Sustainable strategies to achieve an industrial titer of bioethanol from lignocellulosic biomass

A Thesis

Submitted for the award of the degree of

DOCTOR OF PHILOSOPHY

by

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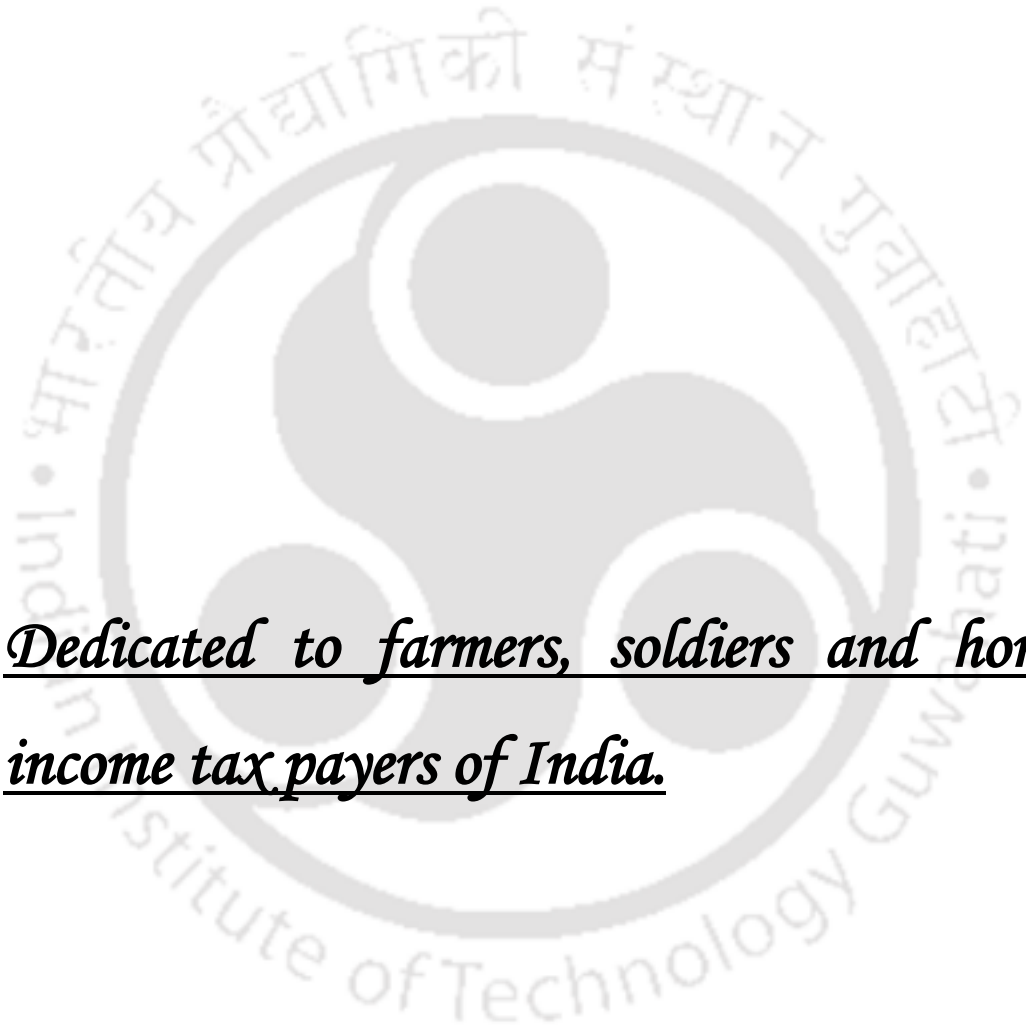

Dedicated to farmers, soldiers and honest income tax payers of India.

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in Centre for the Environment, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the esteemed supervision of **Professor Venkata Dasu Veeranki and Professor Vaibhav V. Goud.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: ……. **Narendra Naik Deshavath**

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Sustainable strategies to achieve an industrial titer of bioethanol from lignocellulosic biomass**" by Mr. Narendra Naik Deshavath for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Centre for the Environment, Indian Institute of Technology Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Date: …… **Narendra Naik Deshavath**

ABSTRACT

Owing to the fossil fuel depletion, bioethanol is found to be an alternative liquid fuel which can be used as a partial or direct substitution for petroleum products. However, bioethanol production from edible sources (like corn, wheat, cassava, sugar beet, and sugarcane) and its usage in the transportation sector can impose food insecurity to the world population. In this regard, lignocellulosic materials are the most potent and promising renewable energy resources for the production of bioethanol which even does not compete with the food. Therefore, the current thesis emphasizes sustainable strategies to achieve an industrial titer of bioethanol production from lignocellulosic biomass via biochemical conversion route. Preliminarily, structural carbohydrates analysis of lignocellulosic materials derived from various sorghum traits has been carried out for the assessment of bioethanol potential. As a result, around 269–329 g of theoretical bioethanol yield was achieved per kg of various sorghum biomass traits. Further, pretreatment of sorghum biomass was carried out at various process parameters such as sulfuric acid concentration (0.2–1 M), reaction time (30–120 min) and temperature (80–121 °C) to maximize the pentose sugar yield with minimized levels of fermentative inhibitors. As a result, around 97.6% of hemicellulose hydrolysis was attained at 121 ºC, 0.2 M H_2SO_4, and 120 min reaction time. Furthermore, pre-hydrolysates are detoxified with calcium hydroxide by the over-liming process to reduce the toxic effect of fermentative inhibitors on *Pichia stipitis*. Moreover, in order to eliminate the sugar loss during the over-liming process, as well as solid waste ($CaSO_4$) generated during the over-liming process by $Ca(OH)_2$, magnesium hydroxide ($Mg(OH)_2$) has been used for neutralization of pre-hydrolysates and their subsequent fermentation by *Pichia stipitis*. Due to the low concentration of fermentative inhibitors in the pre-hydrolysates, a significant ethanol yield of about 0.40-45 g_p/g_s and ethanol

conversion efficiency of about 76-88% was achieved during the fermentation of both detoxified and neutralized hydrolysates.

After the pretreatment, a major fraction of residual biomass contains cellulose followed by lignin. Therefore, pretreated biomass was subjected to the enzymatic hydrolysis process for the conversion of cellulose into glucose units. Different concentrations of cellulase (a biocatalyst) such as 20–80 mg of cellulase protein/g of cellulose have been investigated for the optimization of glucose yield from the pretreated biomass. Due to the presence of recalcitrant lignin in the pretreated biomass, only 54.4% cellulose conversion efficiency was attained at cellulase protein loading of 60 mg. However, in order to decrease the citric acid inhibition effect on glucose fermenting yeast namely *Saccharomyces cerevisiae*, enzymatic hydrolysis of pretreated biomass was carried out at lower citrate buffer strengths (5 mM and 0.5 mM) with the solid loading of 1–5% (w/v). The cellulose conversion efficiency results were found to be strongly agreeable with that of the standard enzymatic hydrolysis (50 mM) process. Moreover, 36.5–43.5 g/L (0.30–0.38 g_p/g_s) and 56.4–62.1 g/L (0.45–0.46 g_p/g_s) ethanol production was attained during the fermentation of enzymatic hydrolysates derived from 5 mM and 0.5 mM citrate buffer strengths, respectively.

Low cellulose conversion (54.4%), low solid loading (5% w/v) and high enzyme dosage (60 mg/g) are the major cost barriers which eventually obstruct the lignocellulosic bioethanol refinery. Therefore, prior to the enzymatic hydrolysis process, pretreated biomass was de-lignified with 1–5% NaOH strengths at 121 ºC for 20 min. As a result, around 81%–98% lignin content was removed from the pretreated biomass. Subsequently, 99.6% of cellulose conversion efficiency was achieved during the enzymatic hydrolysis by employing 40 mg of cellulase protein/g of cellulose at 10%

(w/v) solid loading. Furthermore, enzymatic hydrolysis process in 0.5 mM citrate buffer strength did not show any detrimental effect on glucose fermenting yeast which ultimately produced an industrial titer of 74.7 g/L ethanol with high ethanol yield (0.46 g_p/g_s) and productivity (2.9 g/L/h).

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Abbreviations and Notations

Abbreviation

AA	Acetic acid
Abs	Absorbance
AH	Acid hydrolysate
ADP	Adenosine diphosphate
AIL	Acid insoluble lignin
AIR	Acid insoluble residue
ASL	Acid soluble lignin
ATP	Adenosine triphosphate
bmr	Brown midrib
CO_2	Carbon dioxide
CDH	Conditioned hydrolysate
CH	Cellulosic hydrolysate
DOE	Department of Energy
DNS	Dinitrosalicylic acid
EBPP	Ethanol Blended Petrol Program
EERE	Energy Efficiency and Renewable Energy
EP	Ethanol productivity
EU	European Union
EY	Ethanol yield
FA	Formic acid
FAO	Food and Agricultural Organization
GHG	Greenhouse gasses
GOI	Government of India
HH	Hemicellulosic hydrolysate

HMF	Hydroxymethyl furfural
HPLC	High pressure liquid chromatography
HV	Heating value
ICE	Internal combustion engines
ICRISAT	International Crops Research Institute for Semi-Arid Tropics
IPPC	Intergovernmental Panel on climate change
IS	International sorghum
kW	Kilo watt
NREL	National Renewable Energy Laboratory
OD	Optical density
ODW	Oven dry weight
PH	Pre-hydrolysate
SO_2	Sulfur dioxide
SRS	Sugar recovery standard
T_{EY}	Theoretical ethanol yield
TP	Technical procedure
YPD	Yeast extract peptone dextrose
YPDX	Yeast extract peptone dextrose xylose
WEC	Word energy council

Notations

Mb	Million barrels
g_p/g_s	Gram product/gram substrate
C_{Cb}	Concentration of cellobiose
C_{Glu}	Concentration of glucose
R_{CC}	Rate of cellulose conversion
CC	Cellulose conversion
DF	Dilution factor
R_{SC}	Rate of sugar consumption
F_{efi}	Fermentation efficiency
MSY	Monomeric sugar yield
M	Molar
mM	Milli molar
min	Minute
g	Gram
R^2	Regression coefficient
h	Hour

Chapter 1:

Introduction and Review of literature

1.1. Importance of bioethanol in the transportation sector

The global demand for energy is increasing rapidly due to industrialization, the growth of the world population as well as a change in lifestyle. At present, around 90% of the world energy demand is achieved by fossil fuels such as petroleum, coal and natural gas and are also considered as the primary energy sources which have been generally used in the transportation sector and generation of electricity. According to the World Energy Council (WEC), petroleum, natural gas, and coal are good sources of energy, which collectively contributes nearly 82% of global energy requirements [1]. These fossil fuels are non-renewable energy sources and likely be exhausted rapidly in the near future at the current rate of usage.

In 1970, the global energy crisis occurred due to the shortage of petroleum resources. Especially in the transportation sector, it is anticipated that the availability of fossil fuel derived petroleum products may not conquer the predicted rate of demand by 2040-2050 [2]. Since, the demand of petroleum products will increase from 85 Mb/day in 2008 to 105 Mb/day in 2030 and might be higher values thereafter [3], correspondingly the number of vehicles will increase to 1.3 billion by 2030 and 2 billion by 2050 [4]. In the 20th century, consumption of energy increased by 13-fold which is faster than that of world population increment [1]. Consequently, the oil price has been increased due to the over consumption of non-renewable energy sources. Moreover, the transportation sector (such as aviation, maritime, road and railways) is one of the largest contributor to greenhouse gas (GHG) emission. It has been noted that the transportation sector alone consumes about 60% of petroleum-based fuels globally and

is responsible for one-fifth of total CO_2 emissions [5]. As a consequence, the concentration of GHGs emission and their accumulation in the biosphere greatly increased [1]. This results in global warming leading to climate change, loss of biodiversity, and a rise in sea level [1,2,6,7].

According to the *Inventory of U.S. Greenhouse Gas Emissions and Sinks 1990–2015,* transportation sector represents 27% of total U.S. GHG emissions in 2015 [8]. Therefore, Office of Energy Efficiency and Renewable Energy (EERE), U.S. Department of Energy (DOE) has targeted to reduce greenhouse gases emissions by 17% and oil imports by 50% within the year of 2020 [8]. On 1st November 1993 with 12 membered states, the European Union (EU) was established to reduce the greenhouse gas emission. Progressively, the number states grown to 28 with a series of enlargement and it is currently known as EU-28. As compared to the 1990s, around 22% reduction in greenhouse gas emission was observed in EU-28 in the year 2015. Further, the European Union has setup a specific target to reduce GHG emissions up to 6% by the usage of 10% renewable energy in the transportation sector by 2020 [9].

The developing country like India consumed about 156 million tons of crude oil, of which 77% was imported in the year of 2007 and it was also projected that it will raise about 6 million barrels per day by 2030. This indicates that India is approaching towards the grieve dependency of oil security (Ministry of Petroleum and Natural Gas, 2007), and will express India as the third largest importer of oil (IEA, 2007) [1]. The Indian government appointed an Intergovernmental Panel on climate change in 1997 (IPPC 1997) to estimate the GHG emission from various types of fossil fuel combusted in the energy sector. In 2007, the energy sector in India emitted 1100 million tons of GHG [10]. In this, around 12.9% of GHG emitted from the transportation sector [11].

In order to decrease the GHG emission from the transportation sector, the Indian government has suggested use of 20% ethanol blended gasoline across the country by 2017 [1]. Currently, the US government has already implemented the ethanol utilization for the transportation purpose with a blending of 8–10%. Around 12% of U.S. gasoline sold contains 8–10% ethanol as a fuel additive [2,7]. Ethanol is a vital element of transportation fuel which can boost octane and reduce carbon monoxide and other toxic emissions. Moreover, ethanol can also be used as a direct substitute for petroleum products which can reduce our dependency on foreign oil. About, 80% of Brazilians are using ethanol blends as transportation fuels for their vehicles. Along with this, small aero-plane engines were also developed to operate using this new technology [7].

1.2. Ethanol

Ethanol (C_2H_5OH) is a transparent, volatile and flammable liquid, having 789 kg/L density at 20.85 °C. While combusting of ethanol will produce a smokeless blue flame which generally invisible in normal light [2].

Table 1.1: Thermal properties of ethanol

Parameters	**Temperature (°C)**
Boiling point	78.22
Flash point	16.45
Auto-ignition temperature	424.85
Heat of combustion	26800 kJ/kg

Thermal properties of ethanol are shown in Table 1.1. Among them, the flash point is one of the most important physical factors which has been used to determine the fire potential and explosion hazards of liquids. The flash point of pure ethanol is

16.45 °C which is lower than that of ambient temperature [2]. Moreover, flash points of ethanol-water mixtures are shown in Figure 1.1 for its safe handling, storage, and transportation.

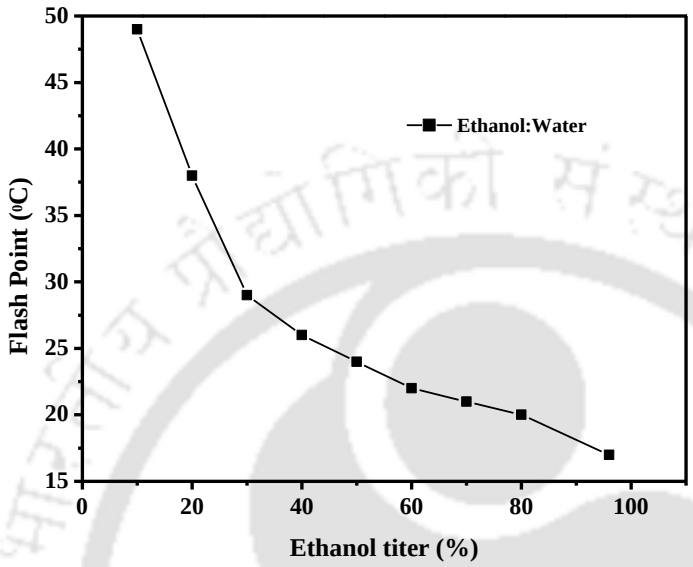

Figure 1.1: Flammable properties or flash points of ethanol-water mixtures

An approximately ~89 mol% ethanol and ~11 mol% water form an azeotrope at atmospheric pressure with the temperature of 77.85 °C [2]. This azeotropic property is a pronounced function of temperature and pressure which vanishes at temperatures below 29.85 °C or pressures below 10 milli bar [2].

1.2.1. Ethanol production processes

Generally, ethanol can be produced by chemical and biological processes. Acid-catalyzed hydration of ethylene forms ethanol which is referred to as a chemical process [2]. Phosphoric acid is the most commonly used catalyst in the chemical process. On the other hand, production of ethanol through fermentation process by using different microbial strains is called as biological process. Under anaerobic conditions, certain

species of yeast (e.g., *Saccharomyces cerevisiae*) and bacteria (e.g. *Zymomonas mobilis*) metabolizes the sugars into ethanol and carbon dioxide [12]. Production of bioethanol from sugars like sucrose, glucose, and fructose is an industrially well-established process and it is found to be a safest and eco-friendly process as well [13]. Since early ages, fermentation process derived ethanol being used in the alcoholic beverages. However, the first usage of ethanol blended gasoline as a fuel occurred in the 1920s and 1930s and was in high demand during World War II because of fuel shortages.

Due to depletion of fossil fuels and global warming concern, many countries such as USA, Brazil, India, China, and several EU member states have already announced commitments to bioethanol programs and challenges to reduce the dependency on fossil fuels derived petroleum products as well as GHG emissions. So far, USA and Brazil have shown the largest commitment to these challenges. U.S. government started an industry in the year of 1970s for ethanol production by using corn as a feedstock and are intended to prepare ethanol blend with gasoline for the transportation purpose [2]. Similarly, Brazil also producing ethanol from sugarcane at a large scale to use it as a transportation fuel. Worldwide, around 66.7 billion liters of bioethanol produced in 2008 and it was enhanced to 88.69 billion liters in 2013 [12]. Brazil and USA are the major contributors of bioethanol producing around 26.72% and 56.72% of the world production, respectively [1]. In addition to this, China is also producing bioethanol from cassava, sugarcane, and yams, while the European Union producing from sugar beet and wheat [1,14]. Currently, India produces 1.3 billion liters of ethanol from wheat, sugarcane, and its derived molasses. Moreover, India has implemented a new policy for the bioethanol production from various renewable resources to reach more than 4 billion gallons per year by 2017 [1,2]. It has been

predicted that bio-ethanol could sink about 60–80% SO_2 and 90% CO_2 when it blends with 95% gasoline [15].

Ethanol having lower heating value (HV) which is two-thirds of gasoline, therefore, it will not affect the internal combustion engines (ICE) when bioethanol is blended with gasoline in a small proportion up to 10–15%. Without modifying the IC engines, around 5–10% of ethanol blend is already being used in several courtiers [7]. But at higher blend ratio, the amount of fuel required is more than that of conventional gasoline which could be due to the lower heating value of ethanol. However, in Brazil, more than 20% of cars runs on ethanol as a sole fuel source [2]. Brazilians have flex-fuel engines which can able to utilize pure ethanol or gasoline or any mixture of both [2]. Similarly, USA developed flex-fuel vehicles which can run on 0–85% ethanol (100–15% gasoline) [2]. The higher ethanol blends are not allowed yet which are believed to be inefficient [2]. The Government of India (GoI) announced Ethanol Blended Petrol Program (EBPP) during the year 2003 for the implementation of 5% ethanol blended petrol (Planning Commission, 2003) [16]. In the year 2008, the GoI announced its National Policy on biofuels targeting 20% blending of ethanol in petrol by 2017 [16]. The proportion of ethanol and gasoline mixtures and their usage in different countries are listed in Table 1.2.

Table 1.2: Common Ethanol-Petrol mixtures

Codes	Composition	Countries	Comments
E5	Max. 5% anhydrous ethanol, min. 95% petrol	Western Europe, India	Regular cars
E10	Max. 10% anhydrous ethanol, min. 90% petrol	USA, Europe, China, India, South Africa	Regular cars
E15	Max. 15% anhydrous ethanol, min. 85% of petrol	USA, South Africa	Regular cars
E25	Max. 25% anhydrous ethanol, min. 75% of petrol	Brazil	Model 2001 and newer vehicles
E85	Max. 85% anhydrous ethanol, min. 15% petrol	The USA and Europe	Flex-Fuel vehicles
E100	Hydrous ethanol (~5.3 wt% water)	Brazil	Flex-Fuel vehicles

1.2.2. Material source for bioethanol production

Several renewable resources like rice, wheat, corn, sorghum grains, sugarcane, cassava, and sugar beet are generally used for the production of bioethanol [1,13]. Sugarcane and sugar beet containing sucrose is much easier to extract, therefore, the production cost of ethanol from sugarcane and sugar beet is lower than that of rice, wheat, corn, sorghum grains, and cassava derived ethanol. Because it does not require hydrothermal treatment (domestically known as cooking) and subsequent enzymatic hydrolysis for the gelatinization of starch and it's conversion into sugars, respectively [2,12].

Large scale production of bio-ethanol is principally depended on the availability of sugar source. The USA is found to be the largest producer of corn in the world, around 96,000,000 acres of land reserved for the corn production [1]. In the years 2013-14, USA produced around 13.02 billion tons of corn. Therefore, USA utilizing corn for the production of bioethanol which is a commercially well-established process and the USA has calmed to be largest ethanol producer in the world [7]. Brazil tops the list of sugarcane producers, with an annual production of 7,39,300 thousand metric tons and found to be the second largest producer of bioethanol [1,7]. In fact, Brazil is the world's largest sugarcane ethanol producer and a pioneer in using ethanol as a motor fuel. The European Union states (EU-28) is the third largest bioethanol producer in the world which currently producing about 4.5 billion liters of ethanol from various cereals and sugar beets [1].

In addition to the sugarcane, sweet sorghum having major credential towards the bioethanol production. Sweet sorghum stalks contain around 75% of juice which is easily extractable like sugarcane. Sweet sorghum having the capability to grow on hot and drought conditions. The water requirement for sweet sorghum is one third that of the sugarcane and 22% less water required than that of corn [17]. In 2007, International Crops Research Institute for Semi-Arid Tropics (ICRISAT) collaborated with Rusni Distilleries established the world's first sweet sorghum-based commercial distillery for the production of ethanol which is located in Andhra Pradesh (currently known as Telangana), India [18].

After the United States, Brazil, and the European Union, China producing more than one billion gallons of ethanol, making it the fourth-largest ethanol producing country in the world. In addition to these, several countries like Germany, Canada,

Sweden, India, and many other countries also producing bioethanol at an industrial level from a various source which is briefly shown in Table 1.3.

Table 1.3: Production of bioethanol from different edible sources at industrial scale

Agriculture crop	Material	Sugar content (%)	Ethanol yield (L/ton)	Potential yield (L/ha)	Reference
Sugar cane	Stalk juice [a]	12-17.6	70-90	6470–6660	[19,20]
Sorghum	Stalk juice [a]	16-28.1	40-86	2062–2595	[21]
	Grains [b]	68-70.7	380	1099	[22]
Sugar beet	Root juice [a]	15-20	95-107	1605–5500	[23]
Cassava	Tuber [b]	35	363-455	4901	[24]
Potato	Tuber [b]	17.5-20	80-100	1600	[25]
Wheat	Grains [b]	65.3-76	376-435	1001–1700	[26]
Corn	Grains [b]	70-72	370-470	4180	[27]

[a] *Sucrose and* [b] *Starch.*

However, production of bioethanol from edible sources like rice, wheat, corn, sorghum grains, sugarcane and its usage in the transportation sector can impose food insecurity to the world population [1]. Moreover, it is anticipated that due to the high demand of ethanol as a fuel in the transportation sectors may eventually increases the prices of the aforementioned edible feedstocks. Therefore, the interest of modern research has been switched to (first-generation biofuels) non-food based ethanol (second generation biofuels) [28]. The USA, Brazil, EU-28, China, India, Canada, and many other countries have already focused on alternative low-cost feedstocks for bioethanol production. In this regard, lignocellulosic materials are found to be one of

the most potential and promising alternative renewable energy resources for the production of bioethanol which is abundant, inexpensive and does not compete with food or feed.

Around the world**,** several types of lignocellulosic feedstocks are abundantly available which includes, inedible agriculture residues (wheat straw, corn stover, sorghum stalks, rice straw, sugarcane bagasse, peanut shell, cotton stalk, sun hemp), forest residue (pine wood, aspen, bamboo, pruning and bark thinning residues of other softwood and hardwoods), grasses (switch grass, *Miscanthus giganteus*, *Panicum virgatum, and* citronella spent biomass) and aquatic weeds (water hyacinth). Among them, inedible agriculture residues such as wheat straw, corn stover, sorghum stalks, rice straw, sugarcane bagasse are the potential and promising feedstocks for the bioethanol production [1,6,12,13,29].

The agriculture residues are generally utilized as animal feed and domestic fuel by direct combustion. The utilization of these crop residues for animal feed is very low [1]. Therefore, a large amount of agriculture crop residue is being disposed of as a waste. Worldwide, approximately 600–900 million tons of rice straw is being produced per year [1], in which, a minor portion is being used as a feedstock for animals and the rest is disposed of by burning. This practice contaminates the ambient air because the burning of lignocellulosic biomass in open field emits greenhouse gasses at a larger volume which could be a principle inducer of global warming. In the United States, about 64–139 million metric tons of corn stover is generated per year, in which only 1–5% of corn stover is collected and used for animal feed and industrial processing, the remaining corn stover is left in the fields as a waste [30]. According to the Food and Agricultural Organization of the United Nations 2017 statistics (FAOSTAT), around

181.8 million tons of agriculture residues (derived from rice, wheat, corn, and sugarcane crops) were burnt in Brazil, China, India and USA in the year 2016 which produced ~15.77 million tons of CO_2 (Table 1.4). In addition to this, around 19.6 million tons of wheat and corn residues were burnt in the European Union (EU-28) countries which produced ~1.7 million tons of CO_2. Open field burning of agriculture residues are already banned in many countries like Western Europe, and recently India has considered it seriously owing to due to deteriorating air quality. Thus, these abundantly available agricultural crop residues need to be converted into useful biofuel products which can give multiple benefits, such as enhancement of indigenous energy sources, strengthening of sustainable energy, boosting of rural economy and eco-friendly system (MNRE, 2008) [16].

Table 1.4: The amount of major agricultural crops residues was burned in the year 2016 and their CO_2 emissions

Agriculture crop residues (Million dry ton/year)					**Million ton/year**
Country	**Corn**	**Rice**	**Wheat**	**Sugarcane**	**CO_2 emission**
USA	35.11	0.07	7.10	0.24	3.73
India	10.20	23.63	12.09	3.22	4.25
China	38.98	16.75	9.74	1.09	5.75
Brazil	14.96	1.07	0.87	6.65	2.03

Before proceeding to bioethanol production it is important to know the chemical and structural composition of lignocellulosic materials. Because there are several factors which majorly hampers the monomeric sugars yield from lignocellulosic biomass and their subsequent conversion into bioethanol. The typical structure of lignocellulosic biomass mainly composed of cellulose, hemicellulose, and lignin.

