.58%.	[6]
2	Rice straw	Aqueous ammonia treatment+ SSF using cellulase and *Candida tropicalis*	-Maximum ethanol concentration was 25.1 g/L, -Yp/s 0.4 g/g	[7]
3	Rapeseed straw	Pretreatment+ saccharification+ferm entation	-Yp/s 0.29 g/g -Ethanol concentration 39.9 g/L -57.9% of theoretical ethanol yield	[8]

6.2 Future perspectives

- Process integration of different chemical and biological processes is required for the lignin utilisation present in lignocellulosic biomasses to increase the economic effectiveness.
- Scaling up of the fractional hydrolysis process must be done to produce low-cost fermentable sugars.
- Scale up strategies for co-culture fermentation process can be developed to achieve higher productivity using lignocellulosic biomass hydrolysate.
- High cell density culture with continuous ethanol removal may improve the overall productivity.

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APPENDIX

APPENDIX-I

List of formulae used to measure the process efficiency (fractional hydrolysis and co-culture fermentation)

1. TRS recovery (%) = $\frac{TRS\ estimated\ \left(\frac{g}{L}\right)}{Initial\ TRS\ \left(\frac{g}{L}\right)} \times 0.9 \times 100$

2. Xylose recovery (%) = $\frac{Xylose\ estimated\ \left(\frac{g}{L}\right)}{Initial\ Xylose\ \left(\frac{g}{L}\right)} \times 0.9 \times 100$

3. Glucose recovery (%) = $\frac{Estimated\ TRS\ \left(\frac{g}{L}\right) - Estimated\ Xylose\ \left(\frac{g}{L}\right)}{Initial\ TRS\ \left(\frac{g}{L}\right) - Initial\ Xylose\ \left(\frac{g}{L}\right)} \times 0.9 \times 100$

4. Saccharification (%) = $\frac{\text{Total recovered sugar } \left(\frac{g}{L}\right)}{\text{Initial carbohydrate content present in the biomass } \left(\frac{g}{L}\right)} \times 0.9 \times 100$

5. Biomass yield coefficient ($Y_{x/s}$) = $\frac{\text{Maximum biomass concentration } \left(\frac{g}{L}\right)}{\text{Initial TRS } \left(\frac{g}{L}\right) - \text{Final TRS } \left(\frac{g}{L}\right)}$

6. Ethanol yield coefficient ($Y_{p/s}$) = $\frac{\text{Maximum ethanol concentration } \left(\frac{g}{L}\right)}{\text{Initial TRS } \left(\frac{g}{L}\right) - \text{Final TRS } \left(\frac{g}{L}\right)}$

7. Sugar consumption (%) = $\frac{\text{Initial TRS} - \text{Final TRS } \left(\frac{g}{L}\right)}{\text{Initial TRS } \left(\frac{g}{L}\right)} \times 100$

8. Ethanol productivity = $\frac{\text{Maximum ethanol concentration } \left(\frac{g}{L}\right)}{\text{Total fermentation time } (h)} \times 100$

9. Sugar consumption rate = $\frac{\text{Initial TRS } \left(\frac{g}{L}\right) - \text{Final TRS } \left(\frac{g}{L}\right)}{\text{Total fermentation time } (h)} \times 100$

10. % theoretical yield or yield efficiency = $\frac{\text{Maximum ethanol concentration } \left(\frac{g}{L}\right)}{\text{Initial TRS } \left(\frac{g}{L}\right) - \text{Final TRS } \left(\frac{g}{L}\right)} \times \frac{1}{0.51} \times 100$

APPENDIX-II

Conversion of 100 g kans grass biomass to ethanol

Total carbohydrate content: 65.5 g

Total carbohydrate content available: 58.95 g (should be ~90% of the total)

Saccharification (%): 84.88 (Using fractional hydrolysis)

TRS recovered: 50.03 g

Ethanol Yield: 0.44 (Using multi-step successive glucose feeding co-culture system)

Final ethanol conversion: 22.01 g or 27.9 mL/100 g of biomass

APPENDIX-III

Data used to estimate Relative specific growth rate (%) in section 5.2

1. Effect of furfurals on *S. shehatae* growth

2-Furfuraldehyde concentration(g/L)	Specific growth rate (h^{-1})
0	0.172
0.1	0.171
0.2	0.169
0.3	0.165
0.4	0.151
0.5	0.136

2. Effect of phenolics on *S. shehatae* growth

Vanillin concentration(g/L)	Specific growth rate (h^{-1})
0	0.172
0.2	0.168
0.5	0.159
1	0.138
1.5	0.125
2	0.107

3. Effect of 5-HMF on the growth of *Z. mobilis*

5-HMF concentration(g/L)	Specific growth rate (h^{-1})
0	0.344
0.2	0.343
0.5	0.33
1	0.304
1.5	0.288
2	0.235

4. Effect of phenolics on the specific growth rate of *Z. mobilis*

Phenolics concentration(g/L)	Specific growth rate (h^{-1})
0	0.335
0.2	0.331
0.5	0.311
1	0.279
1.5	0.22
2	0.1

PUBLICATIONS

1. **Mishra A,** Kumar A, Ghosh S. Energy assessment of second generation (2G) ethanol production from wheat straw in Indian scenario. 3 Biotech 2018;8:1422.

2. **Mishra A,** Ghosh S. Bioethanol production from co-culture fermentation using *Candida shehatae* and *Zymomonas mobilis* from kans grass biomass. Journal of Fundamentals of Renewable Energy and Applications 2018;8:50.

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1. **Archana Mishra** and Sanjoy Ghosh "Bioethanol production from co-culture fermentation using *Candida shehatae* and *Zymomonas mobilis* from kans grass biomass" in 9th Annual Congress and Expo on Biofuel and Bioenergy, Dubai, UAE.
2. **Archana Mishra** and Sanjoy Ghosh "Saccharification of Kans grass biomass by fractional hydrolysis process using various inorganic acids" in BESCON-2017, NSIT, New Delhi (India).
3. **Archana Mishra** and Sanjoy Ghosh "2G ethanol production by a novel fractional hydrolysis technique and cofermentation process" in BESCON-2017, NSIT, New Delhi (India).
4. **Archana Mishra,** Kartik Gehlot and Sanjoy Ghosh "Bioethanol production by fractional hydrolysis and cofermentation" in "Bioenergy Urja-Utsav- 2017" to represent DBT-Pan IIT work organized by MoPNG, Pune (India).
5. **Archana Mishra,** Sneha Khalkho and Sanjoy Ghosh "Estimation of inhibitory compounds tolerance levels of Zymomonas mobilis and Pichia stipitis" in Bioprocessing India-2016, CIAB, Mohali (India).
6. **Archana Mishra,** N.C. Mishra, and Sanjoy Ghosh "Bioethanol production from mixed wastes by fungi" in "Genesis-13", HBTI, Kanpur (India).

International Travel Grants

- Received a travel grant from IIT Roorkee to attend "9th Annual Congress and Expo on Biofuel and Bioenergy" on the theme Biofuel and Bioenergy for Future on the topic "Bioethanol production from co-culture fermentation using *Candida shehatae* and *Zymomonas mobilis* from kans grass biomass" authored by **Archana Mishra** and Sanjoy Ghosh held on April 16-17, 2018, Dubai, UAE.