Along with these, the fewer amount of metals (especially Si), organic acids (acetic acid and glucuronic acids), water soluble (weakly bounded structural carbohydrates, nitrates) and ethanol soluble (waxes and chlorophyll) extractives are also present in the lignocellulosic biomass [31, 32]. Cellulose and hemicellulose are the polymeric carbohydrates which yield different types of hexose and pentose sugars upon the hydrolysis. Whereas, lignin is a three-dimensional methoxylated polyphenolic compound which principally covers cellulose and hemicellulose [33]. Therefore, lignin restricts the depolymerization of cellulose and hemicellulose during the lignocellulosic biomass hydrolysis. These polymers are associated together with various types of bonds (hydrogen bonds, covalent bond, ether bonds) and form a hetero-matrix structure [33]. The complexity and rigidity of the hetero-matrix vary with respect to the composition of lignocellulosic constituents which mainly depends on the type, species, and even source of the biomass [32]. For instance, the percentage of cellulose is higher in hardwood biomass, whereas leaves and agriculture residues like wheat straw have more amount of hemicellulose. In addition to this, agricultural residues and grass have lower lignin content than softwood and hardwood biomass (Table 1.5) [6,34]. Moreover, the ratio of lignocellulosic constituents within the species varies with corresponding soil type, stage of the growth and other environmental factors.

Table 1.5: Chemical composition of different lignocellulosic biomass

Forest feedstocks	Cellulose	Hemicellulose	Lignin	References
Hardwood				
Bamboo	43	23.1	26.2	[35]
Aspen	49	18.2	25.6	[36]
Poplar	49.7	12.3	16.4	[37]
Eucalypt wood	43.4	15.3	25.5	[38]
Oak	41.3	28	24.3	[39]
Softwood				

Pine wood	42	14.7	26.3	[40]
Spruce	38.5	4.6	50.6	[41]
Douglas fir	40.9	18.41	29.30	[42]
Agriculture feedstocks				
Rice husk	30.1	13.5	22.4	[43]
Rice straw	36.1	24.7	16.8	[44]
Wheat straw	40.5-42.8	25-26	18-19	[45,46]
Corn stover (stalk, leaves and husk)	35.6-41.2	26.1-37.4	10.7-21	[47,48]
Corncobs	32.6-37.4	31.7-34.2	16.9-19.2	[49,50]
Sorghum stalks	31-36.8	22-29.7	13.9-22	[51,52]
Banana waste (rachis and pseudostem)	20.1-26.1	9.6-11.2	10.1-10.8	[43]
Sugarcane bagasse	32-45	20-32	17-22	[53,54]
Pineapple leaf	46.17	19.28	2.8	[55]
Cotton stalks or stems	39.5	22.3	19.8	[56]
Coconut coir	34.8	18.3	35.8	[57]
Sunflower stalks	35.8	19.7	14.9	[58]
Sunn hemp	37	21.3	13.8	[52]
Grassy feedstocks				
Switch grass (*Panicum virgatum* L.)	29.9	25.9	14.8	[59]
Elephant grass (*Pennisetum purpureum*)	22.6	20.9	19.4	[60]
Citronella spent biomass*	28.1	32.2	18.7	[61]
Miscanthus giganteus	45.3	27.1	9.8	[62]

** After extraction of essential oil from citronella*

1.2.3. Process for bioethanol production

Unlike edible materials such as sugarcane and starchy materials, bioethanol production process from lignocellulosic biomass is somewhat difficult. Generally, bioethanol production process from sugarcane involves, simple extraction of sugarcane juice (which contains sucrose) followed by fermentation (by hexose sugar utilizing yeast) (Figure 1.2a). During the fermentation of sucrose, *Saccharomyces cerevisiae*

produces invertase (enzyme) which effectively cleaves the α-1, 2- the glycosidic bond between the glucose and fructose. These hexose sugars are subsequently fermented to produce bioethanol [2,13]. In the case of the starchy type of materials, cooking (85–105 ºC) is the primary step which gelatinizes the starch, thereafter, it has been hydrolysed by amylase (enzyme) at 30–60 ºC for the production of glucose units which are further subjected to fermentation process [2,13] (Figure 1.2b). Unlike the sucrose fermentation, starch hydrolysis and bioethanol production cannot be carried out by a single microorganism, where, the gelatinized starch hydrolysed by commercially available amylase enzyme which is produced by bacterial species namely, *Bacillus amyloliquefaciens* and *B. licheniformis*. Thereafter, starch hydrolysis derived glucose molecules can be fermented by yeast species such as *Saccharomyces cerevisiae*. However, due to the simplest production of hexose sugars from sucrose and starch, these processes are industrially well-established and are producing bioethanol at commercial scale [2,13].

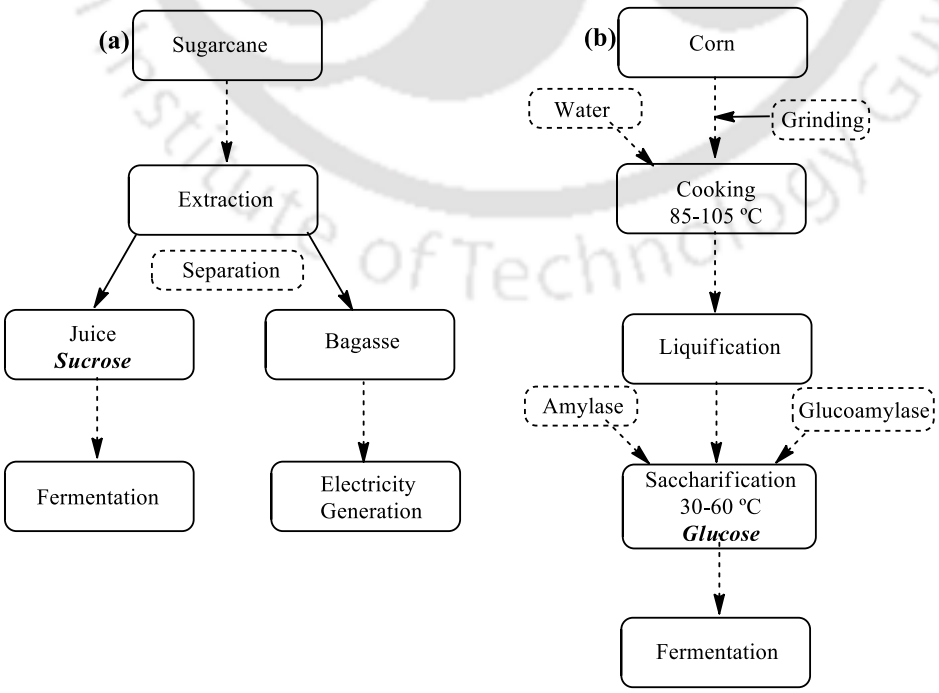

Figure 1.2: A process flowchart for bioethanol production from (a) sugar crops and (b) starchy materials

However, due to the structural rigidity, bioethanol production process from lignocellulosic biomass is difficult than that of sucrose (sugarcane) and starch-containing materials (corn, wheat, rice, sorghum grains, and cassava). Several processes were investigated for the efficient hydrolysis of lignocellulosic biomass for the production of fermentable sugars and their conversion into bioethanol. Among them, pretreatment and subsequent enzymatic hydrolysis of lignocellulosic biomass are found to be more efficient and most widely used process (Figure 1.3).

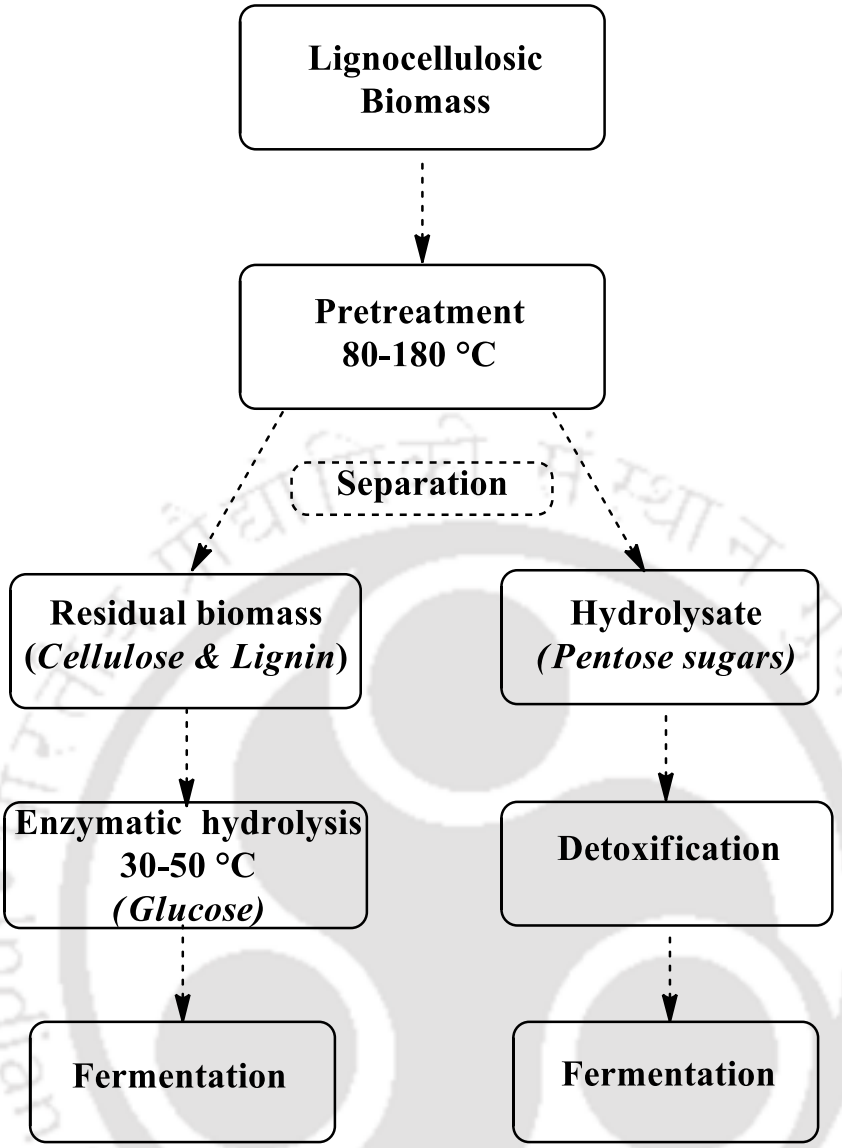

Figure 1.3: Process flowsheet of lignocellulosic biomass conversion into bioethanol

Several pretreatment techniques such as steam explosion, ionic liquid, dilute acid, biological, hydrothermal, organo-solvent, concentrated acid and alkali pretreatment have been investigated to disrupt the lignin structure for effective hydrolysis of polymeric carbohydrates constituents of lignocellulosic biomass. Among them, dilute acid pretreatment is the most effective and widely used method which hydrolyses the most of hemicellulosic fraction and makes the residual biomass containing cellulose more amenable towards the enzymatic hydrolysis. The

concentration of acid range is usually maintained below 5 wt% and the process can be carried out at a temperature range of 120 ºC to 180 ºC with corresponding pressure [63,64]. Several types of inorganic acids such as sulfuric acid (H_2SO_4), nitric acid (HNO_3), phosphoric acid (H_3PO_4), hydrochloric acid (HCl), ferric chloride ($FeCl_3$), aluminium chloride ($AlCl_3$), copper chloride ($CuCl_2$) have been used as acid catalysts and tested on different types of lignocellulosic biomass [64,65]. Among them, sulfuric acid is found to be one of the widely used catalysts and is investigated on all types of lignocellulosic materials such as agricultural residues, grasses, aquatic weeds, softwood and hardwood [63,64,66]. Apart from sulfuric acid, chlorides containing acid catalysts (HCl, $FeCl_3$, $AuCl_3$, and $CuCl_2$) also showed a significant effect on hemicellulosic hydrolysis, but the hydrolysate fermentability found to be hindered due to the presence of chlorides and metal ions. It is a known fact that the presence of metal and chloride ions deter microbial metabolic growth, which results in lower ethanol yield and productivity. However, metals can be removed in the form of metal hydroxides from the hydrolysate by using ultrafiltration process, whereas, removal of chloride ions can be a difficult process. This removal (metals and chlorides) processes eventually increase the ethanol production cost [67].

In addition to the chloride containing acids, HNO_3 and phosphoric acids are also strong catalysts which are able to produce significant levels of hemicellulosic sugars, nevertheless, no reports are observed on bioethanol production from hemicellulosic hydrolysate [64]. While comparing all the inorganic acids, sulfuric acid is found to be a significant catalyst and numerous work has been reported on dilute sulfuric acid pretreatment of lignocellulosic biomass. Unlike the chloride ions, sulfate can be easily removed through precipitation. In this process, $Ca(OH)_2$ or $CaCO_3$ are generally used to neutralize the acid hydrolysate that results in $CaSO_4$ precipitate which can be easily

removed through the filtration or centrifugation. Use of 0.2 µm nylon membrane in the filtration process plays a dual role which includes, separation of $CaSO_4$ and sterilization of the hydrolysate [68]. Usually, xylose-containing hydrolysate is not subjected to heat sterilization, which generally occurs at 121 ºC [69]. At this temperature, the initiation of xylose decomposition results in the formation of furfural which induces toxicity towards the fermenting organism. Apart from Ca based alkaline agents, NaOH and KOH are also used to neutralize the acid hydrolysate, but the resulted sodium sulfate (Na_2SO_4) and potassium sulfate (K_2SO_4) are soluble in hydrolysate [70]. It is a known fact that the presence of sulfate ions along with salts significantly hampers the microbial growth which leads to lower ethanol yield and productivity. Therefore, Ca based alkaline agents, especially $Ca(OH)_2$ is being used for the separation of sulfate ions from hydrolysate. In addition to this, the resultant $CaSO_4$ is used as a soil conditioner. Due to these advantages, dilute sulfuric acid pretreatment and its derived hydrolysate neutralization (or sometimes over-liming) by $Ca(OH)_2$ is the most widely practiced method for pretreatment of lignocellulosic biomass and hemicellulosic ethanol production, respectively. Therefore, dilute acid pretreatment is a well-established and most widely used approach for the pretreatment of lignocellulosic biomass [13,30,63,64,66].

After the pretreatment, a substantial amount of cellulose present in the residual biomass can be converted into glucose units by the action of an enzyme called cellulase. In order to achieve a good percentage of enzymatic digestibility of cellulose content, removal of other polymers namely hemicelluloses and lignin is necessary through certain procedural conditions. In the quest to deduce these optimal conditions, various accompanying factors are also affected. For instance, along with monomeric sugars, several types of sugar decomposition products, sugar-oligomer, organic acids, and

phenolic compounds are also formed during the pretreatment process. Except for the monomeric sugars, compounds that are derived from lignocellulosic biomass hydrolysis are generally considered as inhibitors. The amount of inhibitory compounds formation is mainly depended on the type of pretreatment conditions used for the hydrolysis of lignocellulosic biomass.

1.2.4. Factors affecting the lignocellulosic biomass conversion into bioethanol

1.2.4.1. *Fermentative inhibitors formation during the pretreatment*

Furfural and 5-hydroxymethyl-2-furaldehyde (HMF) are the decomposition products of hexose and pentose sugars. These are the common products which are generally formed during the dilute sulfuric acid pretreatment of lignocellulosic biomass. Further, degradation of HMF takes place under extreme pretreatment conditions like prolong reaction time, high temperature and acid concentration to form levulinic and formic acids [71]. Similarly, furfural also undergoes further degradation to produce formic acid and upon condensation forms resins. Acetic acids are also found to be present in the pre-hydrolysates which are generally formed due to the hydrolysis of acetylated hemicellulose [71].

During the acid pretreatment, a large number of phenolic compounds are formed because of the splitting of β-O-4 ether and other acid-labile linkages in lignin polymers. Phenolic compounds of wood such as 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, syringaldehyde, syringic acid, and Hibbert's ketones are the most commonly formed compounds during the dilute acid pretreatment [72,73]. In addition, *p*-coumaric acid and ferulic acid are also derived during the pretreatment of annual crops residues [71]. In addition to this, a fraction of the phenolic compounds also originated from

extractives of lignocellulosic biomass. For instance, pyrogallol and gallic acid are formed from hydrolysable tannins of hardwoods. Apart from the phenolic compounds, non-phenolic aromatic compounds like benzoic acid, benzyl alcohol, cinnamic acid, cinnamaldehyde, 3,4-dimethoxy-cinnamic acid, and *para*- and *ortho*- toluic acid are also found to be present in the hydrolysates [71]. Moreover, hydrolysates are also expected to contain *para*- and *ortho*- benzoquinones as it also has hydroquinone and catechol in it. Apart from these, small aliphatic aldehyde compounds are also likely to be formed during the pretreatment step. These are volatile in nature thus; they are likely to evaporate. Further investigation is underway to determine the importance of aliphatic aldehydes. It has been recently found that small aliphatic aldehydes are ubiquitous in lignocellulosic biomass after the acid pretreatment [74].

Furthermore, by-products in the form of metal ions can also be seen during dilute acid pretreatment of lignocellulosic biomass. The formation of heavy metals like copper, nickel, chromium, and iron is mainly due to the corrosive action of acids on the equipment. These metal ions can prove to be inhibitory to the microbes [75].

1.2.4.2. *Inhibition of cellulolytic enzymes*

The non-productive association of cellulolytic enzymes on the solid fractions like lignin and residual hemicellulose can hinder the catalytic action of the enzymes [76]. Moreover, in order to obtain high ethanol titer, high substrate loading about 10–20% (w/v) and above is generally used during enzymatic hydrolysis that reduces the rate of cellulose hydrolysis and deters the conversion efficiency. The presence of high lignin content restrict the cellulose accessibility, thermal instability of enzyme and its inactivation [77] are the major factors that are likely to reduce the rate of cellulose hydrolysis during the high substrate loading. Additionally, soluble carbohydrates and

aromatic molecules in the pretreatment liquid also result in cellulase inhibition. Another common inhibitory effect is found in the form of product inhibition of cellulolytic enzymes by monosaccharides like glucose and disaccharides such as cellobiose. A new study has shown that oligosaccharides derived from the xylan and mannan show inhibitory effect on cellulolytic enzymes [78,79].

Aromatic substances can have adverse effects on the reaction as well. Aromatic solubilized phenolics may negatively impact enzymatic saccharification. Addition of sulfur oxyanions like sulfite and dithionite which can react with several aromatic compounds, thus, eliminate the inhibitory effects on cellulolytic enzymes. These sulfur oxyanions do not react with sugars. When sulfite and dithionite have been replaced with sodium borohydride which inhibits the fermenting microorganisms does not inhibit the cellulolytic enzymes [80].

From the aforementioned discussion, it is clear that aromatic compounds play major role in the inhibition of cellulolytic enzymes. It can be also deduced that hydrophobic interactions between the aromatic substances and the cellulolytic enzymes are the main reason for this kind of inhibition. The higher is the hydrophobic nature of the phenolic substances, the more is the inhibitory effect and this is indicated from fractionation by washing with hot and cold water [81]. Though further studies are necessary to understand the total extent of the inhibition of cellulolytic enzymes, data obtained so far direct towards the roles by hemicellulose- and cellulose-derived carbohydrates like mono-, di- and oligo-saccharides, and aromatic substances such as phenolic compounds [71].

1.2.4.3. *Inhibitory effects on microorganisms and strategies to counteract inhibition problems*

The by-products formed during acidic pretreatment of the lignocellulosic biomass can be categorized based on their chemical origin, function, and effect on the microbes used for subsequent fermentation. The common aliphatic carboxylic acids like acetic acid, formic acid, and levulinic acid and furan aldehydes such as furfural and 5-HMF exhibits toxicity towards the microbes [71]. However, these compounds can show the inhibition effect on microbes when they are present at high concentrations in the pretreatment derived hydrolysates. Larsson et al., (1999) observed the inhibitory effect on *S. cerevisiae* when the concentrations of acetic acid, formic acid, and levulinic acid reached 100 mM in acid hydrolysates of Norway spruce tree [73]. Softwood hydrolysates are found to have lower acetic acid concentrations due to less acetyl content. However, the production of formic acid and levulinic acid occurred due to the decomposition of sugar degradation products (Furfural and 5-HMF). However, several methods have been well established for the conditioning of hydrolysates. These include overcoming, ion-exchange, steam stripping, treatment with activated carbon, solvent extraction, and microbial acclimation, to improvise the fermentation efficiency of pre-hydrolysates [82]. Among them, overcoming with calcium hydroxide is the most economical and widely used method for detoxification which assists in removal of furans such as furfural and 5- hydroxymethyl furfural (HMF) from the pre-hydrolysates. However, certain limitations have been reported in the literature that the overliming process of pre-hydrolysate leads to sugar loss which generally caused when the pH is significantly elevated and also it is not an effective way to reduce toxicity caused by organic acids such as formic acid and acetic acid [68,73,83,84].

Therefore, it is important to employ pretreatment processes in which the formation of these acids is minimized. Thus, the concentration of aliphatic carboxylic acids in the pre-hydrolysates of softwood may not be sufficient to cause inhibition but enough to stimulate the ethanol production. This occurs due to effect of increased demand for ATP or less efficient production of ATP because of uncoupling of the respiratory system and the oxidative phosphorylation of ADP, which ultimately increases ATP generating glycolytic activity at the expense of microbial cell biomass formation [71]. Fermentation of hardwood and agriculture residues derived hydrolysates are more difficult because these feedstocks have high acetyl content that deters the microbial metabolic growth, thus leads to low ethanol yield and lower productivity [85].

Apart from the pretreatment, citrate buffer strength (50 mM) used during the enzymatic hydrolysis of pretreated biomass shows inhibition effect on microbial growth rates due to the chelation of trace elements, which are essential for optimal growth of ethanologenic yeast [86]. Moreover, on a commercial scale point of view, conducting the enzymatic hydrolysis in 50 mM citrate buffer may not be feasible [86]. Apart from the citrate buffer, some studies have used ammonia hydroxide to adjust the pH of pretreated biomass about 4.8–5.5 to carry out the enzymatic hydrolysis, but the use of ammonia require reconstruction of the wastewater treatment section as the stillage contains high levels of ammonium salts [87]. Moreover, several studies have used NaOH and KOH to adjust the acid pretreatment derived biomass for subsequent enzymatic hydrolysis which in turns forms sulfate salts that significantly shows the inhibition effect on ethanol-producing microbes [70]. Furthermore, NREL report (NREL/TP-5400-69001-September 2017) stated that vehicles running on ethanol blends (a mixture of ethanol and gasoline) showed some problems caused by sulfate

salts precipitating from the fuel. Investigators identified sodium sulfate and ammonium sulfate are predominant salts that are found in the engines. NREL experimental evidence demonstrates that the distillation process will not eliminate sulfite, which can distill with ethanol, and be concentrated in the fuel ethanol product [88].

1.2.4.4. *Selection of microorganisms for bioethanol production from lignocellulosic hydrolysates*

Moreover, industrially important microbial strains such as *Saccharomyces cerevisiae* or *Zymomonus mobilis* are unable to ferment the pentose sugars due to the lack of pentose assimilation pathway in their metabolism. Besides the hexose fermenting microbes, several types of naturally occurring yeast species such as *Candida shehatae*, *Pichia stipitis*, and *Pachysolen tannophilus* are able to efficiently ferment both glucose and xylose into ethanol [89]. However, these dual substrate utilizing yeasts are unable to produce the industrial titer of ethanol. Generally, in the food-based distillery industries, hexose sugar fermenting yeast, *Saccharomyces cerevisiae* can able to produce around 8-12% (v/v) of ethanol, whereas, *Candida shehatae* and *Pichia stipitis* are not able to reach to this industrial titer. This could be mainly due to the product (ethanol) inhibition effect. Generally, the growth of *Saccharomyces cerevisiae* gets inhibited when the ethanol production level reached above 8–12% (v/v) [90], whereas, *Candida shehatae* and *Pichia stipitis* growth has been inhibited at around 2–3% (v/v) [91]. Therefore, utilizing the *Candida shehatae* and *Pichia stipitis* for the production of bioethanol from xylose and/glucose is not an economically feasible process.

Even, some researchers have investigated the co-cultivation process by using *Pichia stipitis* and *Saccharomyces cerevisiae* for the fermentation of lignocellulosic

biomass derived pentose (xylose) and hexose sugars to achieve high ethanol titer. Unfortunately, this co-cultivation process was not successful because *Pichia stipitis* starts the consumption of xylose only after the complete consumption of glucose. This could be due to the repression of xylose uptake by glucose [92]. For example, while implementing the co-cultivation method to the medium containing (g/L) xylose-20 and glucose-100, both yeasts such as *Pichia stipitis* and *Saccharomyces cerevisiae* effectively utilize the glucose and produce the ethanol titer of 51 g/L (based on theoretical ethanol yield g_p/g_s). It is a known fact that the growth of *Pichia stipitis* usually inhibited when the ethanol concentration of fermentation medium reaches 20 g/L and above [91]. Hence, the xylose concentration of 20 g/L remains unconverted in the fermentation medium.

To overcome these problems, researchers have developed genetically modified microbial strains which can able to produce bioethanol by utilizing both the hexose and pentose sugars. Introducing the *Pichia stipitis* containing genes such as XYL1 and XYL2 (which encodes xylose reductase and xylitol dehydrogenase) into *S. cerevisiae* facilitates the xylose assimilation [93,94]. This recombinant *S. cerevisiae* strain containing xylose reductase and xylitol dehydrogenase genes can metabolize xylose *via* pentose phosphate pathway and produces ethanol titer up to 8–12% (v/v) by utilizing the pure mixture of xylose and glucose. However, during the fermentation of lignocellulosic biomass derived pentose and hexose sugars, the ethanol concentration does not increase beyond 6-7.7% (v/v), which could be due to the formation of several fermentative inhibitors during lignocellulosic biomass hydrolysis [66]. In addition to this, Guadalupe Medina et al., (2010) suggested the deletion of *S. cerevisiae* genes that encode for glycerol-3-phosphate dehydrogenase and expression of an acetylating acetaldehyde dehydrogenase from *E. coli* into *S. cerevisiae* leads to convert acetic acid

to ethanol and remove glycerol synthesis in anaerobic yeast cultures [95]. Wei et al., (2013) integrated the pathway for ethanol formation from acetic acid by expressing the acetylating acetaldehyde dehydrogenase [96].

Microbial strains with high tolerance towards inhibitory products can be identified from industrial and natural environments. In general, *S. cerevisiae* isolated from grape marc showed better resistant towards aliphatic carboxylic acids and furan aldehydes. Apart from the inhibitory tolerance, it is also important that the particular microorganism is suitable in terms of its productivity at industrial levels. Furthermore, the genetic structure of microbes was altered with the help genetic engineering technique to improve the inhibitory tolerance towards the lignocellulosic hydrolysates. Recombinant *S. cerevisiae* containing a gene for expression of laccase from white rot fungus *Trametes versicolor* showed better resistance to spruce wood hydrolysate [97]. Through the genetically engineering, a furfural resistance *E. coli* was developed which exhibits improved resistance to the sugarcane bagasse hydrolysate [98]. A slight increase in the ethanol yield was observed when *S. cerevisiae* was overexpressed with transaldolase and alcohol dehydrogenase. This was achieved when the yeast was cultured in a lignocellulosic hydrolysate containing ample amount of yeast extract and peptone [99]. Even though the amount of furfural present in the hydrolysate was only 7.8 mM but increase in the yield was a result of the improved performance in the presence of furfural [99]. Sanda et al. (2011) engineered *S. cerevisiae* in order to increase the activity of transaldolase and formate dehydrogenase so that there is increased resistance to acetic acid and formic acid. The efficiency of this genetically modified strain was studied on rice straw hydrolysate [100].

In addition to genetic engineering, strain selection, metabolic engineering, and evolutionary engineering have also been investigated by several authors to improve microbial tolerance. However, the performance of these microbial strains is rarely used for comparative studies against other microorganisms or against the well-established alkaline detoxification methods. A comparative study with a modified microbe and other detoxification processes with regard to fermentation of a spruce wood hydrolysate showed that engineered microorganism significantly enhanced its inhibitory resistance [101]. However chemical detoxification is a more potential approach to obtain fermentation result which can be comparable with that of medium containing no inhibitors [71].

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Perspective and motivation of thesis

The conversion of polymeric carbohydrates constituents of lignocellulosic biomass into monomeric sugars and their subsequent conversion into ethanol has been hindered by several factors which eventually increases the minimum selling price. Factors such as incomplete holocellulose conversion, sugar decomposition during pretreatment, high enzyme dosage, high substrate loading during the enzymatic hydrolysis, slow fermentability of pentose and hexose sugars, low ethanol productivity and ethanol yield during the fermentation are the major cost barriers that obstructing the biochemical conversion route. Therefore, efforts have been made to optimize the pretreatment conditions for maximum pentose sugar yield with minimum fermentative inhibitors and subsequent enzymatic hydrolysis of residual biomass at high solid loading with improved cellulose conversion efficiency. Moreover, minimization of fermentative inhibitors effect on pentose and glucose fermenting yeasts for the production of industrial titer of bioethanol with better ethanol productivity and ethanol yield.

Objectives of the thesis

The following objectives have been framed to achieve the aforementioned goals.

- Compositional analysis of different lignocellulosic biomass derived from various sorghum traits
- Optimization of dilute sulfuric acid pretreatment process parameters for maximum pentose sugar yield with a minimum level of fermentative inhibitors.
- Production of xylulosic ethanol from pretreatment derived hydrolysates with enhanced ethanol yield.

- Enzymatic hydrolysis of pretreated biomass at high solid loading without effecting the cellulose conversion efficiency.
- An industrial titer of cellulosic ethanol production from enzymatic hydrolysates with higher ethanol yield and productivity.

Chapter 2:

Assessment of theoretical bioethanol potential of lignocellulosic biomass derived from various sorghum traits

Abstract

Determination of structural carbohydrates (cellulose and hemicellulose) content of lignocellulosic biomass is an essential step for the predictive analysis of its bio-ethanol potential. Therefore, removal of non-structural carbohydrates and other components, and analysis and determination of the structural carbohydrates helps in accurate assessment of theoretical ethanol yield. Therefore, the methods of National Renewable Energy Laboratory (NREL) are adopted for the determination of structural carbohydrates contents of lignocellulosic biomass derived from various genetically modified sorghum traits. In the present study, water and ethanol soluble extractives of sorghum biomass was removed by a two-step soxhlet extraction process. Thereafter, extractive free biomass used for determination of structural carbohydrates and lignin analysis. As a result, 29.2–36.3% of cellulose and 22.2–29.7% of hemicellulose was found in the genetically modified sorghum biomass traits. However, the height structural carbohydrates content (645 g/kg of biomass) was found in the IS 11861/bmr, whereas lowest (527 g/kg of biomass) was found in the IS 23253/bmr6 sorghum traits. Therefore, according to the theoretical ethanol yield (based on stoichiometry), one-gram sugar can able to produce 0.51 g of ethanol. Therefore, around 269 g to 329 g of bioethanol can be produced per kg of various genetically modified sorghum biomass traits.

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2.1. Introduction

Lignocellulosic materials are found to be one of the most potential alternative renewable energy resources for the production of biofuels which are abundant, inexpensive and does not compete with food or feed. Inedible agriculture residues, forest residues and grasses are the major source of lignocellulosic materials [1,28]. Among them, agriculture residues derived from annual crops such as corn (*Zea mays*), sugarcane (*Saccharum officinarum*), wheat (*Triticum aestivum*), rice (*Oryza sativa*) and sorghum (*Sorghum bicolor (L.) Moench*) are found to be the most promising and potential lignocellulosic source for the production of biofuel [48].

In tropical, subtropical and arid regions of India, China, Mexico, United States, Southern Africa and other developing countries where the agronomic severity conditions prevail, sorghum (*Sorghum bicolor (L.) Moench*) is found to be one of the most promising crop for food (sorghum grains) and bioenergy (sorghum stalks) applications [102]. It can be cultivated on infertile soils that are unfavorable for other crops, including maize [103]. These potential features enabled the development of novel sorghum traits via genetic mutation called brown midrib (*bmr*), which resulted in decreased lignin content with altered lignin composition [102]. Phenotypical identification of *bmr* genes present in the sorghum plant could be characterized by brown coloration in mid-leaf veins. The bmr mutants were first identified by Porter (1977) in sorghum [104]. Currently, there are seven known *bmr* loci in sorghum, but *bmr*-6 and *bmr*-12 are the most widely used in breeding programs [122,123]. The phenotype associated with *bmr*-6 and *bmr*-12 is due to mutations in the genes encoding enzymes that catalyse the last two steps of lignin biosynthesis, cinnamyl alcohol dehydrogenase 2, and caffeic o-methyltransferase, respectively, such that lignin content of *bmr* sorghums is reduced relative to the wild-type. Both *bmr*-6 and *bmr*-12 were

introgressed following marker assisted back cross breeding to produce popular grain and forage sorghum lines for enhanced digestibility of dry matter [107,108].

However, after the harvesting the grains (edible source) from sorghum crop, an abundant amount of lignocellulosic material (stalks and leaves) remains in the agriculture filed which are being wasted through burning off in the agriculture filed itself, which leads to emission of greenhouse gasses. Therefore, utilization of lignocellulosic biomass for the production of various types of biofuels like bioethanol [77], bio-oil [109], bio-butanol [110], bio-methane [111] and bio-hydrogen [112] can give multiple benefits such as enhancement of indigenous energy source, strengthening of sustainable energy, boosting of rural economy and environment system. Among the biofuels, bioethanol production from lignocellulosic biomass gained a momentum towards their utilization in transportation sector [2]. However, general assessment of bioethanol potential of a lignocellulosic biomass is purely depends on its structural carbohydrate content [113]. Generally, cellulose, hemicellulose and lignin are the major structural constituents of lignocellulosic biomass.

Cellulose and hemicellulose are the polymeric carbohydrate constituents of lignocellulosic biomass. Cellulose is a homo-polymer which is made up of glucose sub-units that are linked together through β- 1, 4 glycosidic bonds [64,114]. This linear polysaccharide linked together through different inter and intramolecular hydrogen bonds, which enable them packed into micro-fibrils [64]. Such polymer is insoluble in water as the linear polysaccharide containing hydroxyl groups are bonded with each other which develops hydrophobic scenario. Noticeably, the typical cellulose structure exists in two forms i.e. crystalline and amorphous. Major portion of cellulose comprises

of crystalline, whereas a small percentage of unorganized chains are grouped as amorphous [64,114].

Hemicellulose is the second most abundant polymeric carbohydrate in the nature and a major component of lignocellulosic biomass. Unlike homologues cellulose, hemicelluloses are hetero-polymeric structure in nature, which is made up of different types of compounds like pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose) and organic acids (acetic, 4-o-methylglucuronic, D-glucuronic and D-galactouronic acids) [34]. The backbone of hemicellulose constitutes either homo-polymer (xylan, glucan and galactan) or hetero-polymer (glucomannan), which are made up of their respective monomeric sugar moieties that are mostly linked by β-1, 4 glycosidic bonds and occasionally β -1, 3 glycosidic bonds [34]. In addition to this, several type of sugars and organic acid substituents are interlinked with xylan or glucan or galactan or glucomannan backbones that prevent the formation of crystalline structures [34,115]. Instead, they form long chains, which can associate with cellulose through hydrogen bonding [32,116]. Hemicellulose forms a complex network with cellulose by holding the micro-fibrils together, probably at specific connections rather than along with the entire length of the micro-fibril [117].

Unlike the cellulose and hemicellulose, lignin does not contain any carbohydrates in their polymeric structure. Lignin is a three-dimensional methoxylated-phenylpropane compound which is uniquely responsible for the structural rigidity of lignocellulosic biomass that mostly covers hemicellulose and cellulose [33]. It is also known to inhibit the hydrolysis of polymeric carbohydrate constituents of lignocellulosic biomass. Therefore, percentage of lignocellulosic biomass hydrolysis mainly depends on their lignin content [113,118].

However, in this chapter, structural carbohydrates content of lignocellulosic materials derived from low lignin sorghum traits (developed through the genetic mutations) were analysed to evaluate their bioethanol potential.

2.2. Materials and Methods

2.2.1. Chemicals

D-(+)-Cellobiose (≥98%), xylobiose (~95%), D-(+)-xylose (99%), L-(+)-arabinose (99%), D-(+)-glucose (≥99.5%), 5-Hydroxymethylfurfural (≥99%) and furfural (99%) were purchased from Sigma-Aldrich, Bangalore (India). Sulfuric acid (98%), formic acid (≥98%) and levulinic acid (98%) were purchased from Merck Pvt. Ltd., India.

2.2.2. Source of lignocellulosic biomass

Lignocellulosic biomass (stalk and leaves) derived from various of genetically modified sorghum traits (Table 1) were collected from experimental farm of International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana state (Latitude 17°27' N: longitude 78°21' E), India. The biomass samples arrive from the field is an intact or semi-intact with soil or other debris and significant moisture content. Proper sample preparation such as washing, sample drying, particle size reduction, and potential sieving can reduce interferences in subsequent compositional analyses.

2.2.2.1. *Preparation of lignocellulosic biomass for hydrolysis*

These materials are washed under running tap water to clean the dirt, fertilizers and pesticides and then dried at 45 ºC using convection oven until the biomass moisture content reached below 10%. This dried stalks were crushed to get small pieces and

further milled and then sieved to get different particle size ranges. According to methods of the preparation of sample for compositional analysis suggested by NREL, biomass fraction retained on the 80 mesh sieve (-20/+80 mesh fraction) has used in the present study [119].

2.2.2.2. *Analysis of total solids*

Generally, when biomass sample exposed to air, moisture content of biomass can be varied which purely based on the relative humidity. Therefore, results of the chemical compositional analysis of biomass should be reported on dry weight basis. In this procedure, a portion of sample (10 gram) is dried at 105 ºC for 4 h and then the moisture content of biomass samples was calculated according to the following equation (Eq. 2.1) [120]. The amount of solid remained after drying (at 105 ºC for 4 h) is categorized as total solids on oven dry weight (ODW) basis.

$$Total\ solid\ or\ Moisture\ (\%) = \left[\frac{(W_1 - W_2)}{W_1} \times 100\right] \qquad (2.1)$$

Where, W_1 is the weight of biomass after sieving and W_2 is the weight of biomass after drying at 105 ºC for 4 h.

2.2.2.3. *Soxhlet extraction*

In addition to the cellulose, hemicellulose and lignin, lignocellulosic biomass also contains serval types of weakly bounded non-structural carbohydrates, proteins, nitrates/nitrites, lipids, waxes and inorganic matter (mostly silicon which becomes ash at 575 ºC). Therefore, it is essential to remove weakly bounded non-structural compounds from the lignocellulosic material prior to structural carbohydrates and lignin analysis which prevent their interference in the later analytical steps. Hence, a

two-stage extraction process was performed to remove water soluble and ethanol soluble compounds. Water soluble materials may include non-structural carbohydrates, inorganic material (metals), and nitrogenous material (proteins and nitrites/nitrates). Ethanol soluble material includes chlorophyll, waxes, or other minor components.

A two-stage soxhlet extraction process was performed with water (12 h) and then ethanol (8 h) to remove extractives like non-structural carbohydrates, nitrites/nitrates, fertilizers, chlorophyll waxes and proteins [121]. After the soxhlet extraction, biomass was oven dried for 48 h at 45 ±3 °C and the amount of extractives removed from the raw biomass is calculated according to Eq. 2.2. Further, the extractive-free biomass was subjected to structural carbohydrates and lignin analysis.

$$Extractives\ (\%) = \left[\frac{(Wi - Wf) \times \%Total\ solids}{100}\right] \times 100 \qquad (2.2)$$

Where, W_i is the initial weight of biomass before soxhlet extraction and W_f is the final weight of biomass after soxhlet extraction.

2.2.2.4. *Determination of structural carbohydrates and lignin*

Structural carbohydrates and lignin analysis of extractive-free biomass was carried out according to NREL protocol (NREL/TP-510-42618) [31]. Briefly, 300 mg of biomass weighed into tared glass tube and added 3 mL of 72% (v/v) H_2SO_4 (density 1.84 g/mL). Use glass rod for proper mixing of biomass and sulfuric acid and then place the tube in water bath at 30 ºC for 1 h. At every 5-10 min, reaction should be mixed using glass rod for homogeneous interaction of acid and biomass for uniform hydrolysis. After completion of first stage of hydrolysis, around 84 mL of distilled water was added into the glass tube. After addition of 84 mL distilled water, sulfuric acid concentration is become 4%. Agitate the glass tube to eliminate the phase

separation between high and low concentration acid layers. Thereafter, second stage of hydrolysis process has been initiated by keeping the glass tube into autoclave (at 121 ºC) for 1 h. Make sure the screw cap of glass tube should be tighten prior to the autoclave.

After completion of second stage hydrolysis, allow the reaction to reach the ambient temperature and then solid liquid fraction was separated by vacuum filtration unit using 0.2 µm nylon filter membrane. Liquid fraction containing monomeric sugars (derived from cellulose and hemicellulose) has been analysed by high performance liquid chromatography (Eq. 2.3) whereas, acid soluble lignin (AIL) determined by UV-Vis spectrometer (Eq. 2.4). Moreover, solid fraction dried at 105 ºC for 4 h for measurement of acid insoluble residue (AIR) and was further kept in muffle furnace temperature about 550–575 ºC for 24 h. Allow the crucible to reach room temperature and measure the ash content and further subtraction of ash from AIR gives acid insoluble lignin (AIL) (Eq. 2.5). Moreover, summation of AIL and ASL gives lignin (Eq. 2.6)

$$S_{rh}\,(mg) = \left[\frac{Conc.sugar\ \text{detected}\ by\ HPLC \times DF \times V_h}{ODW_{300\,mg\ of\ sample}}\right] \times 1000\ mg\ of\ sample \qquad (2.3)$$

Where, S_{rh} is sugars received from the extractive free biomass during the hydrolysis, DF is the dilution factor and V_h is the volume of hydrolysate.

$$ASL\ (\%) = \left[\frac{UVabs \times Volume\ of\ \ hydrolyzate \times Dilution\ factor}{\varepsilon \times ODW_{sample} \times Path\ length}\right] \times 100 \qquad (2.4)$$

Where, UV_{abs} is the average UV-Vis absorbance for the sample at appropriate wavelength, ε is the absorptivity of hydrolysate at specific wavelength (320), ODW_{sample} is the weight of sample in mg and Path length of UV-Vis cell in cm.

$$AIL\ (\%) = \left[\frac{AIR - Ash}{ODW_{sample}}\right] \times 100 \tag{2.5}$$

$$Lignin\ (\%)_{ext.\ free} = \%ASL + \%AIL \tag{2.6}$$

2.2.3. Sugars recovery standard (SRS)

It is a known fact that, due to the combined action of temperature, pressure and acid concentration, sugars decomposition can be initiated. Therefore, to correct the loss of sugars during the second stage of hydrolysis, a set of pure sugars standard are subjected to second stage of acid hydrolysis. Sugar standards includes glucose, xylose and arabinose. Sugar standards concentration should be chosen to most closely resemble the concentrations of test sample. An appropriate amount of each sugar are weighed into different glass tubes containing 87 mL of 4% H_2SO_4 solution (3 mL of 72% (v/v) H_2SO_4 solution plus 84 mL of distilled water becomes 87 mL with an acid concentration of 4%). This glass tube kept in autoclave (121 ºC with 15 lbs autogenous pressure) for 1 h. After completion of reaction allow the conical flask to reach the room temperature and an aliquot of 20 mL withdrawn from the SRS solution and neutralized with $CaCO_3$. The resulted $CaSO_4$ is separated through 0.2 µm nylon membrane and then analysed by HPLC to determine the loss of sugars during the second stage of acid hydrolysis. According to the following equations (Eq. 2.7 and Eq. 2.8) percentage of sugar loss during the hydrolysis can be calculated which is further substituted for the correction of corresponding sugar concentration values (loss of sugars) during the hydrolysis of biomass sample (Eq. 2.9).

$$SRS\ (\%) = \left[\frac{Conc.\ of\ sugars\ \text{detected}\ by\ HPLC, mg/mL}{Knwon\ Conc.\ Sugar\ before\ hydrolysis,\ mg/mL} \right] \times 100 \qquad (2.7)$$

$$Sugar_{correction}\ (mg) = \frac{S_{rh}}{SRS(\%)/100} \qquad (2.8)$$

$$Total_{sug.conc}\ (mg/g)_{ext.\ free} = S_{rh} + Sugar_{correction} \times Anhydro\ correction \qquad (2.9)$$

Anhydrous correction should be employed to calculate the sugars released per gram of lignocellulosic biomass. The anhydrous correction for glucose and pentose sugars (xylose and arabinose) is 0.90 (162/180) and 0.88 (132/150), respectively. Eventually, Eq. 2.9 shows sugar concentration on basis of extractive free biomass. Since, raw biomass (without soxhlet extraction) used for bioethanol production process, calculation of sugar and lignin content per gram of raw biomass is important for the assessment of theoretical ethanol yield of particular lignocellulosic biomass. Therefore, according to the following equations (Eq. 2.10 and Eq. 2.11), sugar and lignin content of raw biomass can be calculated.

$$Sugars_{\text{extractive biomass}}\ (mg/g) = (Total\ sug.conc_{ext.\ free}) \times \left(\frac{100 - \%Extractive}{100} \right) \qquad (2.10)$$

$$Lignin\ (\%)_{\text{extractive biomass}} = (\%Lignin_{ext.\ free}) \times \left(\frac{100 - \%Extractive}{100} \right) \qquad (2.11)$$

2.2.4. High performance liquid chromatography

Each sample was filtered through a 0.2 μm filter and appropriate dilution was made with Milli Q water. Quantitative analysis of the sugars (sucrose, cellobiose, glucose, fructose, xylose and arabinose), sugar decomposition products (furfural, 5-hydroxymethylfurfural, formic acid and levulinic acid) and acetic acid was performed

using Varian 210 HPLC system and refractive index (RI) detector (355) (Varian, The Netherlands). The separation was achieved by Meta Carb - 87H carbohydrate column (300 × 6.5 particle size 8µm) which was maintained at 60 °C and 9 mM sulfuric acid was used as an eluent at a flow rate of 0.5 mL/min. RI detector was maintained at 50 °C for all the compounds. HPLC peaks were identified by authentic standards based on specific retention time of each compounds.

2.3. Results and discussion

2.3.1. Compositional analysis of different sorghum biomass varieties

According to NREL procedures, the amount of water and ethanol extractives of sorghum biomass varieties were accounted to be 15–28.7%. Moreover, around 2.5–3% of non-structural carbohydrates like sucrose, glucose and fructose were found in the water extractives. During the structural carbohydrates and lignin analysis (Table 2.1), it was observed that, cellulose (29.2–36.3%) found to be a predominant homo-polymer, which is generally made up of glucose subunits. Whereas, hemicellulose (22.2–27.1%) is a hetero-polymeric structure made-up of acetylated xylan, arabinan and glucuronic acid, which has been found to be the second major component of sorghum biomass varieties of present study. This type of hemicellulose is referred as Arabinoglucuronoxylans (AGXs) or arabino-4-O-metylglucuronoxylans, which are the common constituent of agriculture crop residues that accounted to be 25-30% [34]. Arabinoglucuronoxylans is made up of D- xylopyronose units linked with β-1, 4-glycosidic bonds to from xylan (homo-polymer) backbone. Xylan have additional substitutes such as arabinofuranose ring, attached through α- 1, 3- glycosidic bonds, 4-*O*-Methyl-D-glucuronic acid and/or glucuronic acid residues cross linked through α- 1, 2- glycosidic bond, and the acetyl group found at xylan C-2 position (Figure 2.1) [115].

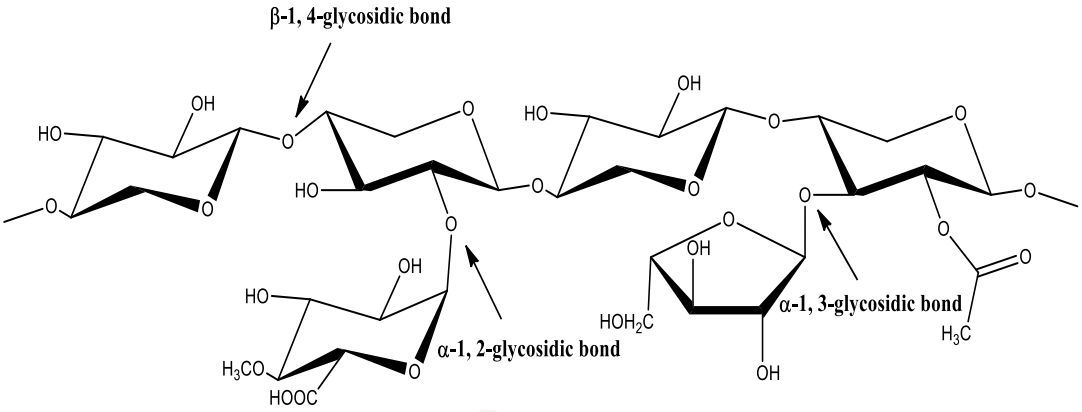

Figure 2.1: Chemical structure of Arabinoglucuronoxylans

However, From Table 2.1, the highest holocellulose (cellulose and hemicellulose) content was observed in IS 11861/bmr (64.5%), whereas the lowest holocellulose content was found in IS 23253/bmr6 (52.75%). Almost similar amount of holocellulose content was observed in IS 23789/bmr 6D, BN 612/bmr 6&12 and N 609/bmr6.

Apart from holocellulose, lignin was found in the genetically modified sorghum biomass varieties in the least amount which accounted to be 14.3–18.3%. Among them, the lowest lignin content was found in IS 11861/bmr, whereas highest lignin content was observed in IS 21549/bmr *6 A*. Generally, lignin is a recalcitrant compound, which stringently restricts the de-polymerization of polymeric carbohydrate (cellulose and hemicellulose) during the pretreatment and enzymatic hydrolysis process as well [113,118,122]. Therefore, presence of lower amount of lignin would be an advantageous characteristic of genetically modified sorghum varieties, which could support the substantial hydrolysis of polymeric carbohydrates.

Table 2.1: Chemical composition analysis of lignocellulosic biomass derived from various genetically modified sorghum varieties

S. NO.	Sorghum lines	Extr.[a] (%)	Cellulose (%)	Hemicellulose (%)[b]	Lignin (%)
1	IS 11861/bmr	15.5	34.8	29.7	14.34
2	IS 23253/bmr6	27.8	29.47	23.27	16.14
3	IS 21549/*bmr 6 A*	15.02	36.3	22.2	18.3
4	IS 23765/bmr 6B	24.28	31.49	25.44	15.72
5	IS 23787/BMR6C	24.92	30.45	24.83	15.99
6	IS 23789/bmr 6D	27.36	32.91	24.57	14.91
7	IS 21891/bmr8	28.78	34.84	23.49	14.63
8	IS 40602/BMR12	27	32.91	25.98	14.82
9	Atlas (NSL 3986)/ Forage variety	21.8	33.43	26.21	16.55
10	BN 605/bmr6	23.6	31.20	25.56	15.05
11	BN 606/bmr12	23.1	32.81	25.56	16.22
12	BN 607/bmr6	22	29.22	25.35	17.75
13	BN 608/bmr12	26	30.74	25.28	14.83
14	BN 612/bmr 6&12	23.64	31.39	26.1	15.68
15	N 609/bmr6	23.42	30.99	26.37	14.86
16	TTX430/Forage variety	21.78	32.9	27.16	16.43

Compositional analysis is based on the dry weight basis at 105 ºC, [a]Extractives, [b]Acetic and glucuronic acids are not included in the hemicellulose calculation which only the summation of xylose and arabinose.

2.3.2. Theoretical bioethanol potential of various genetically modified sorghum biomass varieties

Several technologies are being investigated to convert the cellulose and hemicellulose into monosaccharides and their subsequent conversion into bioethanol through fermentation. However, bioethanol potential of a lignocellulosic material is mainly depends on its holocellulosic content. Therefore, in the present study, around 52.7% to 64.7% holocellulose content was observed in the genetically modified sorghum biomass varieties. (Table 2.1). Based on the following stoichiometric

equations (Eq. 2.12 and Eq. 2.13), the maximum theoretical ethanol yield (g_p/g_s) form one gram of substrate (C_6 or C_5 sugar) is 0.51 g. Moreover, From the Eq. 2.12 & Eq. 2.13, one mole of glucose can be able to produce 2 mole of ethanol, whereas 3 mole of pentose sugar (xylose or arabinose) can able to produce 5 mole of ethanol. Therefore, theoretical bioethanol potential of various genetically modified sorghum biomass varieties are calculated by the following equations (Eq. 2.14 and Eq. 2.15) and listed in table 2.2.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \quad (2.12)$$

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2 \quad (2.13)$$

$$T_{EY}\ (kg/ton) = C_S\ (kg/ton) \times \frac{0.51\ kg\ ethanol}{kg\ sugar} \quad (2.14)$$

$$V\ (\text{L/ton}) = \frac{T_{EY}\ (kg/ton)}{\rho\ (kg/L)} \quad (2.15)$$

Where, T_{EY} represents the theoretical ethanol yield, C_s is the concentration of sugars (C_6 or C_5) present in the lignocellulosic biomass, 0.51 is theoretical ethanol yield (g_p/g_s) constant, V, volume (L); and ρ, is ethanol density (0.789 at 20 ºC kg/L).

Table 2.2: Assessment of theoretical bioethanol potential of lignocellulosic biomass derived from various genetically modified sorghum varieties

S. No.	Sorghum lines	Cellulose (kg/ton)	Hemicellulose (kg/ton)	T_{EY} (kg/ton*) Eq. 2.14	T_{EY} (L/ton*) Eq. 2.15
1	IS 11861/bmr	348	297	328.95	416.9
2	TTX430/Forage variety	329	271	306.3	388.2
3	Atlas (NSL 3986)/ Forage variety	334	262	304.1	385.2
4	IS 21549/*bmr 6 A*	363	222	298.3	378
5	BN 606/bmr12	328	255	297.6	377.2
6	IS 21891/bmr8	348	234	297.4	377
7	IS 40602/BMR12	329	259	296.6	376
8	BN 612/bmr 6&12	313	261	293.1	371.6
9	IS 23789/bmr 6D	329	245	293.1	371.5
10	N 609/bmr6	309	263	292.5	370.7
11	IS 23765/bmr 6B	314	254	290.3	367.9
12	BN 605/bmr6	312	255	289.4	366.8
13	BN 608/bmr12	307	252	285.8	362.3
14	IS 23787/bmr6C	304	248	281.9	357.3
15	BN 607/bmr6	292	253	278.3	352.7
16	IS 23253/bmr6	294	232	268.6	340.4

From the Table 2.2, among the all genetically modified sorghum biomass varieties, IS 11861/*bmr* having highest holocellulose content, which consequently leads to higher theoretical ethanol yield.

2.3.3. Factors affecting the bioethanol production

However, in a practical way, there are several factors that principally hampers the hydrolysis of holocellulose into monomeric sugars and their subsequent conversion

into ethanol. For instance, sugar decomposition during the pretreatment process, incomplete enzymatic hydrolysis due the interference of recalcitrance lignin. Apart from fermentable sugars (glucose, xylose, arabinose etc), products such as furans, organic acid and phenolic compounds derived during the lignocellulosic biomass hydrolysis process are considered as fermentative inhibitors that deter the microbial metabolic growth, which led to lower the fermentation efficiency.

Currently, Department of Energy-National Renewable Energy Laboratory (NERL) USA producing bioethanol at pilot scale level from corn stover as lignocellulosic substrate, which contains (kg/ton) glucan- 374, xylan- 211 and arabinan- 29 (total: 614 kg/dry ton). Therefore, according to the theoretical ethanol yield calculations (Eq. 2.14 and 2.15), around 313.14 kg or 396.8 litres of bioethanol have produced from per ton of corn stover [66]. However, in a practical way, according to NREL technical report (NREL/TP-5100-61563) 2014, dilute sulfuric acid pretreatment (160–190 ºC for 1–10 min with 0.3–0.4 wt%) and subsequent enzymatic hydrolysis (19–33 mg of protein/gram of cellulose at 50 ºC for 1–4 days) of corn stover was performed to maximize the hydrolysis of hemicellulose and cellulose into monomeric sugars. At these conditions, NREL conducted pilot scale demonstration (5 batches) and the average values of hemicellulose and cellulose conversion are represented in the Table 2.3.

Table 2.3: Bioethanol potential of corn stover according to the NREL report: NREL/TP-5100-61563.

Structural Carbohydrates	I_{sc} (kg/ton)	S_{uob} (%)	Monomeric sugar yield (kg) Eq. 2.16	F_{efi} (%)	Ethanol (kg/ton) Eq. 2.17	Ethanol (L/ton) Eq. 2.15
Cellulose	374	14.8	318.40	95	154.26	195.51
Xylan	211	15	176.35	82	73.74	93.46
Arabinan	29	8	26.68	55	6.12	7.75
Total	614	NA	521.43	NA	234.12	296.72

In this process around 85.2% of cellulose and 85% xylan and 92% arabinan convert into monomeric sugars. Therefore, at an average of 14.8% of cellulose, 15% of xylan and 8% of arabinan are unavailable for ethanol conversion process (Table 2.3). This could be due to the loss of sugars and unconverted holocellulose (cellulose and hemicellulose) during the pretreatment and enzymatic hydrolysis processes, respectively. Furthermore, the ethanol conversion efficiency from glucose, xylose and arabinose was found to be 95%, 82% and 55%, respectively (Table 3.3). This eventually, produces around 266.4–276.3 litres of bioethanol from 1 ton of dry corn stover (Eq. 2.16, Eq. 2.17 and then Eq. 2.15). Worldwide dilute acid pretreatment followed by enzymatic hydrolysis of lignocellulosic biomass is the most preferred method. Therefore, according to the above calculation, an average bioethanol potential of any lignocellulosic biomass can be predicted according to the following equations.

$$MSY\ (kg/ton) = \left[I_{sc}\left(1 - \frac{S_{uob}}{100}\right)\right] \tag{2.16}$$

$$EY\ (kg/ton) = \left(\frac{MSY \times F_{efi} \times T_{EY}}{100}\right) \tag{2.17}$$

Where, *MSY* is the monomeric sugar (glucose or xylose or arabinose) yield obtained after pretreatment and enzymatic hydrolysis, I_{sc} is the initial sugar content present in the biomass, S_{uob} is the percentage of unobtainable sugars which includes sugars loss (decomposition of glucose or xylose or arabinose) during the pretreatment, sugar oligomers formed during the pretreatment and enzymatic hydrolysis and holocellulose remained after pretreatment, and subsequent enzymatic hydrolysis. *EY*, ethanol yield; T_{EY}, theoretical ethanol yield (which is constant 0.51 g_p/g_s); F_{efi}, fermentation efficiency (%).

2.4. Summary

According to the NREL procedures, lignocellulosic biomass derived from various genetically modified sorghum traits contains 57.2–64.5% of structural carbohydrates. This equivalent to 572–645 kg/ton of sorghum biomass. Therefore, according to the theoretical yield of ethanol, around 269–329 kg of bioethanol can be obtained per ton of lignocellulosic material derived from various genetically modified sorghum traits. Among all sorghum traits, IS 11861/bmr contains highest amount (645 kg/ton of biomass) of structural carbohydrates, thus in the further study, IS 11861/bmr source used as a model lignocellulosic substrate for the production of bioethanol.

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Chapter 3:

Optimization of pretreatment process for the production fermentable sugars from sorghum biomass: Influence of furfural and 5-hydroxymethylfurfural in reliable estimation of reducing sugars by DNS assay

Abstract

The present study investigates various pretreatment process parameters to optimize the reducing sugars yield from sorghum (IS 11861/*bmr*) biomass (stalks and leaves). According to the 3, 5-Dinitrosalicylic acid (DNS) assay, the maximum reducing sugars yield was attained at 121 °C, 120 min, and 0.2 M sulfuric acid concentration. Decomposition of reducing sugars was also observed with further increasing the sulfuric acid concentration at 121 °C. Therefore, high-performance liquid chromatography (HPLC) was used for the quantification of individual sugars and their decomposition products. HPLC analysis revealed that DNS assay showed higher reducing sugars concentration than actual sugars present in the pre-hydrolysates. It might be attributed that, sugar decomposition products such as furans could also react with 3, 5-dinitrosalicylic acid to produce more color intensities which eventually increases the reducing sugar concentration. Thus, a detailed study has been carried out to evaluate the influence of active carbonyl compounds like furfural and 5-hydroxymethylfurfural (degradation product of pentose and hexose sugars, respectively) in the overall estimation of reducing sugars (glucose, xylose, and arabinose) by DNS method. The results of the study revealed that due to the presence of furans in the pre-hydrolysate the reducing sugars estimation was found to be 68%

higher than actual sugars. The current findings clearly demonstrated that use of DNS assay for quantifying reducing sugars in the presence of furans is not appropriate.

3.1. Introduction

Pretreatment, enzymatic hydrolysis, and fermentation are one of the most successful and wieldy practicing biochemical conversion route for the production of bioethanol from lignocellulosic biomass. Owing to the structural rigidity of lignocellulosic biomass, pretreatment should be employed prior to the enzymatic hydrolysis. Pretreatment is an essential step which breaks down the lignin barrier, hydrolyse most of the hemicellulosic fraction and makes the residual biomass containing cellulose more accessible and amenable for enzymatic hydrolysis [123–125]. The energy requirement for pretreatment process is typically based on the amount of lignin present in the lignocellulosic biomass. Moreover, a sustainable pretreatment process is generally based on its cost-effectiveness and maximum hemicellulose hydrolysis with less carbohydrate degradation. Pretreatment can be carried out by physical (steam explosion), thermo-chemical (dilute acid and dilute alkali), hydrothermal (hot water) and biological (microorganisms or its allied enzymes) methods [64]. In spite of having limitations, dilute sulfuric acid pretreatment is considered as an effective and inexpensive method for treating all types of biomass. The pretreatment of lignocellulosic biomass yields different types of reducing sugars such as hexose (glucose), pentose sugars (xylose and arabinose), along with sugar degradation products (furfural, 5-HMF), organic acids (acetic acid, glucuronic acid, formic acid, and levulinic acid) and lignin-derived phenolic compounds. Except for sugars, other compounds derived from the lignocellulosic biomass hydrolysis are generally considered as fermentative inhibitors which inhibit the microbial growth during the bioethanol production [126].

Different colorimetric methods have been well-established for the estimation of reducing sugars present in the pretreatment derived hydrolysate which includes DNS

[127] and Nelson – Somogyi [128,129]. Since few decades, DNS assay has been most widely used for the quantification of reducing sugars derived from lignocellulosic biomass pretreatment [130–137]. However, several problems impeded with the DNS assay is its inaccuracy, where the different reducing sugars show dissimilar color intensities [138]. Moreover, DNS assay is not suitable for quantifying single sugar in the presence of other mixture of sugars [139]. Moreover, the presence of unwanted impurities such as phenol in DNS reagent preparation increases the color intensity of a sample which leads to overestimation of sugars concentrations [140]. Therefore, phenol-free DNS reagent has been implemented to correctly estimate the reducing sugars [140–142]. DNS method specially designed for the quantification of single reducing sugar, have also been adopted for quantifying the mixture of reducing sugars (pentose and hexose) which are common products of lignocellulosic biomass hydrolysis [138,139,143]. Although, DNS method is not suitable for quantifying the mixture of sugars it is still being used for the total reducing sugars estimation [142,144]. Additionally, compounds such as xylose, arabinose, glucose, furfural and 5-HMF formed during the lignocellulosic biomass pretreatment process are having a common free carbonyl group [92,145,146]. DNS is prone to reduction to corresponding 3-amino-5-nitrosalicylic acid thereby oxidizing the carbonyl center to a carboxylic acid [127]. Thus, it is indeed a redox reaction involving a color change from yellow to brick red. However, the origin of the color change is the reduced form of DNS, irrespective of the source of the carbonyl group. Therefore, we predict that during the estimation of reducing sugars by DNS method, decomposition products of reducing sugars such as furfural and 5-HMF may also interfere to show higher color intensity than actual sugars concentration.

However, the present chapter focused on pretreatment of sorghum (bmr IS 11861) biomass to optimize the reducing sugars yield and effect of pretreatment severity on reducing sugars decomposition was also investigated. In addition, the error of estimation between three individual reducing sugars such as glucose, xylose, and arabinose has been investigated through plotting the calibration curve by DNS method. To avoid inaccuracies in estimation, the reactivity of furfural and 5-HMF towards DNS have also been considered. Moreover, the estimation of reducing sugars mixture has been performed using the DNS method to evaluate the percentage of error against the calibration plot of single reducing sugars alone. Furthermore, a synthetic hydrolysate has been prepared with respect to the lignocellulosic biomass pre-treatment to investigate the feasibility of using the DNS method for quantifying the reducing sugars in the presence of furfural and 5-HMF. Finally, to validate the proposed hypothesis, pretreatment derived hydrolysate was analyzed by the HPLC method and the eluted reducing sugars and furans concentrations were comprehensively compared with that of DNS results.

3.2. Materials and methods

3.2.1. Chemicals

D-(+)-xylose (99%), L-(+)-arabinose (99%), D-(+)-glucose anhydrous (≥99.5%), 5-hydroxymethylfurfural (≥99%) and furfural (99%) 3,5-dinitrosalicyclic acid (98%) were purchased from Sigma-Aldrich, Bangalore (India). Sodium potassium tartrate (99.98%) and Sulfuric acid (98%) were purchased from Merck India, Pvt, Ltd.

3.2.2. Compositional analysis of sorghum (bmr IS 11861) biomass

Lignocellulosic biomass derived from sorghum (IS 11861/bmr) crop was used in the present study and its chemical composition is shown in Table 3.1.

Table 3.1: Composition of SBMR IS11861 biomass

Chemical composition	Raw biomass (%)[a]	Residual biomass (mg) [b]	Acid-treated (%) [a]
Water Extractives	13.32	-	-
Glucose	1.44	-	-
Fructose	1.47	-	-
Ethanol Extractives	2.23	-	-
Cellulose	34.8	275.3	56.2
Hemicellulose	29.7*	6.86 [c]	1.4 [c]
Lignin	14.3	120	24.5

** Xylan- 27%, Arabinan- 2.7%; [a] Per gram of biomass, [b] Per 490 mg of acid pretreatment derived residual biomass, [c] Xylan,*

3.2.3. Dilute acid pretreatment of biomass

Sorghum biomass pretreatment was performed with different sulfuric acid concentrations *viz.*, 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1 M for 2 h reaction time at 80±2 °C, 100±2 °C (using water bath Labtech, LOB-506D, India) and 121±1 °C (Equitron, 7407PAD, India), with 5% (w/v) solid loading. At every 30 min interval, the reaction was stopped and allowed to reach to the room temperature and then an appropriate amount of sample (1 mL) was withdrawn from the pre-hydrolysate for the estimation of reducing sugars by DNS method [127]. The reducing sugars yield (Y_{RS}) can be calculated by the following equation (Eq. 3.1).

$$Y_{RS}\ (mg/g) = \frac{\text{sugar (mg/mL)} \times \text{dilution factor} \times \text{total volume of hydrolysate (mL)}}{\text{initial weight of biomass (g)}} \quad (3.1)$$

At the optimum reducing sugars released condition, solid and liquid fractions were separated through 0.2 µm nylon membrane filter. The solid fraction was washed with distilled water to attain neutral pH and then dried at 45 ±3 °C for 48 h. Further,

the dried biomass used for the compositional analysis to evaluate the amount of cellulose, hemicellulose, and lignin remained after the pretreatment process.

3.2.4. DNS assay

3.2.4.1. *3, 5-Dinitrosalicyclic acid reagent preparation*

The method described below was used for the preparation of 3,5-dinitrosalicylic acid [127]. 1.6 g NaOH was added to 75 mL of distilled water under continuous stirring and then 3, 5-Dinitrosalicyclic acid was added to it. Finally, 3 g of sodium potassium tartrate was added and the remaining volume was filled with distilled water to make 100 mL of DNS reagent. Phenol was not used in the preparation of DNS reagent which unnecessarily increases the color intensities of the measured concentration range of compounds [140–142].

3.2.4.2. *Preparation of stock solutions*

A series of calibration standards were prepared and quantified. The concentration range of each and individual reducing sugar (glucose, xylose, and arabinose) and furans (furfural and 5-HMF) are listed in Table 3.2. In addition, a mixture of reducing sugars (glucose, xylose, and arabinose) medium was prepared (Table 3.3). Furthermore, synthetic hydrolysate medium was prepared which contains a mixture of reducing sugars and furans (furfural and 5-HMF) and are listed in Table 3.3.

Table 3.2: List of reducing sugars and furans for making of four pointed calibration curve

Components	Concentration range (mg/mL)
Glucose	0.4–1.2
Xylose	0.4–1.2
Arabinose	0.4–1.2
Furfural	0.4–1.2
5-HMF	0.4–1.2

Table 3.3: Composition of sugar mixture and synthetic hydrolysate

S. No.	Reducing sugars (A) (mg/mL)			Furans (B) (mg/mL)		MRS (mg/mL)	SH (mg/mL)
	Xyl.	Gluc.	Arab.	Furfural	HMF	(A)	(A+B)
1	0.1	0.1	0.1	0.1	0.1	0.3	0.5
2	0.2	0.2	0.2	0.2	0.2	0.6	1
3	0.4	0.4	0.4	0.4	0.4	1.2	2
4	0.6	0.6	0.6	0.6	0.6	1.8	3

MRS, Mixture of reducing sugars; SH, Synthetic hydrolyzate; Xyl, Xylose; Gluc, Glucose; Arab, Arabinose; HMF, Hydroxymethyl furfural.

3.2.4.3. *Reducing sugar estimation procedure*

DNS assay was carried out by placing 1 mL of standard or test sample in a 25 mL test tube and then 3 mL of DNS reagent was added. The reaction mixture was heated in boiling water for 5 min [127]. The absorbance of test samples was measured at 540 nm using 2 mm path length quartz cuvettes in the UV-vis spectrophotometer (Agilent, Carry 100, USA). A blank test was performed in 1 mL of distilled water instead of the test sample.

3.2.4.4. *HPLC analysis*

Pre-hydrolysate (pretreatment derived hydrolysate) containing sugars and their decomposition products (at optimum reducing sugar released condition) were quantitatively analyzed by HPLC. The detailed specifications and operating conditions of HPLC are shown in section 2.2.4.

3.3. Results and Discussion

3.3.1. Effect of pretreatment parameters on reducing sugars yield

Dilute sulfuric acid pretreatment process produces significant amount of reducing sugars from sorghum stalks which were estimated by using the DNS method [127]. The concentrations of reducing sugars increased with increase in the acid concentration and reaction time at 80 °C and 100 °C (shown in Figure 3.1a and 3.1b). As the increase in the sulfuric acid concentration and temperature enhances the catalytic effect of pretreatment reaction which leads to increases the reducing sugars yield from the sorghum biomass. As a result, 261 mg and 297 mg of reducing sugars yield were attained at 80 °C and 100 °C, respectively in 120 min reaction time with 1 M sulfuric acid concentration. At 121 °C, both increasing and decreasing trend of reducing sugars yield was observed at different sulfuric acid concentrations with corresponding reaction time (Figure 3.1c).

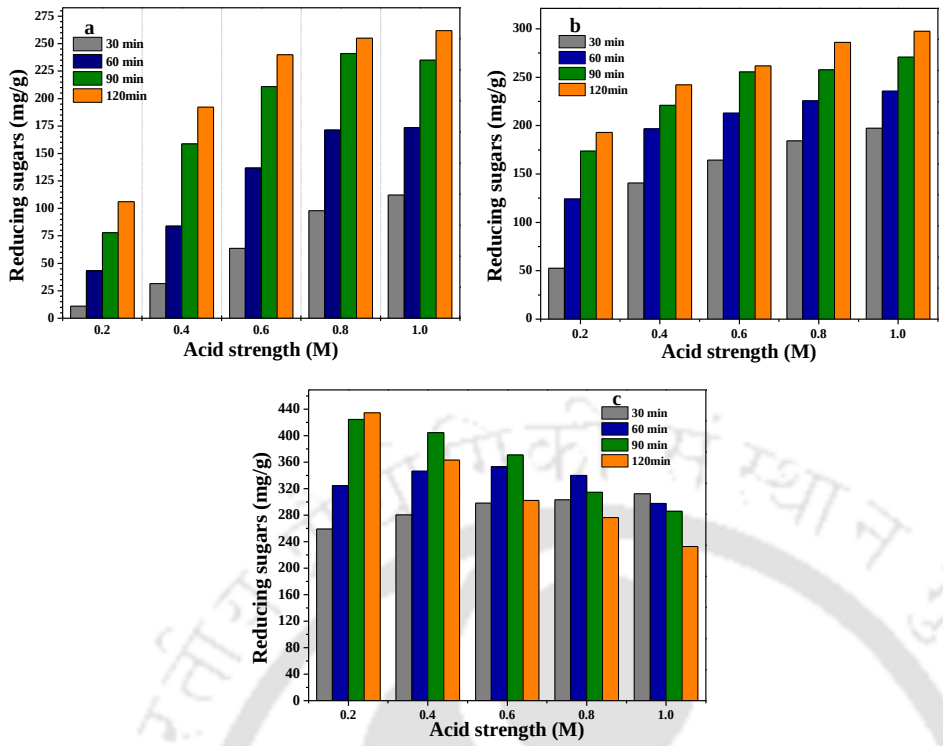

Figure 3.1: Effect of temperatures (a) 80 °C, (b) 100 °C and (c) 121 °C, reaction times and sulfuric acid strengths on reducing sugars yield.

As shown in Figure 3.1c, with increase in the pretreatment reaction time from 30 to 120 min, in the presence of 0.2 M sulfuric acid concentration, the amount of reducing sugars gradually increased from 259–434 mg/g of biomass at 121 °C. These obtained result indicated that the total possible reducing sugars yield was higher at this condition (i.e., 121 °C, 120 min, and 0.2 M H_2SO_4). However, further, increase in the sulfuric acid strength at 121 °C significantly affects the reducing sugars yield and concurrently their decomposition (Figure 3.1c). Moreover, decomposition of reducing sugars was also increased with an increase in pretreatment reaction time. From Figure 3.1c, it can be seen that low acid strength (0.4 M H_2SO_4) required longer reaction time (90 to 120 min), whereas high acid strength (1 M H_2SO_4) required shorter reaction time (60 to 120 min) for the degradation of sugars. The maximum degradation of reducing sugar was observed at 121 °C, 1 M H_2SO_4 and 120 min. However, the maximum

reducing sugars yield (434 mg/g) was attained at 121 °C, 0.2 M H_2SO_4 for 120 min. (Figure 3.1c). Utilization of low acid strength might result in a negligible quantity of sugar decomposition. Hence, pretreatment at low acid strength is the most feasible method for fermentable sugars production from lignocellulosic biomass. Further, the increase of pretreatment severity (in-terms of increase in reaction time and sulfuric acid strength) synergistically influence the degradation of reducing sugars into fermentative inhibitors [52].

In the previous published reports it is stated that an increase in the pretreatment reaction severity, pre-hydrolysate containing hexose and pentose sugars undergoes dehydration to forms 5- HMF and furfural, respectively [33]. These are the potential fermentative inhibitors which lead to decrease the microbial growth rate during the bioethanol production [84]. Therefore, prior to the fermentation of pre-hydrolysate, it is important to know the number of fermentative inhibitors that are formed during a pretreatment process, especially at optimum conditions. It is a known fact that furfural, 5-HMF, acetic acid, formic acid, and levulinic acid are the major potential fermentative inhibitor that deters the microbial metabolic growth during the fermentation of pre-hydrolysate. According to Delgenes et al., (1996), 2 g/L of furfural, 5 g/L of 5-HMF and 11.9–15 g/L of acetic acid are the inhibiting concentrations of yeast species. In addition to this, quantification of pentose and hexose sugars is important for culturing the specific microorganisms for bioethanol production. Unlike *Pichia stipitis*, *Saccharomyces cerevisiae* and *Zymomonas mobilis* are not able to convert the pentose sugars into ethanol. Moreover, according to the previous literature reports, most of the hemicellulose hydrolysis take place during the dilute acid pretreatment of lignocellulosic biomass which ultimately yields xylose and arabinose (pentose sugars). However, it has been difficult to identify and quantify the specific fermentative

inhibitors using Ultraviolet-Visible (UV-Vis) spectrophotometer as pre-hydrolysate contains different types of reducing sugars, acid soluble lignin derivatives and other hemicellulose-derived chemical constituents. Therefore, optimum pretreatment condition (121 ºC, 0.2 M H_2SO_4 120 min) derived pre-hydrolysate has been analyzed by HPLC using a RI detector for the quantification of each and individual fermentative inhibitors and sugars.

HPLC analysis revealed that around 45 mg of glucose, 16 mg of arabinose and 219 mg of xylose were released per gram of sorghum stalks. Along with this, 4.6 mg of furfural (a pentose sugar decomposition product) and 5.4 mg of 5-HMF (a hexose sugar decomposition product) and 24 mg of acetic acid (was derived from acetylated xylan) [52]. When comparing the quantification of reducing sugars by HPLC (280 mg/g) and DNS (434 mg/g) methods, a huge difference of reducing sugars yield about 154 mg/g of sorghum biomass was observed. This might be due to the amount of fermentative inhibitors such as furfural and 5-HMF may also react with 3, 5-dinitrosalicylic acid to yield high color intensities.

The basic principle of DNS assay includes the reduction in one of the nitro groups in 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid and subsequent oxidation of the aldehyde groups in substrates to carboxylic acids [127]. However, with employment of different sugars, the production of 3-amino-5-nitrosalicylic acid is not uniform [147] and hence different sugars yield dissimilar color intensities [138], which suggests that the chemistry of the test demands detailed scrutiny. Since, the lignocellulosic materials are heterogeneous in nature, consequently different types of reducing sugar formation can occur during the pre-treatment [148]. Reducing sugar decomposition products such as furfuraldehyde (furfural) and 5-

Hydroxymethylfurfuraldehyde (5-HMF) are also formed as by-products during the pre-treatment process depending upon the reaction temperature, pressure, and type of catalyst being used for hydrolysis [123,132,148–150]. Xylose, arabinose, glucose (reducing sugars), cellobiose, xylobiose (disaccharides), acetic acid, gluconic acid (organic acids), furfural and 5-HMF (reducing sugars decomposition products) are the general pre-treatment products of any lignocellulosic material [92,148]. Apart from the above-listed disaccharides and organic acids, the aldehyde is found to be a common functional group present in the reducing sugars as well as furans. Being reducing in nature, DNS could also oxidize the aldehyde group in furfural and 5-HMF along with that in the reducing sugars. Therefore, to discriminate the effect of DNS on the sugars and by-products, we performed a thorough analysis. A typical set of calibration standard curves of glucose, xylose, arabinose, furfural, and 5-HMF were made by using the DNS assay (Figure. 3.2).

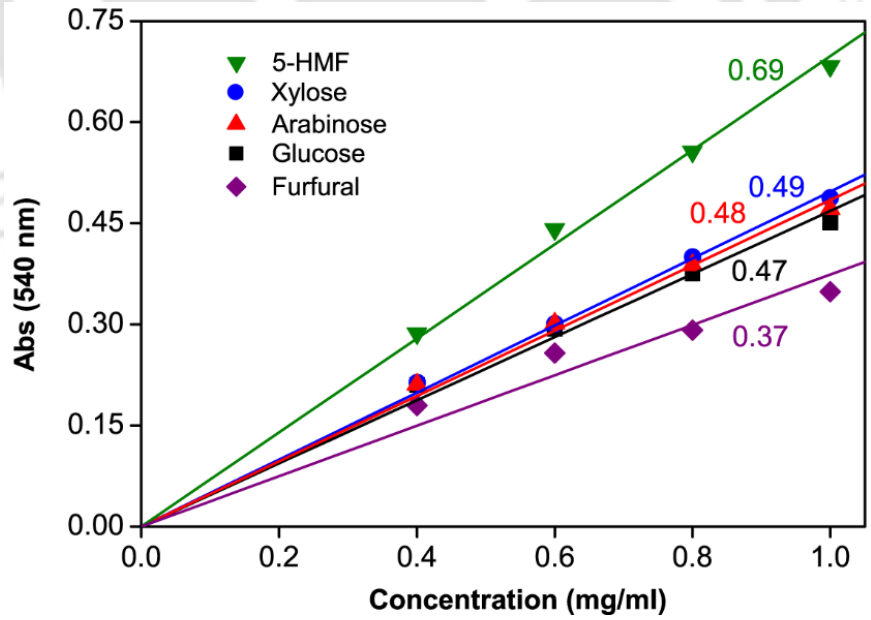

Figure 3.2: Absorbance against concentration plot for various aldehyde-containing compounds in DNS assay. All the absorbance values were recorded at 540 nm at room temperature.

As expected, dissimilar color intensities were observed when DNS reagent was reacted with different reducing sugars and furans. It is understood that aldehyde groups of reducing sugars and furans are oxidized to carboxyl groups, simultaneously 3,5-dinitrosalicylic acid reduces to 3-amino-5-nitrosalicylic acid under alkaline conditions and exhibits different color intensities. The highest error of estimation obtained from all the reducing sugars components tested was less than 1.5%. However, for the most practical purposes, this error margin should be considerable and acceptable for monitoring the reducing sugars concentration. Based on these results, DNS method was also examined for the mixture of reducing sugars and the results were in quite acceptable error range (<1.5%) with correspondence to standard calibration curves of each and individual reducing sugars (Figure 3.3).

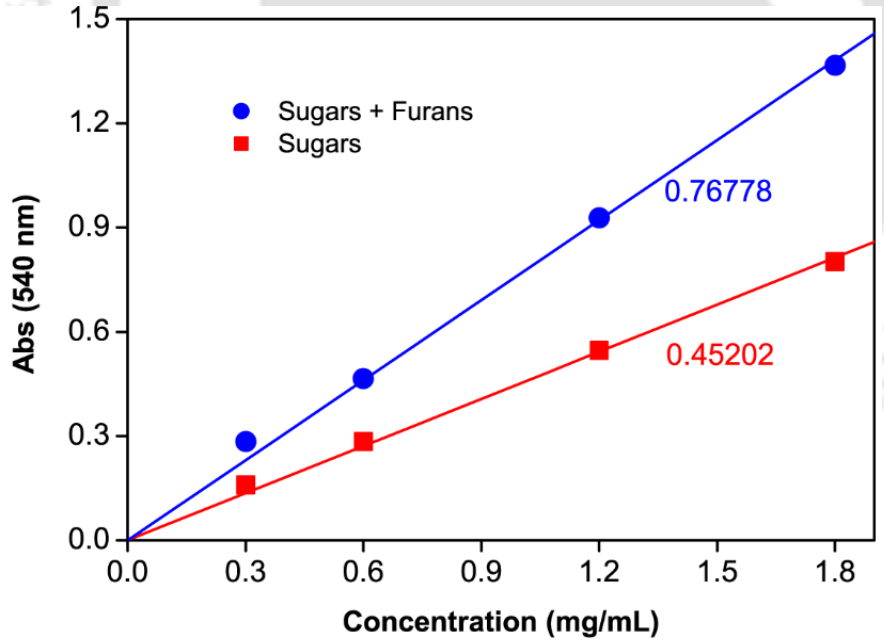

Figure 3.3: Absorbance against concentration plot for synthetic hydrolysate (blue line) and a mixture of sugars (red line), keeping sugar concentration constant. All the absorbance values were recorded at 540 nm at room temperature.

Apart from the reducing sugars, we observed that furfural and 5-HMF also reacted separately with 3,5-dinitrosalicylic acid to yield different color intensities. This encouraged us to investigate the feasibility of DNS method for a mixture of reducing sugars in the presence of furans and thus quantify the exact estimation of sugars. Since past few decades, DNS is the most widely used method to monitor the production of reducing sugars from the pre-treatment of lignocellulosic biomass. However, the interference of furfural and 5-HMF in the said process remained unnoticed. Mechanism of the formation of furfural and 5-HMF from pentose (xylose and/or arabinose) and hexose (glucose and/or fructose) sugars includes dehydration of the reducing sugars with subsequent loss of three water molecules [151]. It is known fact that the decomposition of pentose and hexose sugars initiated when the pre-treatment reaction has been conducted in a pressurized batch reactor at elevated temperatures (>121 ºC) by using different types of catalysts [92,148,150,152,153]. Generally, pre-treatment of lignocellulosic biomass is conducted either by hydrothermal process (where water act as a catalyst at subcritical state) [64,136,153,154] or thermochemical process (where dilute mineral acids act as a catalyst) [146,148–150,152] for the significant hydrolysis of polymeric carbohydrates. The temperature range for hydrothermal pretreatment and dilute acid pre-treatment would be 160–220 ºC and 121–160 ºC, respectively with correspondence to the reaction pressure [64,136,146,148–150,152,153]. Therefore, a significant amount of furan formation is expected to occur when the lignocellulosic biomass pretreatment is conducted at these elevated temperatures. Based on the aforementioned literature, synthetic hydrolysate medium was prepared which contains a mixture of reducing sugars and furans (Table 3.3). The concentration range of reducing sugars used in this synthetic hydrolysate medium was similar to that of the

aforementioned mixture of reducing sugars medium (Table 3.3). Since the concentration of the reducing sugars in both the synthetic mediums was kept constant for absorption spectroscopy, the corresponding absorbance for the two mediums was expected to be similar. However, enhancement of color intensity was observed in the furan containing synthetic hydrolysate medium (Figure 3.3). As evident from the concentration against absorbance plot in Figure 3.3, DNS assay for the furan containing synthetic hydrolysate gives higher absorbance values at a definite concentration. Therefore, this surge in absorbance in the furans containing synthetic hydrolysate can be attributed to the reaction of furfural and 5-HMF with 3,5-dinitrosalicylic acid. As such, almost 68% enhancement in absorbance was observed during the quantification of reducing sugars in the presence of furans. Thus, the detailed analysis of two synthetic hydrolysate media infers an overestimation in the quantification of reducing sugars by DNS method in the presence of furans. Henceforth, it is suggested that use of DNS method results in the imprecise estimation of reducing sugars present in the pre-hydrolysates of lignocellulosic biomass. Precise sugar estimation is more important for the prediction of bioethanol yield for the development of a specific bio-refinery process.

For instance, according to the DNS assay, per gram of sorghum biomass produces 434 mg of reducing sugar which is equivalent to 434 kg/ ton of sorghum biomass. Whereas, the actual reducing release (analyzed by HPLC) was found to be 280 mg/g or 280 kg/ton of sorghum biomass. Based on the following stoichiometric equation (Eq. 3.2), the maximum theoretical yield (g_p/g_s) of ethanol per gram of substrate (C_6 or C_5 sugar) is 0.51 g. Therefore, according to the Equation 3.3, 280 kg of reducing sugar (detected by HPLC) can be converted into 142.8 kg of ethanol, whereas 434 kg of reducing sugar (detected by DNS method) can be converted into

221.3 kg of ethanol. Therefore, about 78.5 kg or 99.5 L/ton (based on Eq. 3.4) miss interpretation has been taking place by employing the DNS method for the quantification of reducing sugars derived from pretreatment of lignocellulosic biomass. Hence, wrong interpretation of ethanol yield significantly hampers the techno-economic viability of a bio-refinery process.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 \tag{3.2}$$

$$T_{EY}\ (kg/ton) = C_S\ (kg/ton) \times \frac{0.51\ kg\ ethanol}{kg\ sugar} \tag{3.3}$$

$$V\ (\text{L/ton}) = \frac{T_{EY}}{\rho} \tag{3.4}$$

Where, C_s is the concentration of sugars (C_6 or C_5) present in the lignocellulosic biomass, 0.51 (g_p/g_s) is theoretical ethanol yield T_{EY} constant, ρ is the ethanol density (0.789 kg/L at 20 ºC).

3.3.2. Mass Balance analysis

As shown in Table 3.1, per gram of raw sorghum biomass (before pretreatment) contains 154 mg of extractives, 348 mg of cellulose, 297 mg of hemicellulose and 143 mg of lignin. Upon the pretreatment of sorghum biomass at optimum condition (0.2 M H_2SO_4, 121 C for 120 min), around 51% of lignocellulosic biomass was solubilized and 490 mg of residual biomass remained after the pretreatment which constitutes 275 mg cellulose, 6.86 mg of hemicellulose and 120 mg of lignin (Table 3.1). Therefore, the percentage of cellulose, hemicellulose, and lignin hydrolysis can be calculated according to the following equation (Eq. 3.5), which found to be 10% cellulose, 97.6%

hemicellulose and 2% lignin hydrolysis was attained at optimum pretreatment condition.

$$\text{Hydrolysis (\%)} = \left(\frac{m_{ub} - m_{pr}}{m_{ub}} \right) \times 100 \quad (3.5)$$

Where, m_{ub} is the mass of the polymer (cellulose/ hemicellulose/ lignin) present in the untreated biomass and m_{pr} stands for the residual amount of polymer after the pretreatment.

The amount of reducing sugars obtained during the pretreatment process and the amount of polymeric compounds present in the residual biomass are found to be conflicting with each other. According to the composition analysis by NREL procedure, 647 mg of holocellulose (cellulose and hemicellulose) is present in the untreated sorghum biomass, whereas solid remains (490 mg) after the pretreatment contains 282.16 mg of holocellulose. Therefore, as per the compositional analysis of untreated and pretreated sorghum biomass, the percentage of holocellulose hydrolysis was found to be 44%. In contrast, when calculating the percentage of holocellulose hydrolysis with corresponding to the reducing sugars yield (434 mg/g detected by DNS method) derived by pretreatment process, the holocellulose hydrolysis was found to be 68%. Moreover, the compositional analysis of biomass before and after pretreatment also support the overestimation of reducing sugars concentration in pre-hydrolysate of lignocellulosic biomass than actual by DNS assay method.

3.4. Summary

The reducing sugars yield was increased with an increase in the reaction time (from 30 to 120 min) at 121 °C and 0.2 M H_2SO_4. Further increase in the sulfuric acid concentrations with respective reaction time decreased reducing sugars yield. This can be attributed that, increasing the pretreatment severity, result in the formation of fermentative inhibitors. Moreover, while quantifying the reducing sugars by HPLC (280 mg/g) and DNS (434 mg/g) method, a huge difference of reducing sugars yield about 154 mg/g of sorghum biomass was observed. This could be due to reaction of furan-aldehydes (furfural and 5-HMF) with 3, 5-dinitrosalisylic acid to produce higher color intensities than actual reducing sugars concentration. As evident from the absorption spectroscopic studies, highest reactivity and absorbance was observed with 5-HMF, whereas the lowest was found with furfural. Moreover, due to the presence of furfural and 5-HMF in synthetic hydrolysate medium, the reduction of DNS to 3-amino-5-nitrosalicylic acid increased, thereby producing higher absorbance values than that of the mixture of reducing sugars alone in the visible region of the absorption spectrum. Albeit the presence of an identical concentration of reducing sugars in both the medium, the overestimation of reducing sugars was found to be around 68% which is principally due to the presence of furfural and 5-HMF. Therefore, the present study concludes that the estimation of reducing sugars by DNS assay is not a feasible and reliable method in the presence of furfural and 5-HMF.

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Chapter 4:

Dilute acid pretreatment of sorghum biomass to maximize the hemicellulose hydrolysis with minimized levels of fermentative inhibitors

Abstract

Based on the chapter 3, it was observed that estimation of reducing sugars by DNS assay is not a reliable method to quantify the accurate reducing sugars yield of pre-hydrolysate (pretreatment derived hydrolysate). Moreover, no trend of decrease in the reducing sugars yield was observed when pre-hydrolysate derived at 121 ºC, 0.2 M H_2SO_4 and 120 min were analysed by DNS assay. However, analysing the similar pre-hydrolysate by HPLC revealed the formation of 4.6 mg of furfural and 5.4 mg of 5-HMF. This can be attributed to the combined action of heat and sulfuric acid which could responsible for initiating the decomposition of pentose and hexose sugars. Therefore, in the present chapter, we used similar process parameters such as sulfuric acid concentration (0.2–1 M), reaction time (30–120 min) and temperature (80–121 °C) for the pretreatment of sorghum brown midrib (SBMR) IS11861 biomass to investigate the effect of pretreatment process on production of monomeric sugars as well as its degradation products formation. Moreover, the main aim of this work also focused to evaluate the optimum pretreatment condition for the maximization of pentose sugars yield with minimized levels of fermentative inhibitors from sorghum biomass. As a result, around 97.6% of hemicellulose was converted into xylobiose (18.02 mg/g), xylose (225.2 mg/g), arabinose (20.2 mg/g) with low concentration of furfural (4.6 mg/g) at the optimum pretreatment condition of 0.2 M sulfuric acid, 121 °C and 120

min. Furthermore, the process parameters were statistically optimized by using response surface methodology (RSM) based on central composite design (CCD).

4.1. Introduction

Hetero-polymeric structure of lignocellulosic material is made up of cellulose, hemicellulose and lignin [155]. Hemicellulose and cellulose are the polymeric carbohydrates which consist of pentose (xylose and arabinose) and hexose (glucose) sugars, respectively. Fractionation and hydrolysis of these polymeric carbohydrates is important for commercialization of bioethanol production. Therefore, pretreatment is an essential step to disrupt the complex network of lignocellulosic material to hydrolyse the most of the hemicellulose fraction and alter the cellulose structure to make it more accessible to the enzymatic hydrolysis. Several pretreatment methods have been developed to depolymerize the lignocellulosic materials which include steam explosion, acid hydrolysis and hot water pretreatment [64]. However, most of these pretreatment methods require high energy input, high temperature and high acid strength, which often results into the formation of toxic compounds such as furfural from pentose sugars and 5- hydroxyl methyl furfural (5-HMF) from glucose [33]. These are the potential toxic compounds which inhibit microbial growth during the fermentation of pre-hydrolysates, thus lead to low ethanol yield and productivity. From the aforementioned literature, the development of pretreatment conditions for maximization of pentose sugars yield along with the minimized level of fermentative inhibitors from sorghum biomass would be a challenging task.

Therefore, the current chapter mainly focused on the development of an effective dilute acid pretreatment process which maximizes the hemicellulose hydrolysis to attain high yield of pentose sugars with minimal concentration of fermentative inhibitors. In addition, response surface methodology (RSM) was employed to determine the effects of various pretreatment parameters on pentose sugars yield and furfural formation.

4.2. Materials and methods

4.2.1. Biomass source

Detailed description about sorghum biomass (IS11861/bmr) and its composition is shown in section 3.2.2 (Table 3.1).

4.2.1.1. *Pretreatment parameters*

As described in chapter 3, similar process parameters were maintained for the pretreatment of sorghum biomass. Briefly, biomass to liquid ratio of 1:20 (w/v) was mixed with different dilute sulfuric acid concentrations, *viz.*, 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1 M and each of them were hydrolysed for 2 h at different temperatures such as 80 ± 2 °C, 100 ± 2 °C and 121 ± 1 °C using water bath (Lauda, Labtech, India) and autoclave, respectively. At every 30 min interval reaction was stopped and the mixture allowed to cool to room temperature. From the reaction mixture, 100 µl of sample was withdrawn and made an appropriate dilution for the quantification of sugars and fermentative inhibitors using high performance liquid chromatography (HPLC). The detailed description about HPLC configuration and operating conditions are described in the section 2.2.4.

4.2.1.2. *Experimental Design*

Based on the preliminary results of biomass pretreatment, the release of pentose sugars and furfural formation were statistically optimized through central composite design (CCD) by Design expert software® trial version 10 (Stat-Ease, Inc., Minneapolis, MN, USA). From the CCD model, 20 distinct experimental conditions were obtained which are summarized in Table 4.1. From the preliminary results, it was found that the pentose sugars yield was higher at 120 °C, 0.2 M and 120 min compared with other conditions. Therefore, for the optimization study three potential variables,

viz. temperature (X_1) = 100, 120, 140 °C; time (X_2) = 90, 120, 150 min; and H_2SO_4 concentration (X_3) = 0.1, 0.2, 0.3 M were used as the independent parameters to maximize the pentose sugars yield and minimize furfural in the pre-hydrolysates.

Table 4.1: Experimental design matrix of CCD model and its corresponding results

Std. Order	Temp. (X_1)	Time (X_2)	Acid Conc. (X_3)	Pentose sugars (mg/g)		Furfural (mg/g)	
				Exp.	Pred.	Exp.	Pred.
1	100	90	0.1	92.32	77.25	0.71	0.099
2	140	90	0.1	186.60	163.74	4.99	5.26
3	100	150	0.1	132.94	110.47	1.21	1.72
4	140	150	0.1	142.57	122.50	6.34	6.07
5	100	90	0.3	112.06	103.66	1.88	2.49
6	140	90	0.3	184.04	178.03	8.38	8.21
7	100	150	0.3	160.45	154.83	2.72	2.78
8	140	150	0.3	168.16	154.75	6.34	7.48
9	86.3	120	0.2	43.12	60.05	0.53	0.47
10	153.6	120	0.2	109.35	132.70	9.35	8.94
11	120	69.54	0.2	197.55	214.34	3.37	3.59
12	120	170.45	0.2	200.40	223.29	5.21	4.52
13	120	120	0.0318	44.31	78.43	1.98	2.33
14	120	120	0.368	121.59	127.75	6.51	5.69
15	120	120	0.2	246.54	244.55	4.56	4.57
16	120	120	0.2	244.26	244.55	4.76	4.57
17	120	120	0.2	247.25	244.55	4.66	4.57
18	120	120	0.2	245.50	244.55	4.46	4.57
19	120	120	0.2	245.89	244.55	4.26	4.57
20	120	120	0.2	244.78	244.55	4.61	4.57

Temp. = Temperature (°C), Acid Conc. = Acid concentration (M), Exp. = Experimental, Pred. = Predicted

From the experimental results, the obtained values of response variables were subjected to a regression analysis to find out the interaction effect of factors on the response using CCD method [156]. The common form of second-order polynomial equation obtained from the regression analysis is depicted in Eq. 4.1. The equation was used to evaluate the effect of independent variables on the response which was further analysed to obtain the optimum pretreatment conditions [157]. Models and regression coefficients were authenticated with an analysis of variance (ANOVA). The significance for any statistical results was established for P-value < 0.05.

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1,i\leq j}^{n} \sum_{j=1,i\leq j}^{n} \beta_{ij} X_i X_j + \varepsilon_{ijk} \qquad (4.1)$$

Where, Y is the response (pentose sugars and furfural yield), β_0 is the constant coefficient β_i is the i^{th} linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the ij^{th} interaction coefficient. X_i and X_j are independent variables. CCD consists of 2^k factorial points, $2k$ axial points (± α), and six central points, where k is the number of independent variables.

4.3. Results and discussion

4.3.1. Effect of pretreatment parameters on the reducing sugars yield

Xylobiose, glucose, xylose and arabinose observed to be the principal sugars formed during the dilute acid pretreatment of SBMR IS11861 biomass. Apart from reducing sugars, pretreatment reaction also produces fermentative inhibitor, such as 5-HMF, furfural, formic acid and acetic acid. Reaction temperature, time and acid concentration are the key parameters which affect the sugars release and their

degradation. The concentration of sugars and fermentative inhibitors are calculated based on the following equation (Eq. 4.2).

$$Sugar\ or\ Inhibitors\ (mg/g) = \frac{conc.\ detceted\ by\ HPLC\ (mg/mL) \times dilution\ factor \times volume\ of\ hydrolysate\ (mL)}{initial\ weight\ of\ biomass\ (g)} \quad (4.2)$$

4.3.1.1. *Pentose sugars yield and their degradation products*

During the dilute acid pretreatment, conversion of hemicellulose into monomeric sugars occurred in two steps which include, 1) cleavage of covalently bonded acetyl groups form xylan backbone and 2) splitting of glycosidic linkages between xylose and arabinose units [52]. Sulfuric acid acts as a catalyst to breakdown the glycosidic linkages present in polymeric carbohydrates. It can be observed that the catalytic effect of sulfuric acid increased with an increase in the temperature. From Figure 4.1, it can be observed that the hydrolysis of hemicellulose increases with the increase in the reaction temperature (80 °C to 121 °C). At 80 °C, 0.2 M H_2SO_4 and 30 min reaction time, 8.8 mg of xylose and 4.08 mg of arabinose were attained. Further increase in the temperature (80 °C to 121 °C) and time (30 - 120 min) at 0.2 M acid concentration, xylose and arabinose concentrations were increased significantly to 225.2 mg and 20.2 mg per gram of sorghum biomass, respectively.

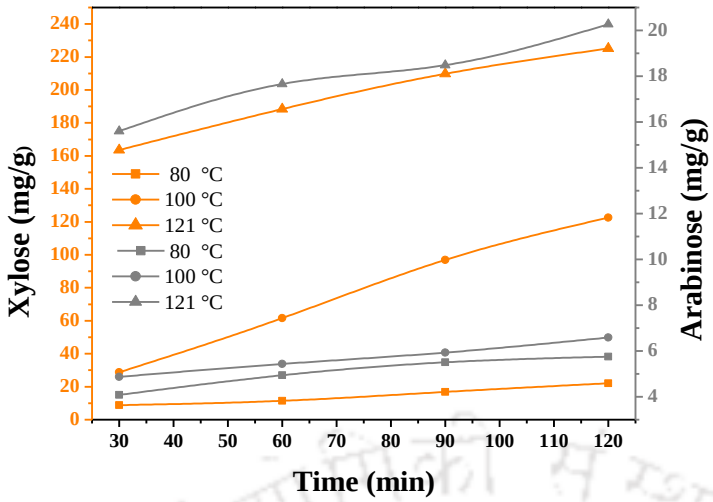

Figure 4.1: Effect of reaction temperature and time on xylose and arabinose sugars yield in the presence of 0.2 M sulfuric acid.

It is also evident from Figure 4.2a and 4.2b, that with an increase in sulfuric acid concentration at 121 °C, xylose and arabinose concentration decreases, which could be due to their decomposition. It has been reported that, xylose can be easily degraded to form furfural at a temperature about 120 °C and above [145]. Similar trend of increased furfural concentration with increase in sulfuric acid concentration and reaction time at 121 °C was seen in the present study (Figure 4.2c). This phenomenon indicates cyclo-dehydration of xylose to form furfural, i.e., removal of three water molecules from xylose are responsible for the furfural formation [52]. Similarly, arabinose being a geometrical isomer of xylose alike results of furfural formation can

be expected [158]. Further, furfural decomposes to form formic acid with an increase in the pretreatment severity [33] as shown in Figure 4.2d.

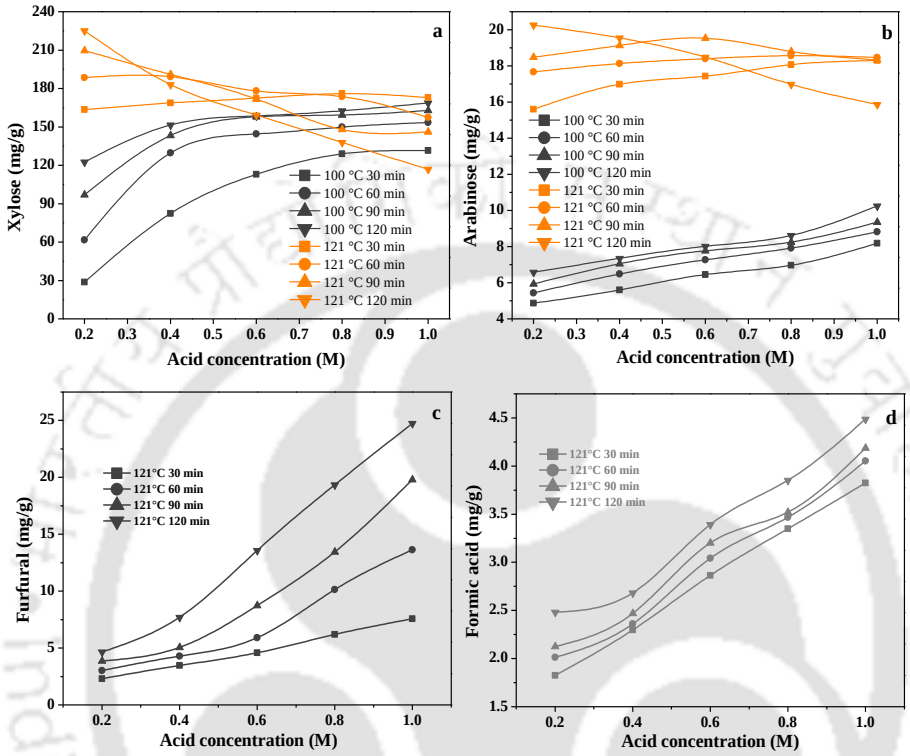

Figure 4.2: Release of pentose sugars from sorghum biomass and their decomposition products (a) xylose, (b) arabinose, (c) furfural and (d) formic acid at different temperatures, acid concentrations and reaction times.

Moreover, the detailed reaction pathway for the conversion of hemicellulose to pentose sugars and their decomposition products are shown in Figure 4.3.

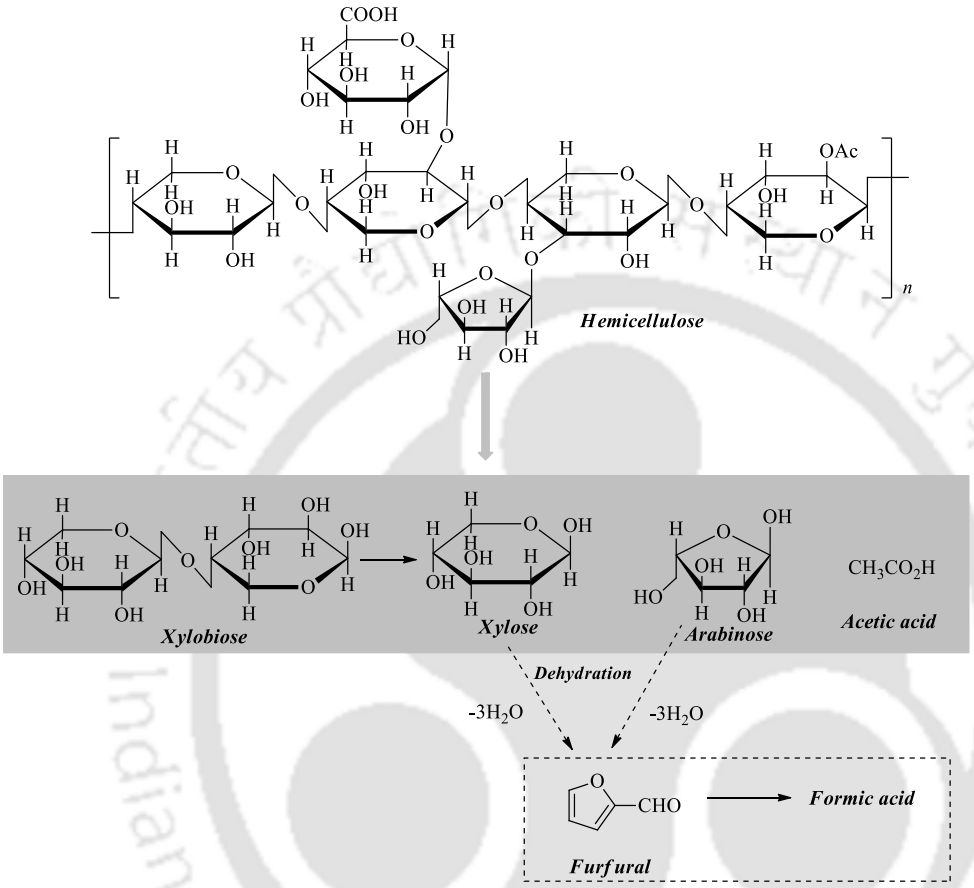

Figure 4.3: Schematic representation of hemicellulose hydrolysis into sugars and their decomposition products.

The optimized condition for hemicellulose hydrolysis has been determined as temperature, 121 °C; time, 120 min; and sulfuric acid, 0.2 M. As a result, 97.6% of hemicellulose is significantly converted into 18.02 mg of xylobiose, 225.2 mg of xylose and 20.2 mg of arabinose with 4.6 mg (or 0.23 g/L) of furfural and 2.3 mg of formic acid. Vancov and McIntosh (2012) reported that, approximately 55% of hemicellulose in the sorghum bicolor straw has been hydrolysed in the presence of 2% sulfuric acid to yield 150 mg/g of xylose at 121 °C and 60 min of pre-treatment time. In another

study, corn stover pre-treated at 200 °C for 14.3 min releases approximately 77.3% of xylose and its oligomers [159]. Kamireddy et al., (2013) studied the dilute acid hydrolysis of sorghum brown midrib (SBMR) and sorghum non brown midrib (SNBMR) in a batch reactor at a temperature ranging from 150 to 160 °C, 1–2% sulfuric acid concentration with the reaction time of 10 to 20 min. According to Kamireddy et al., (2013), xylose yield of 95 % and 91% was observed in SNBMR and SBMR, respectively, with a varying furfural concentration of 0.75 to 3.4 g/L for SBMR and 0.68 to 3.81 g/L for SNBMR. Compared to literature, the method used in the present study is considered as an efficient method for hemicellulose hydrolysis of lignocellulosic biomass, which yields maximum xylose and arabinose with minimal concentration of sugar degraded products. Furthermore, the obtained xylobiose, xylose and furfural concentrations at selected pretreatment parameters are shown in Figure 4.4.

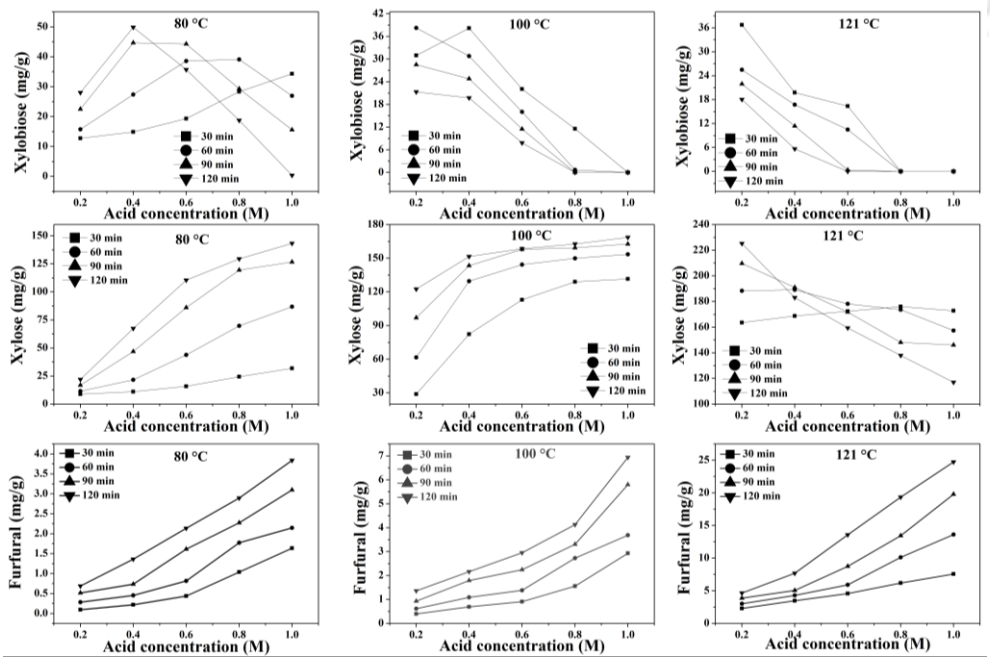

Figure 4.4: Different concentrations of xylobiose, xylose and furfural at selected pretreatment parameters

In addition to this, acetic acid is one of the most encountered by-products obtained during the acid pretreatment which is derived from the hemicellulose constituent of acetylated xylan [73]. The formation of acetic acid initiated at the beginning of hydrolysis reaction can be seen in Figure 4.5.

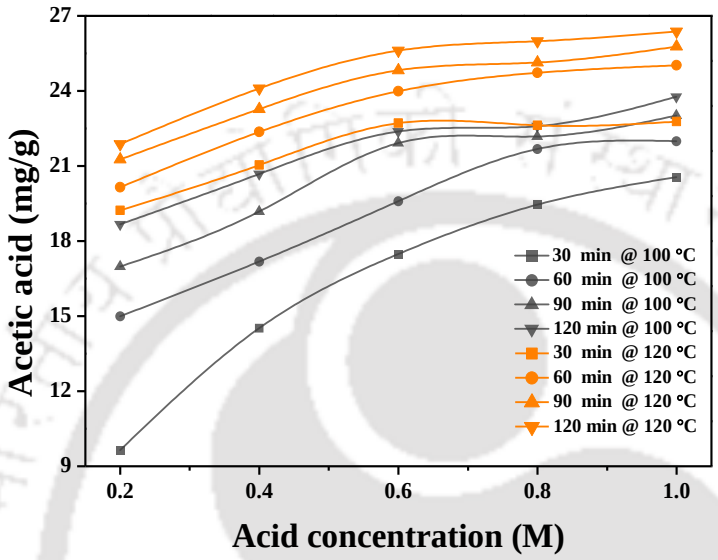

Figure 4.5: Acetylated xylan to acetic acid production at 100 °C and 121 °C with different sulfuric acid concentrations

4.3.1.2. *Hexose sugar yield and its degradation products*

Along with hemicellulose hydrolysis, acid pretreatment can also hydrolyse the cellulose polymer of sorghum biomass to produce glucose units. During the acid pretreatment, cellulose hydrolysis was found to be comparatively lower than that of hemicellulose. From Figure 4.6a, shows low level of glucose yield during sorghum biomass hydrolysis. Generally, cellulose exists in two forms in the lignocellulosic biomass, i.e. amorphous and crystalline cellulose [114]. The percentage of crystalline cellulose is higher than amorphous cellulose. It is a known fact that the crystalline cellulose is thermodynamically stable due to the presence of strong intra and inter-linked hydrogen bonds between the glucan chains [114]. This might be a reason for the

low concentration of glucose yield during the acid pretreatment. Apart from that some fraction of glucose and 5-HMF were also observed at 121 °C. 5-HMF is a dehydration product of glucose. With the increase in reaction time at 0.2 M acid concentration, 5-HMF formation was found to increase. Further, increase in acid concentration and reaction time, decreased 5-HMF concentration (Figure 4.6b). This could be due to the decomposition of 5-HMF into levulinic acid and formic acid [33]. From the above discussion, it is clear that all the three process parameters, viz. temperature, acid concentration, and time have a significant influence on the hydrolysis. Nonetheless, the combination of process parameters i.e. 121 ºC, 0.2 M H_2SO_4, and 120 min yields less concentration of 5-HMF (5.1 mg).

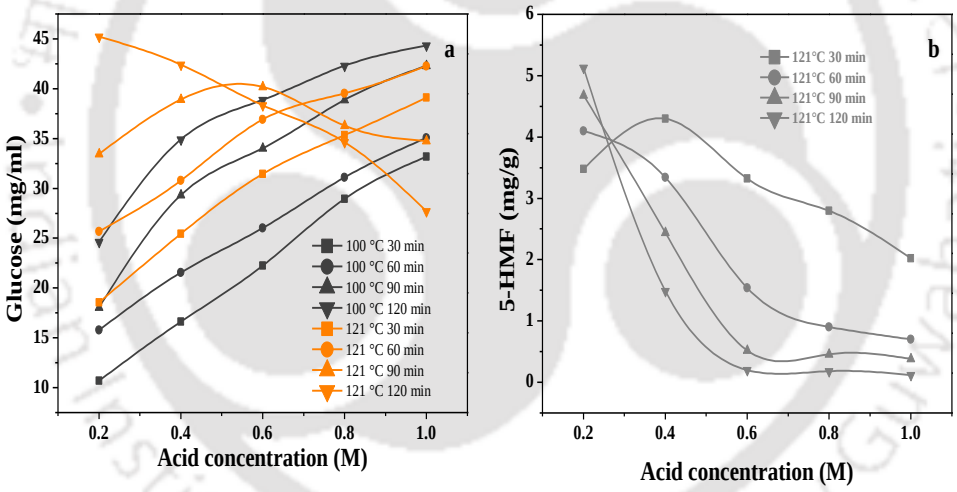

Figure 4.6: Glucose release from sorghum biomass and its decomposition product (a) glucose and (b) 5-HMF at different reaction times, acid concentrations and temperatures

4.3.2. Statistical impact of pretreatment parameters on pentose sugars release and furfural formation

Response surface methodology (RSM) is a statistical approach to analyse the importance of each individual pretreatment parameter and their interactions on

response variables. RSM has several advantages such as consumes less time and can investigate the various numbers of factors at a time with a minimum number of experiments. Central composite design (CCD) is one of the most popular models to optimize the independent variables. According to the CCD model, each and every factor of the experiments is simultaneously varied with all possible combinations to determine effect of variable interaction on the response. In the present study, CCD model has been employed and executed to determine the influence of pretreatment temperature, time and acid concentration on pentose sugars and furfural formation. Such analysis could be extremely useful in the conversion of lignocellulosic biomass into fermentable sugars and their degradation for further production of biofuels and value added products.

4.3.2.1. *ANOVA analysis*

Analysis of variance (ANOVA) demonstrated that the developed quadratic model for pentose sugars and furfural are the most significant, as their P-values are less than 0.05 (Table 4.2 and 4.3). The individual pretreatment parameters and their interaction effects on response variables were determined by the regression coefficients (R^2) as 0.94 and 0.95 for pentose sugars and furfural, respectively. The regression model equation resulting from ANOVA analysis in terms of coded factors for response variables are given below in Eq. 4.3 and 4.4.

$$\text{Pentose Sugars (mg/g)} = 244.55 + 21.60X_1 + 2.48X_2 + 14.66X_3 - 18.61X_1X_2 - 3.03X_1X_3 + 4.49X_2X_3 - 52.39X_1^2 - 8.99X_2^2 - 50.01X_3^2 \quad (4.3)$$

$$\text{Furfural (mg/g)} = 4.57 + 2.52X_1 + 0.27X_2 + C - 0.25X_1X_2 + 0.089X_1X_3 - 0.38X_2X_3 + 0.050X_1^2 - 0.18X_2^2 - 0.20X_3^2 \quad (4.4)$$

Table 4.2: ANOVA analysis for pentose sugars release quadratic model

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F
Model	81181.25	9	9020.14	18.60	< 0.0001
X_1-Temperature (C)	6371.57	1	6371.57	13.14	0.0047
X_2-Time (min)	84.16	1	84.16	0.17	0.6858
X_3-Acid Conc. (M)	2936.26	1	2936.26	6.05	0.0336
X_1X_2	2771.66	1	2771.66	5.72	0.0379
X_1X_3	73.40	1	73.40	0.15	0.7054
X_2X_3	161.16	1	161.16	0.33	0.5770
X_1^2	39553.22	1	39553.22	81.56	< 0.0001
X_2^2	1165.68	1	1165.68	2.40	0.1521
$X_3^{2\,2}$	36049.57	1	36049.57	74.33	< 0.0001
Residual	4849.78	10	484.98		
Lack of Fit	4843.66	5	968.73	790.54	< 0.0001
Pure Error	6.13	5	1.23		
Cor Total	86031.04	19			

Table 4.3: ANOVA analysis for quadratic model of furfural formation

Source	Sum of Squares	DF	Mean Square	F-Value	p-Value Prob > F
Model	103.94	9	11.55	26.10	< 0.0001
X_1-Temperature (C)	86.44	1	86.44	195.36	< 0.0001
X_2-Time (min)	1.03	1	1.03	2.32	0.1585
X_3-Acid Conc. (M)	13.70	1	13.70	30.97	0.0002
X_1X_2	0.51	1	0.51	1.16	0.3067
X_1X_3	0.064	1	0.064	0.14	0.7125

X_2X_3	1.16	1	1.16	2.63	0.1359
X_1^2	0.035	1	0.035	0.080	0.7830
X_2^2	0.47	1	0.47	1.06	0.3282
X_3^2	0.55	1	0.55	1.25	0.2890
Residual	4.42	10	0.44		
Lack of Fit	4.27	5	0.85	28.09	0.0011
Pure Error	0.15	5	0.030		
Cor Total	108.36	19			

From the results (Table 4.2 and 4.3), pretreatment temperature and acid concentration showed significant effect on the response variables (pentose sugars and furfural), whereas the reaction time showed less impact on both the responses. It was also noticed that there is a viable interaction effect between pretreatment temperature and time on pentose sugars yield. 3D response surface and contour plots illustrated that the interaction effect of independent variables on pentose sugars and furfural yield. The significant effect on the response variable can be observed by varying two factors at a time and keeping the other factor at a constant level. These plots are extremely important in order to investigate and understand the interaction effects between the two factors on the response. Figure 4.7a shows the interaction between temperature and acid concentration, in which the maximum pentose sugar yield increase at the center of the region (zero level). On the other hand, with an increase in the acid concentration at high temperature decreases the pentose sugars yield. Figure 4.7b and 4.7c indicates the interaction between pretreatment temperature and acid concentration with time on pentose sugars yield. The concentration of pentose sugars was increased at a fixed zero level of temperature and time. Through varying the affecting variables such as

temperature and time levels at a constant acid concentration leads to decrease the pentose sugars concentration. This could be due to the formation of sugar degraded product such as furfural.

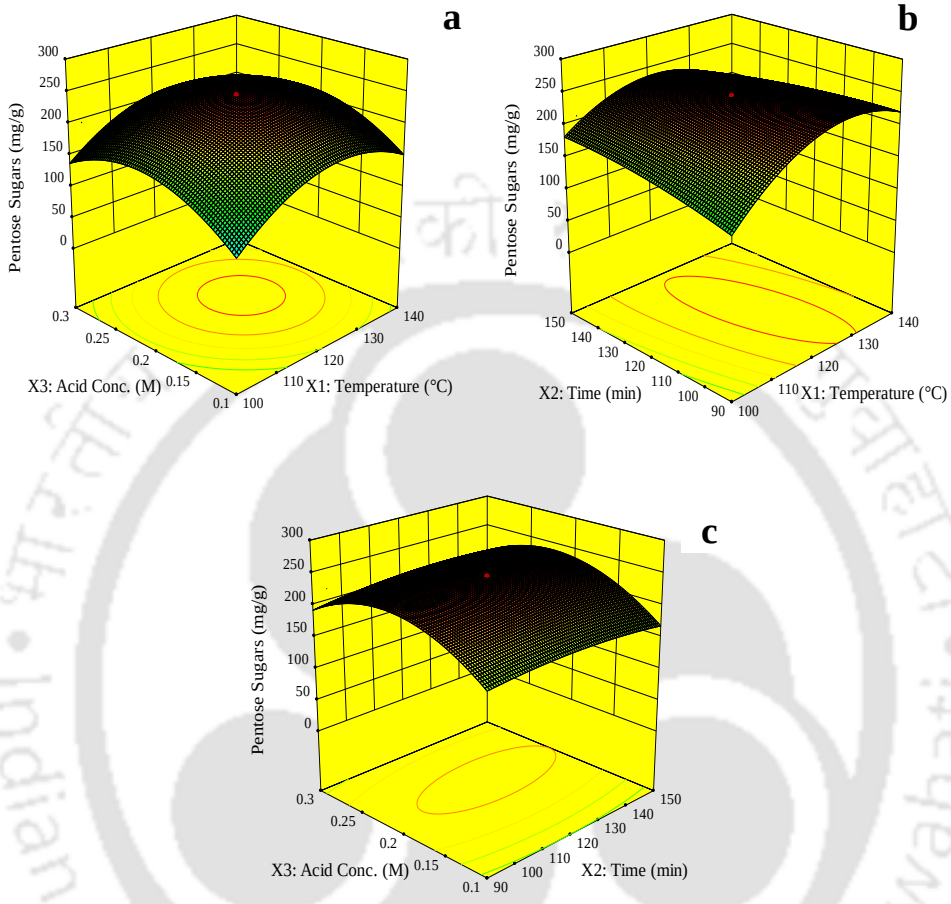

Figure 4.7: Response surface (3D) and counter plots indicating the interaction effect of independent variables on pentose sugars release and their decomposition; (a) acid concentration and temperature (b) time and temperature (c) acid concentration and time

The effect of temperature, time and acid concentrations on the formation of furfural are also shown in 3D response surface plots (Figure 4.8). The interaction of time with temperature (Figure 4.8a) and acid concentration with temperature (Figure 4.8b) continuously enhances the furfural concentration. On the other hand, increasing and then slightly decreasing trend was observed in the furfural concentration during the

interaction between acid concentration and time in the surface plot Figure 4.8c. Due to the prolonged pretreatment time, furfural decomposed into formic acid.

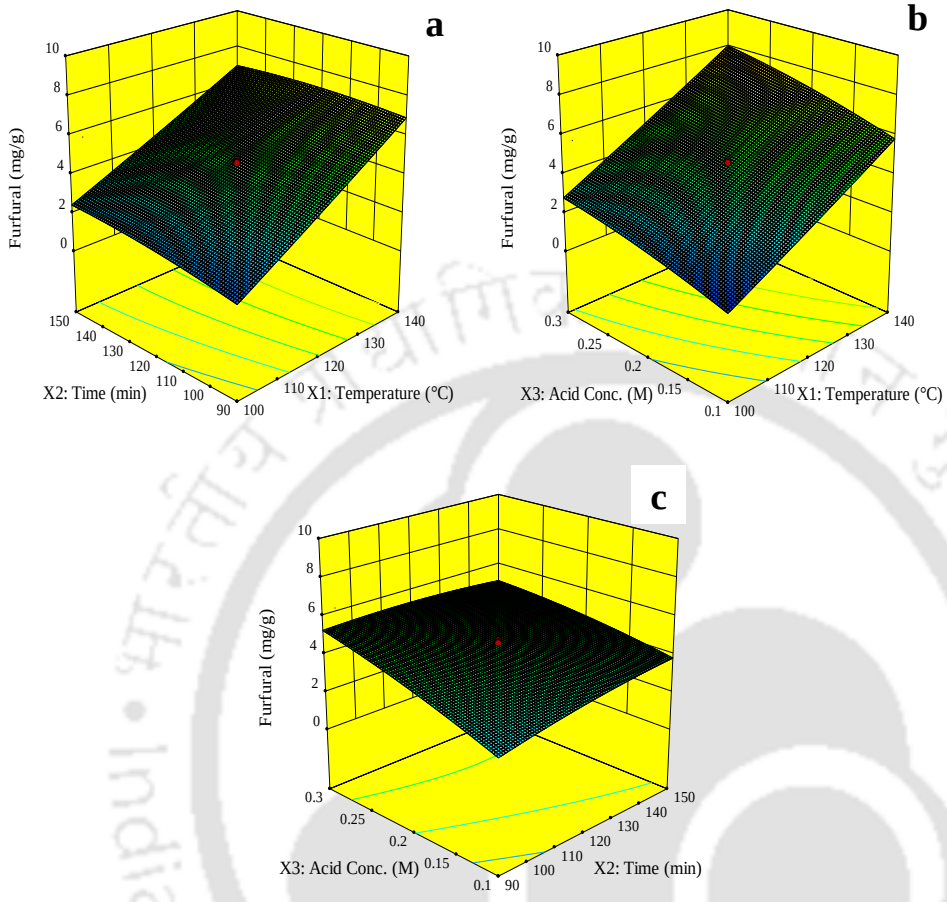

Figure 4.8: Response surface (3D) and counter plots representing the interaction effect of independent variables on furfural formation; (a) time and temperature (b) acid concentration and temperature (c) acid concentration and time

4.3.2.2. *Validation of predicted response at the optimum condition*

From the Table 4.1, the predicted optimum condition for maximum pentose sugars yield (241.2 mg/g) along with the minimal furfural concentration (4.57 mg/g) was obtained at T= 120.3 °C, t= 102.3 min, and C= 0.215 M acid concentration. In order to validate the predicted optimum condition responses, additional experiments were performed to examine the suitability of the model equation. From the experimental study, the pentose sugars and furfural concentrations were found to be

244.7 mg/g and 4.66 mg/g, respectively at the similar operating conditions. This indicates that the predicted model is in close agreement with the pentose sugars and furfural concentrations. The results of current study validated that, this model can be effectively applied on the hemicellulose hydrolysis of sorghum biomass for the production of maximum pentose sugars with low concentration of furfural. Pre-hydrolysate containing a high concentration of pentose sugars (especially xylose) and low concentration of fermentative inhibitor (furfural) may enhance the fermentation efficiency during the synthesis of bio-based products. Such analysis could be useful for designing a lignocellulosic biomass conversion process for the bio-refinery platform in a techno-economical prospective.

4.4. Summary

The optimum dilute acid pretreatment condition (121 °C, 0.2 M H_2SO_4 and 120 min) significantly hydrolysed the hemicellulose in the SBMR IS11861 biomass, with 97.6% conversion efficiency, and minimal fermentative inhibitors. The presence of low concentration of fermentative inhibitors would certainly enhanced the hydrolysates fermentation efficiency. The present optimization study showed a very good agreement between the experimental and predicted values of pentose sugars and furfural concentrations.

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Chapter 5:

Development of dilute sulfuric acid pretreatment method for the enhancement of xylose fermentability

Abstract

Based on the optimization study (chapter 4), 121 ºC was considered as the optimum temperature to hydrolyse the maximum hemicellulose content (~ 97.6%) of sorghum biomass. Moreover, fermentative inhibitors concentration was also found to be minimum at 121 ºC. Hence, the further pretreatment studies of sorghum biomass (IS 21549/*bmr 6 A*) has been conducted at 121 ºC for 2 h reaction time. Since lignin content of sorghum traits IS 21549/*bmr 6 A* was found to be higher among all the sorghum traits, the sulfuric acid concentration about 0.1–0.4 M has been used to evaluate the maximum hemicellulose hydrolysis, pentose sugars yield and fermentative inhibitors formation. As a result, 94.6% of hemicellulose was converted to yield 3 mg of xylobiose, 150.2 mg of xylose, 14.3 mg of arabinose and 4.26 mg of furfural per gram of biomass at 121 °C with 0.2 M sulfuric acid for 120 min. In addition to this, pre-hydrolysate liquors were detoxified with calcium hydroxide at different pH levels (i.e., 8.5 to 11.5) for investigation of fermentative inhibitors removal and their subsequent impact on xylulosic ethanol production. Consequently, around 91% ethanol conversion efficiency was achieved during the fermentation of pre-hydrolysate which was detoxified at pH 11.5. Moreover, 0.46 g_p/g_s ethanol yield and 0.48 g/L/h ethanol productivity were achieved within 24 h incubation time of *Pichia stipits* NCIM 3498.

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5.1. Introduction

Pretreatment of lignocellulosic biomass is a vital step to unlock the lignocellulosic complex structure. Most of the pretreatment process is aimed to hydrolyse maximum of the hemicellulosic fraction of lignocellulosic biomass to make residual biomass more amenable towards the enzymatic hydrolysis. In this regards, dilute-acid pretreatment is the most effective and widely used method, which is proven to be fast as well as cost-effective method [64]. This process significantly hydrolyses the hemicellulose to yield pentose sugars along with a few other sugars from the lignocellulosic material. Xylose is a principle sugar formed during dilute acid pretreatment. The main disadvantage of dilute acid pretreatment was sugar degradation which leads to fermentative inhibitors formation such as furans (Furfural, 5-hydroxymethyl furfural), which are further converted into organic acids (formic acid and levulinic acid). Along with this, acid soluble lignin-derived phenolic compounds are also formed [34]. These compounds show an inhibition effect on microbial growth during the fermentation process [84,160]. Therefore, conditioning of pre-hydrolysates (pretreatment derived hydrolysates) is an essential step which is employed prior to the successful fermentation of pre-hydrolysate [101].

Several methods have been well established for the conditioning of pre-hydrolysates. These include over-liming, ion-exchange, steam stripping, treatment with activated carbon, solvent extraction, and microbial acclimation, to improve the fermentation efficiency of pre-hydrolysates [68,101,161]. Among them, over liming with calcium hydroxide is the most economical and widely used method for detoxification which assists in the removal of furans such as furfural and 5-hydroxymethyl furfural (HMF) from the pre-hydrolysates [83,162]. However, certain limitations have been reported in over liming process i.e., sugar loss is generally caused

when the pH is significantly elevated and also it is not an effective way to reduce toxicity caused by organic acids such as formic acid and acetic acid.

However, xylulosic ethanol production at high yield from pre-hydrolysates would be helpful for the commercialization of lignocellulosic biofuels production process [163]. A number of naturally occurring yeast species such as *Candida shehatae*, *Pichia stipitis*, and *Pachysolen tannophilus* are able to efficiently ferment both glucose and xylose into ethanol [164]. Among them, *Pichia stipitis* exhibits good potential towards the industrial application, because it ferments xylose with a high ethanol yield [165]. Therefore, the present study focused on the development of sorghum biomass pretreatment method with dilute sulfuric acid at stipulated parameters for maximum xylan conversion with minimized xylose decomposition. Additionally, validation of developed pretreatment process was done by fermentation of conditioned hydrolysate using *Pichia stipitis*.

5.2. Materials and methods

5.2.1. Dilute sulfuric acid pretreatment

Sorghum biomass (IS 21549/bmr 6 A) has used in the present study and its chemical composition is shown in Table 5.1.

Table 5.1: Chemical composition of sorghum biomass (IS 21549/*bmr 6A*)

Composition	Untreated (%)	Treated (%)
Water extractives	12.1	-
Ethanol extractives	2.92	-
Cellulose	36.3	58.2
Hemicellulose	22.2[a]	2.44[b]
Lignin	18.2	32.6

[a]Xylan- 19.7%, Arabinan- 2.5%; [b]Xylan- 2.17%, Arabinan- 0.27%;

In the previous chapters, we performed pretreatment studies in screw cap conical flask which were kept in an autoclave for a period of 2 h. At an interval of every 30 min, the reaction was terminated and cooled until it reaches the room temperature. Therefore, to complete the pretreatment reaction it took almost 8 h, which includes 30 min to raise the temperature to 121 ºC from ambient temperature, thereafter 30 min of pretreatment reaction and further 60 min for decreasing the reaction temperature from 121 ºC to ambient. Therefore, in the present study, we assembled a pretreatment setup to withdraw a sample in between from reaction mixture without disturbing the pretreatment process. Consequently, the time required to perform 2 h pretreatment reaction has been reduced from 8 h to 3.5 h.

Pretreatment process is conducted in a portable autoclave (Equitron, 7407 PAD, India) which is generally used for sterilization purpose in R&D laboratories. The autoclave has a working chamber of 300 (Ø) X 230 (D) mm with 16 L capacity which has been equipped with an industrial grade energy efficient ring type heater with a heating rate of 4.8 ± 0.2 ºC /min (which is immersed in 3 L of distilled water), exhauster valve (for pressure release), vacuum breaker, pressure regulating device, safety valve, pressure gauge and drain valve. Making no physical alternations to the existing autoclave, we have simply used the drain valve as the sampling port. The schematic diagram of the autoclave is shown in Figure 5.1. A factory preset temperature of 121 ºC generates 15 lbs pressure which sufficiently provides heat to the pretreatment reactor vessel.

1 L of Duran glass bottle [101 (O.D.) X 225 (h) mm] was used as pretreatment reactor vessel which contains 30 g of biomass in 600 mL (solid to liquid ration is 1:20 w/v) of sulfuric acid at different molar concentrations from 0.1 M–0.4 M H_2SO_4. This

glass bottle having a screw cap with one male hose stainless steel connector (DURAN® Stainless steel 1-port connector cap GL 45, Germany) on both the top and bottom assemblies for flexible tubing. Both the ports were connected with silicone tube (Thermo-Scientific, Masterflex®, USA). Inlet port was connected to 0.2 µm solvent filter (Chrome Tech, A-242, USA) and submerged in the reaction mixture. The outlet port was connected to inlet port of drain valve with the help of silicon tube. Due to the autogenous pressure, the sample could easily pass through the sampling port (external port of drain) when the screw type valve was slightly loosened. Therefore, at every 30 min interval, around 5 mL of sample was collected from the reaction mixture.

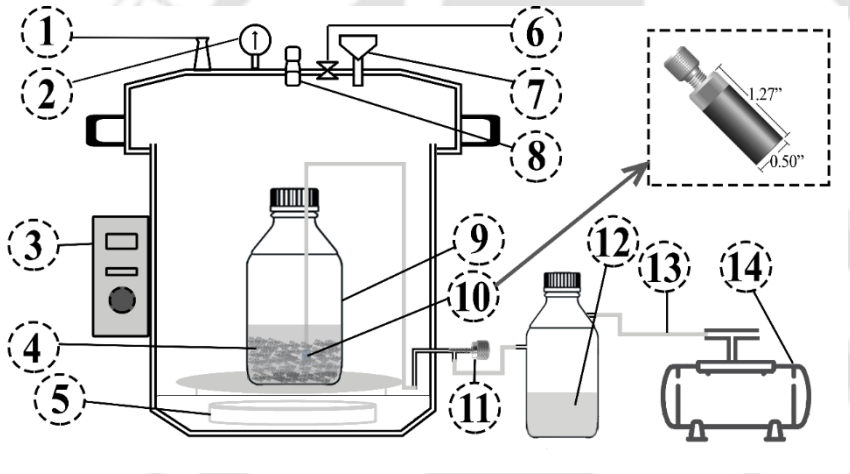


Figure 5.1: Pretreatment reaction setup *1) Pressure safety valve, 2) pressure gauge, 3) PAD controller, 4) reaction mixture, 5) heater, 6) pressure regulating device, 7) exhauster valve, 8) vacuum breaker, 9) reactor vessel, 10) inlet solvent filter, 11) screw type drain valve, 12) pre-hydrolysate collection vessel, 13) silicon pipe and 14) vacuum pump*

Around 100 µL of the sample was taken and upon appropriate dilution was subjected to HPLC analysis to estimate sugars and fermentative inhibitors. According to the following equation (Eq. 5.1), the concentration of sugars and fermentative inhibitors have been calculated.

$$Sugars or\ Inhibitors\ (mg/mL) = Concentration\ \text{detected}\ by\ HPLC \times DF \qquad (5.1)$$

At optimum pretreatment condition, the entire chamber is allowed to reach ambient temperature and thereafter pre-hydrolysate was extracted from the reaction mixture by applying vacuum, as depicted in Figure 5.1. The schematic illustration of this entire pretreatment process and separation of pre-hydrolysate from the reaction mixture has been shown in Figure 5.1. The amount of sugar released per gram of biomass (on the dry weight basis at 105 ºC) has been calculated according to Eq. 5.2.

$$Sugars\ (mg/g) = \left(\frac{Sugars\ (mg/mL) \times total\ volume\ of\ prehydrolyzate\ (mL)}{Initial\ weight\ of\ biomass\ (g)} \right) \quad (5.2)$$

The liquid fraction was subjected to detoxification process, whereas the solid residue recovered after pretreatment was washed with distilled water until it reaches to neutral pH and then dried at 45 ºC for 48 h. The residual carbohydrates and lignin content present in the pretreated solid residue are analyzed according to the NREL procedure [31]. The percentage of cellulose and hemicellulose hydrolysis after pretreatment process has been calculated according to the following equation (Eq. 5.3).

$$\text{Hydrolysis (\%)} = \left(1 - \frac{\text{Conc. of sugars in the residual weight of biomass}}{\text{Conc. of sugars in the initial weight of biomass}} \right) \times 100 \quad (5.3)$$

5.2.2. Detoxification of pre-hydrolysates

During the detoxification process, the unconditioned pre-hydrolysate or acid hydrolysate was divided into four fractions and each fraction was treated with calcium hydroxide [$Ca(OH)_2$] to attained different pH levels i.e., 8.5, 9.5, 10.5 and 11.5. First, the unconditioned pre-hydrolysates were heated to 50 °C and held at this temperature for 15 min. This was followed by slow addition of $Ca(OH)_2$ to reach the different targeted pH range and then continued the agitation for 30 min. These over-limed hydrolysates were centrifuged to separate the calcium sulfate ($CaSO_4$) sludge and pH

of supernatants were readjusted to 6 (which is a cultivation pH of *P. stipitis*) with 10N sulfuric acid. Thereafter, these liquors are considered as conditioned hydrolysates. These conditioned hydrolysates were concentrated under reduced pressure by using rotary evaporator (Buchi, Rotavapor-R-210, and Switzerland). The concentrated hydrolysate was stored for xylulosic ethanol production.

5.2.3. Microorganism

Pichia stipitis NCIM 3948 (Same as CBS 6054) strain was procured from National Collection of Industrial Microorganisms (NCIM) Pune, India. This strain was supplied on MGYP agar slants, which contained (g/L): 3, Malt extract; 10, Glucose; 3, Yeast extract; 5, peptone; and 20, agar; and, pH was maintained at 6.4–6.8.

5.2.3.1. *Seed culture preparation*

Modified YPDX media was prepared based on the ratio of glucose and xylose (i.e., 1:4) present in the conditioned hydrolysate. The composition of modified YPDX media includes (g/L): yeast extract-10, peptone-20, glucose-5, xylose-15, agar-20, and was autoclave sterilized. *P. stipitis* was cultivated on modified YPDX agar plates and incubated at 30°C for 48h. A colony from the plate was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of filter-sterilized YPDX liquid growth medium and incubated in a shaking incubator with an agitation speed of 120 rpm at 30 °C for 18 h. The pH of YPDX medium was maintained at 6. Further, cells were harvested by centrifugation at 8000 rpm for 10 min and re-suspended in sterile distilled water to adjust the final concentration of 40 g/L which is served as inocula for bioethanol production. Cell growth was observed by measuring the absorbance at 600nm (OD_{600}) using UV-Visible spectrophotometer (Agilent, Cary 100, USA).

5.2.3.2. *Fermentation*

The fermentation was performed in sterile 250 mL Erlenmeyer flasks containing 100 mL of fermentation medium which includes 2 mL of 50X concentrated nutrient solution (1.7 g of yeast nitrogen base, 1 g of urea and 6.56 g of peptone in 20 mL of water), 5 mL of inocula gives an initial cell concentration of 2 g/L and, added an appropriate quantity of hydrolysate to reach desired volume. Initial pH of the media was adjusted to 6 and incubated at 30 °C with 120 rpm. All fermentation samples were taken periodically for HPLC analysis.

5.2.4. Analytical methods

Each sample was filtered using a 0.2 μm filter and appropriate dilution was made with Milli Q water. Sugars, fermentative inhibitors, and ethanol quantitative analysis were performed using a Varian 210 HPLC system. The detailed HPLC specifications and operating conditions are described in section 2.2.4. Moreover, the total phenolic content present in the samples were also determined by the Folin–Ciocalteu [166] method using gallic acid as a standard.

5.3. Results and discussion

5.3.1. Monomeric sugars yield during the pretreatment of biomass

Determination of the efficiency of a pretreatment process is naturally dependent on the hemicellulose hydrolysis of lignocellulosic biomass. During the pretreatment of sorghum biomass, xylose (a major hemicellulose component) found to be the predominant sugar present in the pre-hydrolysate liquors. However, at high pretreatment severity conditions, the amount of xylose concentration was decreased due to decomposition of xylose into furfural.

However, the present study was focused on xylose yield into pre-hydrolysate liquors with respect to pretreatment parameters. Sulfuric acid concentration shows a synergetic effect on hemicellulose hydrolysis. From Figure 5.2a, it can be seen that xylose content increased drastically from 96.02 mg to 150.2 mg due to increase in the sulfuric acid concentration and reaction time, i.e., 0.1 M to 0.2 M at 30 min to 120 min, respectively. Further increase in the sulfuric acid concentration (0.3–0.4 M) showed a slight decrease in the xylose concentration at 60 to 120 min, which could be due to the cyclo-dehydration of xylose. Therefore, high acid concentration effectively hydrolysed the hemicellulose to release xylose units and concurrently influence its decomposition into furfural. From Figure 5.2b it is evident that 0.4 M sulfuric acid concentrations yield more amount of furfural compared with other acid concentration.

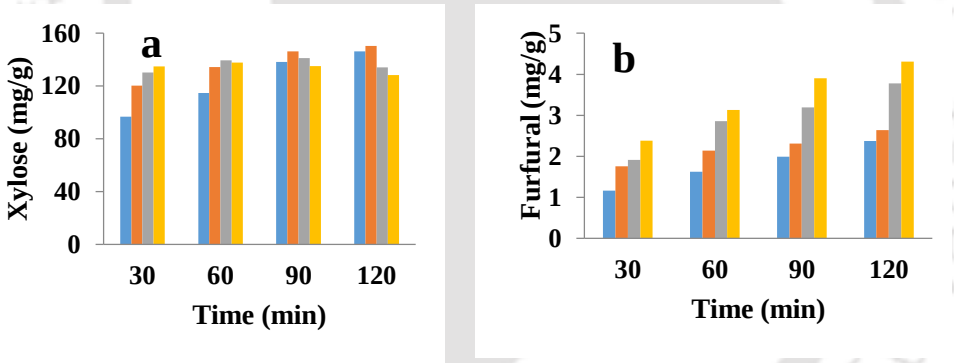


Figure 5.2: Effect of pretreatment parameter on a) xylose release and b) furfural formation

Blue, Orange, Grey and yellow bars indicates 0.1 M, 0.2 M, 0.3 M and 0.4 M sulfuric acid, respectively.

For instance, 150.2 mg of xylose yield was observed at 121 ºC, 0.2 M acid concentration and 120 min pre-treatment, whereas, 0.4 M H_2SO_4 yield 128.4 mg xylose under otherwise similar condition. Moreover, compositional analysis of pretreated biomass samples (by NREL procedure) derived at 0.2 M and 0.4 M H_2SO_4 revealed that the decomposition of pentose sugars increased with corresponding sulfuric acid concentration (Table 5.2).

Table 5.2: Composition analysis of residual biomass according to NREL procedure

Components (mg)	Residual biomass	
	121 °C, 0.2M, 2h (per 546 mg)	121 °C, 0.4M, 2h (per 528 mg)
Cellulose	319	315.5
Xylan	12.2	4.1
AIL*	175.4	171.8

** Acid insoluble lignin*

$$Xylan \text{ conversion } (\%) = \frac{(I_x - R_x)}{I_x} \times 100 \quad (5.4)$$

$$Xylose\ decompostion\ (\%) = \frac{[I_x - (L_{x,xb} + R_x)]}{I_x} \times 100 \quad (5.5)$$

Where, I_x is the initial amount of xylose present in the untreated biomass, R_x stands for the amount of xylose present in the residual biomass derived from pretreatment and $L_{x,\,xb}$ is the concentration of xylose and xylobiose present in the pre-hydrolysate liquor.

Theerarattananoon et al., (2010) in their study have used pressurized batch reactor for the pretreatment of different sorghum biomass varieties at 140 °C and 2% (v/v) H_2SO_4. The results of their study revealed xylan conversion of about 82% in grain sorghum, 83% in BMR sorghum, 84% in photosensitive sorghum, 87% in forage sorghum and 90% in sweet sorghum along with 19.6 to 38% of xylose decomposition (Table 5.3). Further, increase in the temperature i.e., 165 °C leads to slightly increase the xylan conversion and concurrently higher xylose (51 to 69.8%) decomposition. The comparative analysis of the results obtained in the present study with that of the literature is shown in Table 5.3. It is very well known that decomposition of xylose

generally initiated at a temperature higher than 120 °C [145]. Pre-treatment at 140 °C and 165 °C showed drastic degradation in the xylose which ultimately results in fermentative inhibitor formation.

Table 5.3: Summary of xylan conversion and xylose decomposition analysis results of the present study with literature

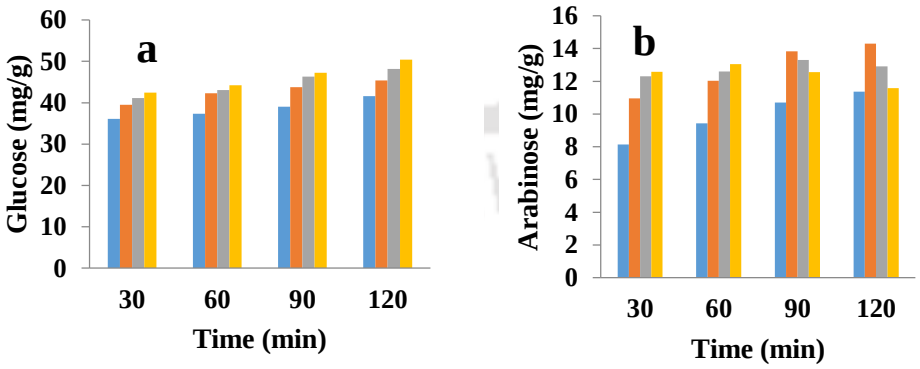
Type of Sorghum Biomass	Pretreatment condition	Xylose (mg/g)*	%XC (Eq. 5.4)	Xylose (mg/g) (L_X)	%XD (Eq. 5.5)
Grain	Untreated	246.0 (I_x)	–	–	–
	140 °C, 30 min [a]	43.4 (R_x)	82.5	152.9	20.3
	165 °C, 10 min [a]	25.8 (R_x)	89.8	99	49.4
BMR	Untreated	261.6 (I_x)	–	–	–
	140 °C, 30 min [a]	42.2 (R_x)	83.8	166.8	19.6
	165 °C, 10 min [a]	29.4 (R_x)	88.4	97	51
Photoperiod sensitive	Untreated	274.0 (I_x)	–	–	–
	140 °C, 30 min [a]	42.6 (R_x)	84.6	154.4	28.4
	165 °C, 10 min [a]	24.2 (R_x)	91.2	81	61.6
Forage	Untreated	297.9 (I_x)	–	–	–
	140 °C, 30 min [a]	36.2 (R_x)	87.8	148.2	38.1
	165 °C, 10 min [a]	25.4 (R_x)	91.6	95	59.7
Sweet	Untreated	268.1 (I_x)	–	–	–
	140 °C, 30 min [a]	26.4 (R_x)	90.2	178.6	23.6
	165 °C, 10 min [a]	24.1 (R_x)	91	56.7	69.8
Present study BMR	Untreated	197.4 (I_x)	–	–	–
	121°C, 120 min [b]	21.7 (R_x)	93.8	150.2	16

** mg/g of biomass; XC, xylan conversion; XD, xylose decomposition; [a] 2% (v/v) H_2SO_4; [b] 0.2 M H_2SO_4 [or equivalent to 1.12% (v/v)]; - Not applicable; ^{I}X Initial xylan present in the untreated biomass; ^{R}X residual xylan present pretreated biomass; ^{L}X xylose concentration in the liquid fraction or pre-hydrolysate.*

Therefore, the present study suggests that, instead of using high temperature and high-pressure batch reactors, utilization of autoclave (121 °C) for pretreatment of sorghum biomass showed significant impact on xylan conversion (93.8%) with a lower

concentration of furfural. In addition to this, use of autoclave for pretreatment of lignocellulosic biomass has several advantages over high energy input batch reactors such as less capital investment, low power consumption, zero maintenance cost and easy to operate.

Apart from xylose and furfural, acid catalyzed pretreatment reaction can also produce different types of sugars and fermentative inhibitors. Sugars like glucose (Figure 5.3a) and arabinose (Figure 5.3b) are derived from cellulose and hemicellulose, respectively. Fermentative inhibitors such as 5-HMF (Figure 5.3c), formic acid (Figure 5.3d), acetic acid and phenolic compounds are derived from glucose, furfural, acetylated xylan and acid soluble lignin, respectively. Release and stability of sugars are depended on the pretreatment severity conditions. As pretreatment severity increases glucose dehydrates to form 5-HMF, furfural decomposes to form formic acid and, the accumulation of phenolic compounds may also increase in pre-the hydrolysate liquors. These are known to be potential toxic compounds which inhibit microbial growth during fermentation [34].



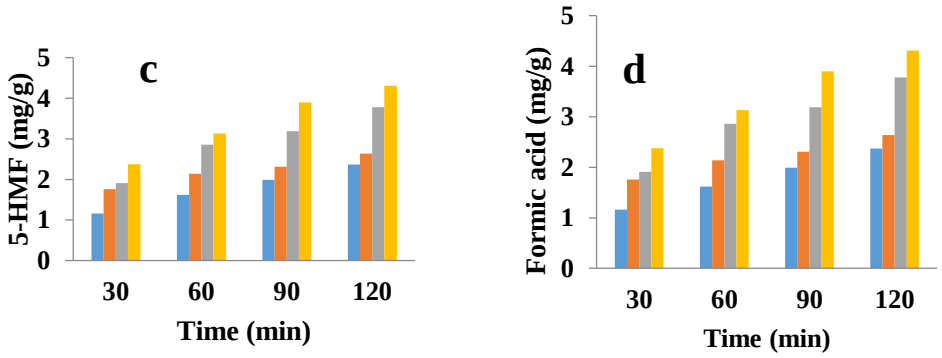


Figure 5.3: Effect of pretreatment parameters on a) glucose, b) arabinose, c) 5-HMF and d) formic acid formation

Blue, Orange, Grey and yellow bars indicates 0.1 M, 0.2 M, 0.3 M and 0.4 M sulfuric acid, respectively.

However, in order to minimize the fermentative inhibitor effect on the microorganisms for efficient fermentation, it is essential to select an optimized condition which gives a comparatively high concentration of sugars with lower amounts of fermentative inhibitors. Therefore, from the results, the optimized sugars yield was obtained at 121 °C, 0.2 M H_2SO_4 and 120 min. At this condition, 209.9 mg/g of maximum sugars yield was obtained which includes xylose (150.2 mg/g) arabinose (14.3 mg/g), glucose (45.4mg/g) along with fermentative inhibitors like, 4.26 mg of furfural, 5 mg of 5-HMF, 2.64 mg of formic acid, 21.3 mg of acetic acid and 3.7 mg of phenolic compounds.

5.3.2. Conditioning of pre-hydrolysates

Detoxification is an essential step prior to the fermentation of pre-hydrolysates. Over-liming is a well-known procedure to reduce the toxicity of pre-hydrolysates through the removal of inhibitory compounds by precipitation which chemically converts the potentially toxic components into less toxic forms that eventually enhance the fermentation efficiency. The effect of various over liming pH levels on furans

(furfural and 5-HMF), organic acids (acetic and formic acids) and phenolic compounds were investigated. The percentage of fermentative inhibitors removal along with sugars loss at different over liming pH levels is shown in Figure 7.3. Moreover, the concentration of sugars and fermentative inhibitors remained after the over liming process are listed in Table 5.4.

Table 5.4: Chemical composition of conditioned hydrolysates before rotary evaporation

Compounds (g/L)	AH	CDH 8.5	CDH 9.5	CDH 10.5	CDH 11.5
Glucose	2.31	2.15	2.09	2.05	1.99
Xylose	10.1	9.43	9.23	8.5	8.01
Arabinose	0.82	0.74	0.72	0.69	0.67
Formic acid	0.65	0.63	0.62	0.60	0.58
Acetic acid	1.62	1.53	1.49	1.45	1.41
HMF	0.3	0.27	0.26	0.25	0.24
Furfural	0.34	0.31	0.28	0.26	0.25
Phenolic (g/L)	0.50	0.47	0.45	0.43	0.41

CDH, Conditioned hydrolysates; AH, acid hydrolysate

Although organic acid concentrations were less affected by over-liming, but a reasonable decrease in the total phenolic compounds and a significant decrease in furan content was observed. Apart from the sugar loss, decrease in the furan concentrations was observed by increasing the over liming pH levels. The effective detoxification was achieved at pH-11.5 which includes 23.7% of furfural, 20% of 5-HMF, 11.9% of formic acid, 12.9% of acetic acid and 20% of the total phenolic compounds reduction (Figure 5.4). Furthermore, pH of all the over-limed hydrolysates was readjusted to 6 with 10N

H_2SO_4 and then concentrated under the reduced pressure by a rotary evaporator. Hereafter, these hydrolysates referred to as conditioned hydrolysates.

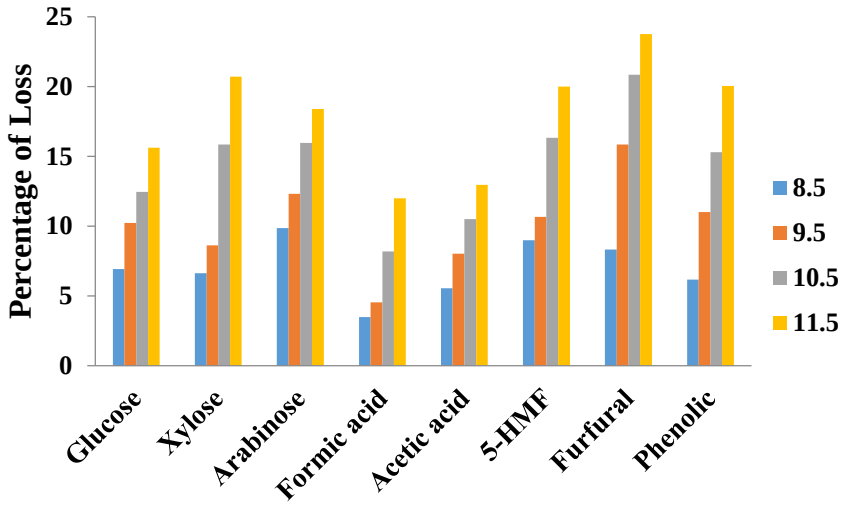


Figure 5.4: Fermentative inhibitors removal along with sugars loss during the over-liming process at various targeted pH levels

5.3.3. Fermentation

The chemical compositions of conditioned hydrolysates (denoted as CDH-8.5, CDH-9.5, CDH-10.5, and CDH-11.5) are summarized in Table 5.5. According to the Delgenes et al., (1996), 2 g/L of furfural, 5 g/L of 5-HMF and 11.9- 15 g/L of acetic acid are the inhibiting concentrations of *P. stipitis* and other yeast species. Thus, in this present study fermentative inhibitors range was restricted below the inhibition limit.

Table 5.5: Chemical composition of different conditioned hydrolysates and synthetic sugars used for fermentation

Compounds (g/L)	CDH 8.5	CDH 9.5	CDH 10.5	CDH 11.5	Synthetic sugars
Glucose	5.37	5.22	5.12	4.97	5.2
Xylose	23.57	23.07	21.25	20.02	20.6
Arabinose	1.85	1.8	1.72	1.67	-

Formic acid	1.59	1.57	1.51	1.45	-
Acetic acid	3.82	3.65	3.47	3.3	-
HMF	0.68	0.67	0.62	0.6	-
Phenolic (g/L)	1.18	1.12	1.08	1.04	-

Furfural was not detected after the rotary evaporation.

5.3.3.1. *Effect of over-liming pH levels on ethanol production*

From the results (Table 5.6), the highest ethanol conversion (91%), ethanol yield (0.46 g_p/g_s) and productivity (0.48 g/L/h) were observed during the fermentation of CDH-11.5 hydrolysate and it was completed in 24 h cultivation time of *Pichia stipitis*. Except the ethanol productivity (0.35 and 0.41 g/L/h) and cultivation time (36 h and 30 h) a substantial range of ethanol yield 0.44 g_p/g_s and ethanol conversion (86.4% and 86.7%) was observed in CDH- 8.5 and 9.5. hydrolysates fermentation. In the case of CDH-10.5 hydrolysate fermentation, approximately similar ethanol concentration (11.6 g/L) and productivity (0.48 g/L/h) was observed within 24 h of fermentation time, but, the ethanol yield (0.44 g_p/g_s) and ethanol conversion (86.3%) was comparatively lower than that of CDH-11.5 hydrolysate fermentation. As compared to the other over liming pH levels (8.5, 9.5 and 10.5), the percentage of fermentative inhibitors removal was found to be higher at pH 11.5 (Figure 5.4). Therefore, highest ethanol conversion (91%) was achieved during the fermentation of CDH-11.5 hydrolysate. Moreover, during the fermentation of synthetic sugars, about 12.3 g/L ethanol production was observed was observed within 24 h of fermentation period and the ethanol productivity and ethanol yield were found to be of 0.48 g/L/h and 0.47 g_p/g_s, respectively. The synthetic sugars fermentation results are meticulously resembling with that of CDH-11.5 hydrolysate fermentation results.

Table 5.6: Fermentation summary results of conditioned hydrolysates and synthetic sugars

Parameters	CDH 8.5	CDH 9.5	CDH 10.5	CDH 11.5	SS*
Ethanol concentration (g/L)	12.76	12.52	11.6	11.6	12.3
Ethanol yield (g_P/g_S)	0.44	0.44	0.44	0.46	0.47
Ethanol productivity (g/L/h)	0.35	0.41	0.48	0.48	0.51
Sugar consumption rate (g/L/h)	0.8	0.94	1.09	1.04	1.07
Cell growth (g/L/h)	0.19	0.23	0.28	0.31	0.31
Max. Ethanol production Time (h)	36	30	24	24	24
Ethanol conversion efficiency (%)	86.4	86.7	86.3	91.2	93.4

**Synthetic sugars*

According to the previous literature reports, slow xylose fermentation with low ethanol yield and low ethanol productivity are the major disadvantages of various sources of hemicellulosic hydrolysate fermentation [77]. A brief literature data of *P. stipitis* on ethanol conversion efficiency, yield, and productivity along with fermentation time of various hemicellulose hydrolysates that are detoxified by different methods are listed in Table 5.7. Even though, performing different detoxification methods like, ion exchange resins [167], trialkylamine extraction [168], over liming and followed by activated carbon adsorption (for the removal of fermentative inhibitors) [169] and microbial adaptation (for reducing the fermentative inhibitor effect on fermenting organism) [161] have not shown significant range of ethanol conversion efficiency, ethanol yield and productivity in a limited time period of fermentation.

Table 5.7: Brief literature summaries of different hemicellulose hydrolysates fermentation using *P. stipitis*

Feed stock	Conditioning	EY (g_s/g_p)	EP (g/L/h)	Time (h)	Reference
Sugarcane Bagasse	Activated charcoal	0.35 (68.6%)*	0.34	70	[170]
Corncobs	detoxification	0.33 (64.7%)*	0.18	44	[171]
Wheat Straw	Overliming	0.41 (80%)*	0.39	48	[172]
Water-Hyacinth	Overliming	0.35 (68.6%)*	0.18	100	[173]
Rice Straw	Overliming	0.37 (72.5%)*	0.22	41	[161]
Sugarcane Bagasse	Ion-exchange resins	0.30 (58.8%)*	0.16	48	[167]
Corn Stover	Tri-alkylamine extraction	0.34 (66.6)*	0.43	48	[168]
Cotton Stalk	Overliming with activated charcoal	0.45 (88%)*	0.28	36	[169]
Sorghum Stalks	Overliming	0.46 (91%)*	0.47	24	Present study

***** *Ethanol conversion efficiency*

As compared to literature reports (Table 5.7), the present study shows potential results such as fermentation was completed at 24 h incubation time with 91% ethanol conversion efficiency followed by 0.46 g_p/g_s ethanol yield and 0.48 g/L/h ethanol productivity. This could be due to the presence of low concentration of fermentative inhibitors in the pre-hydrolysate that does not deter the microbial growth during the fermentation of conditioned hydrolysate.

5.3.3.2. *Sequential substrate utilization and Cell growth*

During the fermentation of conditioned hydrolysates, xylose consumption initiates only after the complete utilization of glucose by *P. stipitis*. As shown in Figure 5.5–

5.5d, complete glucose consumption was observed at 6 h of fermentation, thereafter, xylose consumption was initiated. This is because glucose is the preferred substrate of *P. stipitis*. Therefore, xylose consumption initiates only after complete consumption of glucose. This could be due to the suppression of xylose uptake by glucose [174].

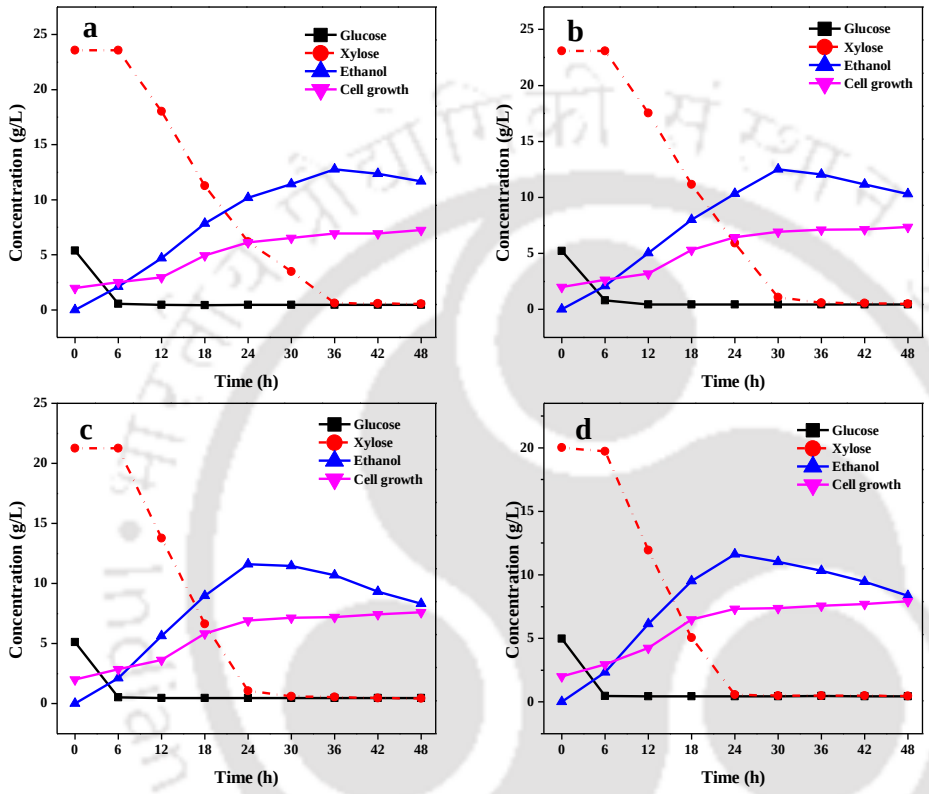


Figure 5.5: Profile of sugars utilization, ethanol production and cell growth during the fermentation of different detoxified/conditioned hydrolysates a) CDH-8.5 b) CDH-9.5 c) CDH-10.5 and d) CDH-11.5

On the other hand, there is no lag phase observed during fermentation of detoxified hydrolysates, because pretreatment derived fermentative inhibitors are not detrimental to the *P. stipitis* growth. Some reports stated that a high concentration of fermentative inhibitors usually deter the microbial growth [84,160]. In that case, microorganisms acclimatized to fermentative inhibitors in which there is neither a cell growth nor a product formation. Interestingly, in the present study, dynamic cell growth was observed until the maximum ethanol production, thereafter, cell growth was found to

be minimal. This is mainly because, *P. stipitis* utilizes glucose and xylose (fermentable sugars) until it reaches the maximum ethanol production, thereafter, ethanol re-assimilation occurred. Apart from that, at the end of maximum ethanol production, comparatively higher cell growth rate (0.31 g/L/h) was observed during the fermentation of CDH-11.5 hydrolysate, whereas, lowest cell growth rate (0.19 g/L/h) was observed in CDH-8.5 hydrolysate fermentation. An increased trend of cell growth rate and cell rate was observed from CDH-8.5 to CDH-11.5 hydrolysates fermentation (Table 5.6 and Figure 5.5a–5.5d). It is a fact that the percentage of fermentative inhibitors removal was high at elevated over-liming pH levels which ultimately enhances the cell growth rate as well as cell concentration. Moreover, an ethanol yield of 0.47 (g_p/g_s) and ethanol productivity of 0.51 g/L/h and cell growth of 0.31 g/L/h was observed during the fermentation of synthetic sugars, which are closely resemble with that of fermentation results of CDH-11.5.

Along with glucose and xylose, *P. stipitis* can also utilize acetic acid and formic acid during fermentation of conditioned hydrolysates (Figure 5.6b and 5.6c). However, product formation from *P. stipitis* is still unknown during the consumption of acetic acid and formic acid, nevertheless, an increase in pH of the fermentation medium is observed. A similar observation (such as acetic acid consumption and increase in pH) was found in the fermentation of hemicellulose hydrolysates of corn stover wood and sugarcane bagasse [165]. Moreover, at the end of maximum ethanol production, *Pichia stipitis* started the consumption of a non-fermentable sugar (arabinose) along with ethanol re-assimilation (Figure 5.6d). A previous study also suggests that *P. stipitis* can utilize arabinose for cell growth but not for ethanol production [172]. Even though this information is out of context in the present work yet it is still being discussed on research retrospect.

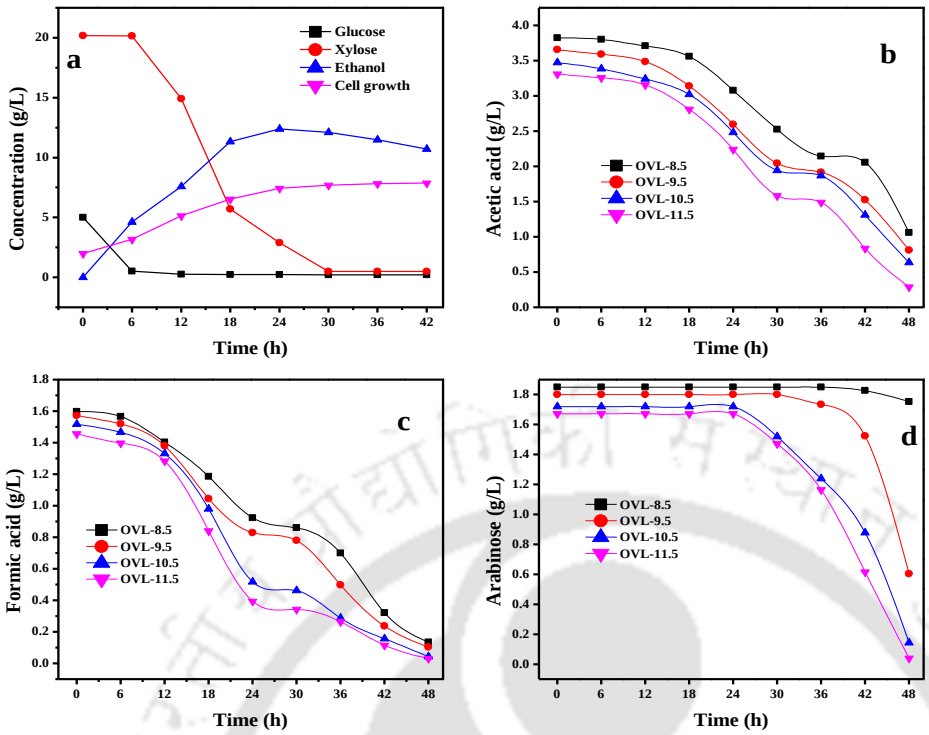


Figure 5.6: a) Fermentation profile of synthetic sugars and utilization of b) acetic acid c) formic acid d) arabinose during the fermentation of conditioned hydrolysates

5.4. Summary

The developed pretreatment conditions significantly hydrolysed the xylan (93.8%) with only 31 mg of xylose decomposition. During the fermentation of conditioned hydrolysates, ethanol conversion was found to be 86% and above. This could be due to the least amount of fermentative inhibitors formed during the pretreatment which have not shown any adverse effect on the fermentability of hydrolysates. Consequently, the highest overall ethanol conversion (91%) followed by 0.46 g_p/g_s ethanol yield and 0.48 g/L/h productivity was attained during the fermentation of CDH-11.5 hydrolysate at 24 h cultivation time of *P. stipitis* 3498. Less amount of xylose decomposition, low concentration of fermentative inhibitors formation and high fermentability of hydrolysates with enhanced ethanol conversion eventually validate the developed pretreatment condition.

Chapter 6:

A sustainable process development towards the industrial titer of bioethanol production from lignocellulosic biomass

Abstract

Production of bioethanol from lignocellulosic biomass at an industrial titer along with high ethanol yield has been a challenging task. The amount of sugar decomposition products formed during the pretreatment process, and the concentration of citric buffer used during the enzymatic hydrolysis was found to be predominant fermentative inhibitors that deter the microbial metabolic growth which consequently lowers the overall ethanol yield and productivity. Therefore, in this chapter, pretreatment and subsequent enzymatic hydrolysis of sorghum biomass were performed at low severity conditions and lower citrate buffer strength, respectively. Around, 94.6% hemicellulose hydrolysis was attained at 121 ºC with 0.2 M sulfuric acid for 120 min at the solid loading of 5% (w/v). In order to decrease the citric acid inhibition effect on glucose fermentation by *Saccharomyces cerevisiae*, enzymatic hydrolysis of pretreated biomass was conducted at lower citrate buffer strengths (5 mM and 0.5 mM) with the solid loading of 1–5% (w/v) and the cellulose conversion results (54.4%) were strongly agreeable with that of the standard enzymatic hydrolysis (50 mM) process. It was also observed that by increasing the solid loading, cellulose conversion efficiency was found to be decreased from 54.4–45.4%. However, around 383 mg of sugar was obtained from 558 mg of holocellulose (cellulose and hemicellulose). Finally, xylulosic ethanol was produced without detoxification of pre-hydrolysate which produced a maximum amount of 19.5 g/L by *P. stipitis*. Moreover, 36.5–43.5 g/L (0.30–0.38 g_p/g_s) and 56.4–62.1 g/L (0.45–0.46 g_p/g_s) ethanol production

was observed during the fermentation of enzymatic hydrolysate derived from 5 mM and 0.5 mM citrate buffer strengths, respectively.

6.1. Introduction

After pretreatment, a large amount of residual biomass gets easily digested but a considerable amount of recalcitrant lignin still remains in the undigested biomass [64]. Subsequent enzymatic hydrolysis should be carried out for cellulose conversion into glucose for cellulosic ethanol production. According to previous literature reports, cellulose conversion efficiency during enzymatic hydrolysis was insignificant at high solid loadings which generally occurs above 10 wt% (on dry weight basis) [85,152], whereas lower solid loading leads to less glucose concentration which cannot fulfil the industrial scale of bioethanol production. Even though high solid loading gives increased glucose concentration, but deters the enzymatic hydrolysis efficiency which ultimately lowers the cellulose conversion [152]. Along with this, a pH range of 4.8–5.5 need to be maintained during the enzymatic hydrolysis by 50 mM citrate buffer, but in a commercial scale point of view, conducting the enzymatic hydrolysis in 50 mM citrate buffer may not be feasible [86]. In addition, 50 mM citrate buffer inhibit the microbial growth rate due to the chelation of trace elements, which are essential for optimal growth of ethanologenic yeast [86].

As per the relevant literature reports, production of xylulosic ethanol from pre-hydrolysates without detoxification and minimization of citrate buffer inhibition effect on glucose fermenting organisms is a challenging task. Therefore, the present study is focused on the pretreatment of sorghum biomass with dilute sulfuric acid for hemicellulose hydrolysis and subsequent enzymatic hydrolysis. Furthermore, citrate buffer strengths of 50 mM, 5 mM and 0.5 mM have been used for enzymatic hydrolysis at different substrate loadings to investigate the effect of citrate buffer strength on sugar release. Presence of significant amount of fermentable sugars in the hydrolysates is necessary to meet the industrial titer of ethanol production, which results in cost

reduction of ethanol distillation process. Therefore, concentrated hydrolysates are prepared from pretreatment and enzymatically derived hydrolysates by rotary evaporator and added to particular fermentation mediums at various concentrations for the optimization of xylulosic and cellulosic ethanol production by *Pichia stipitis* and *Saccharomyces cerevisiae*, respectively. In addition, the effect of non-detoxified pre-hydrolysate and citrate buffer concentration on microbial growth and ethanol yield have also been investigated.

6.2. Materials and methods

6.2.1. Dilute sulfuric acid pretreatment

According to chapter-5, optimised pretreatment condition of sorghum biomass (IS 21549/*bmr 6 A*) was obtained as 121 ºC, 0.2 M, and 120 min. Therefore, pre-hydrolysate derived from the sorghum biomass (IS 21549/*bmr 6 A*) has been used in the present study which contains (g/L), cellobiose- 0.49, xylobiose- 0.19, glucose- 2.09, xylose- 9.16, arabinose- 0.76, acetic acid- 1.46, formic acid-0.2, 5-HMF- 0.27, and furfural- 0.34.

6.2.2. Enzymatic hydrolysis

The pretreatment derived residual biomass containing cellulose was hydrolysed by cellulase (Celluclast 1.5L) at different protein concentrations (20–80 of cellulase protein/g of cellulose) for the optimization of glucose yield. Protein content in the cellulase was determined according to the Folin-Ciocalteu method [15]. The optimum cellulose converting protein concentration was used for further optimization of solid loading of the pretreated biomass (viz., 2% 3% 4% 5%). Solid loading beyond 5% was not considered due to low density of pretreated biomass, (Figure 6.1). The enzymatic hydrolysis experiments were performed in 50 mM, 5 mM and 0.5 mM citrate buffer

(pH 4.8) at 50 °C, 120 rpm, and 120 h. An aliquot of 0.1 mL of sample was withdrawn every 24 h from the reaction mixture and boiled for 10 min for enzyme inactivation and then analyzed by HPLC.

Figure 6.1: Momentum of solid loading of pre-treated biomass in enzymatic hydrolysis medium.

6.2.3. Preparation of pretreatment and enzymatically derived hydrolysates for the fermentation process

Pre-hydrolysate was neutralized with $Ca(OH)_2$ at 50 °C and the resulted precipitate of $CaSO_4$ was removed at maximum extent through vacuum filtration using 0.2 µm nylon membrane filter. Finally, filtrate pH was adjusted to the cultivation pH (i.e., 6) of *P. stipitis* with 10N sulfuric acid. Unlike the pre-hydrolysate, no additional processing was required for enzymatic hydrolysate. In order to achieve desired sugar concentrations for efficient ethanol production, neutralized pre-hydrolysate and enzymatic hydrolysate were concentrated under reduced pressure by using rotary evaporator (Buchi, Rotavapor-R-210, and Switzerland) or traditional distillation method. The amount of power consumption required for both the aforementioned evaporation processes was further subjected for cost effective analysis. Thereafter, the concentrated hydrolysates referred to as hemicellulosic (pre-hydrolysate) and cellulosic

(enzymatic) hydrolysates. These hydrolysates were filter sterilized and then, used for the bioethanol production.

6.2.4. Microorganisms

Pichia stipitis NCIM 3948 and Saccharomyces cerevisiae NCIM 3090 were procured from National Collection of Industrial Microorganisms (NCIM) Pune, India. The strains were supplied on MGYP agar slants, which contained (g/L): 3, Malt extract; 10, Glucose; 3, Yeast extract; 5, peptone; and 20, agar; and, 6.4–6.8 pH.

6.2.4.1. *Seed culture preparation*

P. stipitis was sub-cultured on YPDX agar plates which contains (g/L): 10, yeast extract; 20, peptone; 5, glucose; 15, xylose and 2, agar. Whereas, *S. cerevisiae* was sub-cultured on YPD agar plates which contains (g/L): 10, yeast extract; 20, peptone; 20, glucose and 20, agar. Both the yeast strains were incubated at 30 °C for 48 h. A colony from both the plates was separately inoculated into 100 mL of filter sterilized YPDX and autoclave sterilized YPD liquid growth medium and incubated in a shaking incubator with agitation speed of 140 rpm at 30 °C for 18 h. pH of both the mediums was maintained at 6. Cells were harvested by centrifugation at 8000 rpm for 10 min and re-suspended in sterile distilled water to adjust the final concentration to 40 g/L which served as inocula for ethanol production. Cell growth was observed by measuring the absorbance at 600nm (OD_{600}) using Agilent Cary 100 UV-Visible spectrophotometer.

6.2.4.2. *Fermentation of hemicellulosic hydrolysates*

The fermentation experiments were performed in sterile 250 mL Erlenmeyer flasks containing 100 mL of fermentation medium which includes, different

concentrations of hemicellulosic hydrolysates, 2 mL of autoclave sterilized 50X concentrated nutrient solution (1.7 g of yeast nitrogen base, 1 g of urea and 6.56 g of peptone in 20 mL of distilled water), and 5 mL of inocula (which gives an initial cell concentration of 2 g/L). Initial pH of the media was adjusted to 6 with 10N H_2SO_4 and incubated at 30 °C and 120 rpm.

6.2.4.3. *Fermentation of cellulosic hydrolysates*

Fermentation experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of fermentation medium which includes different concentrations of cellulosic hydrolysates, 4 mL of 25X YP (10 g of yeast extract and 20 g of peptone in 40 mL of distilled water) nutrient solution, and 6 mL of seed culture (which gives initial concentration of 1.6 g/L on cell dry weight basis). Initial pH of the fermentation broth was maintained at 5.5 and incubated at 30 °C, 150 rpm and 30 h. All fermentation samples were taken periodically for HPLC analysis.

6.2.5. Analytical methods

Each sample was filtered through a 0.2 µm syringe filter and appropriate dilution was made with Milli Q water. Quantitative analysis of the sugars, fermentative inhibitors and ethanol was performed using Varian 210 HPLC system (detailed HPLC specifications and operating conditions are shown in the section 2.2.4.

6.3. Results and discussion

6.3.1. Optimization of enzyme loading for maximum cellulose hydrolysis

The effect of enzyme loading (20 mg, 40, 60 and 80 mg protein/g of cellulose) was investigated by performing the enzymatic hydrolysis at 1% (w/v) substrate loading for 120 h. Maximum cellulose conversion was achieved at 72 h reaction time, i.e.,

35.2%, 44.1%, 54.4% and 54.5% corresponding to 20, 40, 60 and 80 mg protein and further increase in the reaction time (120 h) showed insignificant effect on cellulose conversion (Figure 6.2a–6.2d).

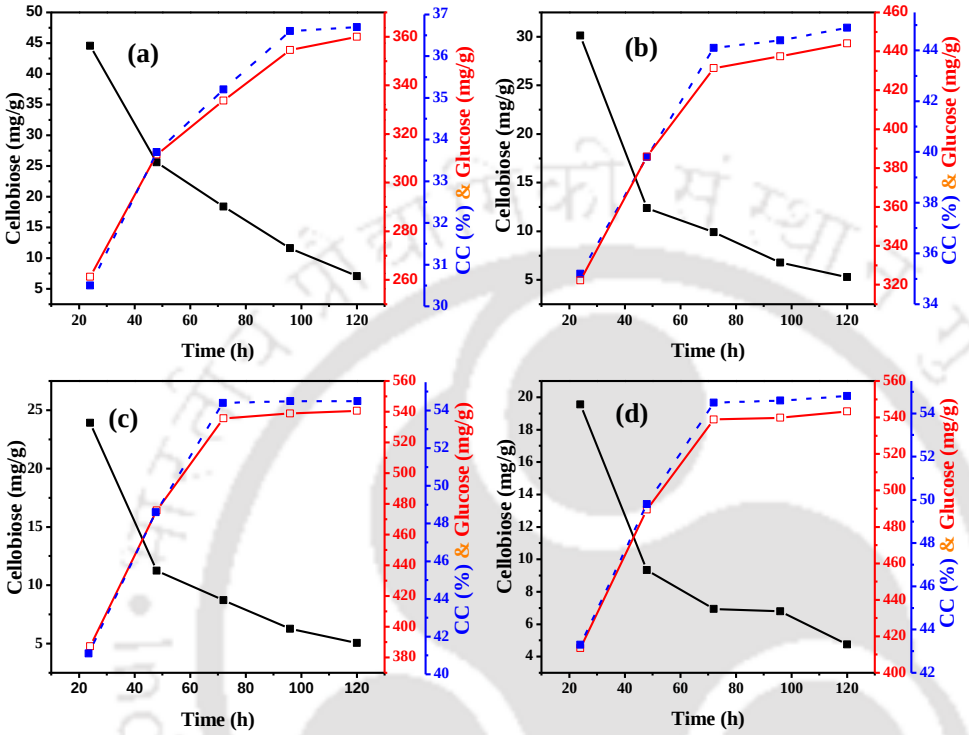


Figure 6.2: Effect of cellulase loading (based on protein concentration/gram of cellulose) a) 20 mg b) 40 mg c) 60 mg and d) 80 mg on glucose yield and percentage of cellulose conversion.

Figure 6.2a–6.2d shows that significant cellulose conversion was achieved during first 24 h of reaction time i.e., 30.5%, 35.2%, 41.1% and 43.3% with 20, 40, 60 and 80 mg protein, respectively. This might be due to easily digestible parts of pretreated biomass being hydrolysed before the recalcitrant part. After 24 h, reduced hydrolysis rates were observed (Table 6.1), which can be calculated according to the following equations (Eq. 6.1 and Eq. 6.2).

$$\text{Cellulose conversion (\%)} = \left(\frac{C_{cb} + C_{glu}}{I_{cell}}\right) \times 100 \quad (6.1)$$

$$R_{cc} = \frac{Cellulose\ conversion\ (\%)}{t_{(h)}} \quad (6.2)$$

Where, C_{Cb} and C_{Glu} represents the concentration of cellobiose and glucose derived from the enzymatic hydrolysis of pretreated biomass, I_{Cell} is the initial amount of cellulose present in the pretreated biomass, R_{CC} and t represents the rate of cellulose conversion at specific time (h), respectively.

Table 6.1: Rate of cellulose conversion with corresponding to time at different cellulase loading

Time (h)	20 mg/g* (cc/h)	40 mg/g* (cc/h)	60 mg/g* (cc/h)	80 mg/g* (cc/h)
24	1.27	1.46	1.71	1.80
48	0.70	0.82	1.01	1.03
72	0.48	0.61	0.75	0.75
96	0.38	0.46	0.56	0.56
120	0.30	0.37	0.45	0.45

** Cellulase loading based on protein concentration/gram of cellulose ; cc, cellulose conversion;*

Apart from the recalcitrant part, there are several factors that can also reduce the hydrolysis rate which include, enzyme inactivation [77], product inhibition and thermal instability of cellulase [77]. However, optimum cellulose conversion (54.4%) was attained at 60 mg protein concentration in 72 h reaction time and almost similar conversion was observed by further increasing the protein loading (i.e., 80 mg leads to 54.5% cellulose conversion). This might be due to the recalcitrant nature of lignin that has impeded further cellulose conversion by restricting the cellulose accessibility, non-specific binding of enzyme and small molecule inhibition [175]. A schematic

representation of lignocellulosic biomass structure and its transformation after the pretreatment and subsequent enzymatic hydrolysis is depicted in Figure 6.3.

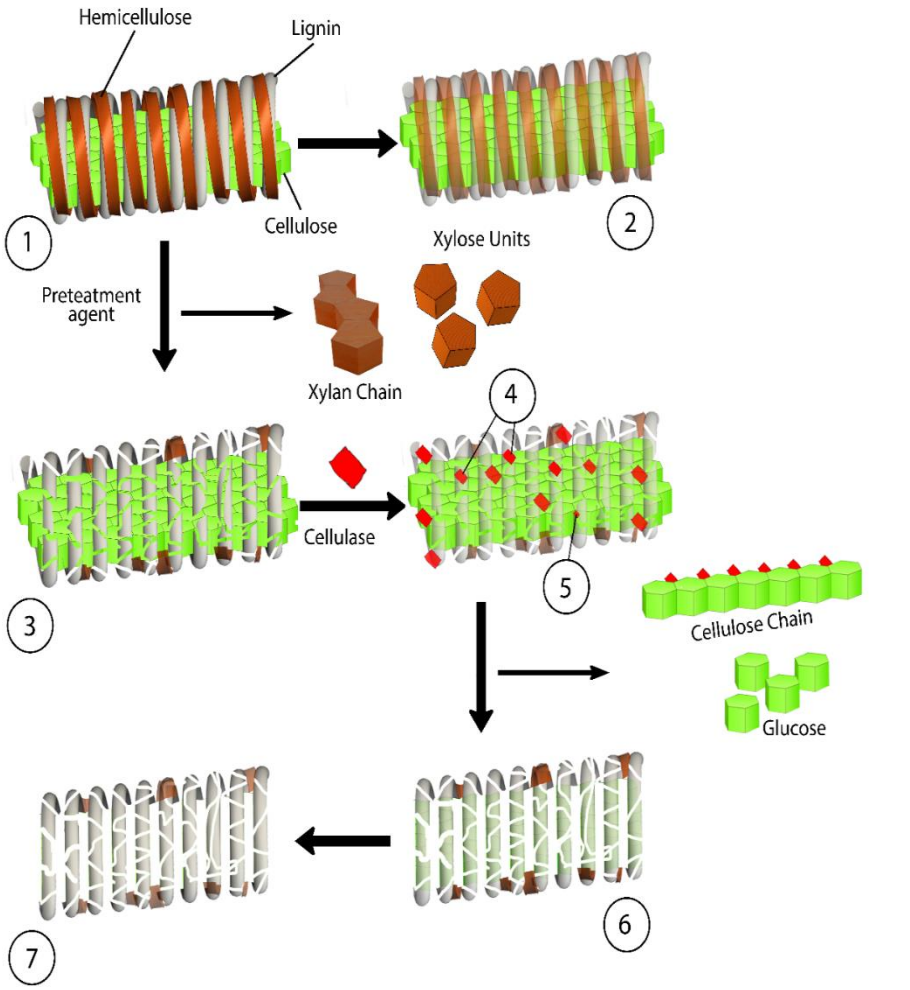


Figure 6.3: Diagrammatic representation of lignocellulosic biomass transformation during the pretreatment and enzymatic hydrolysis process *(1) Structure of lignocellulosic biomass (2) lignocellulosic biomass shown in transparent way to elucidate the hemicellulose and lignin enclosed cellulose (3) Impact of pretreatment of lignocellulosic biomass that hydrolyses most of hemicellulose and also imposed crakes on lignin structure (4) Lignin restricting the cellulose accessibility which infers Non-productive binding of cellulase (5) Cellulase penetration through the lignin cakes and (6) hydrolyses cellulose (7) Invisibility of cellulose that surrounded by lignin.*

As shown in the Figure 6.3, pretreatment hydrolyses most of the hemicellulosic fraction and imposed crakes on lignin structure which commonly known as lignin disruption. Therefore, during the enzymatic hydrolysis, cellulase can easily hydrolyse

the freely accessible cellulose portion which are induced by pretreatment process, whereas small fraction of cellulose and hemicellulose is still remained after the pretreatment and subsequent enzymatic hydrolysis. This could be due the presence of cellulose and hemicellulose within the lignin matrix which cannot be accessed by the catalyst (Figure 6.3). As evident from the Table 6.2, percentage of lignin remained after the pretreatment and subsequent enzymatic hydrolysis is comparatively higher than that of hemicellulose and cellulose. In addition to this, a minor portion of residual xylan present in the pretreated biomass was also converted into xylose (Table 1) which might be due to the presence of xylanase (262 Units/mL) and β-xylosidase (98.3 Units/mL) activities in the cellulase (Celluclast 1.5L®) [176].

Table 6.2: Compositional analysis of sorghum biomass samples before, after pretreatment and after enzymatic hydrolysis

Chemical composition	Before pretreatment (mg/g)	After pretreatment (mg/0.546 g)	After enzymatic hydrolysis (mg/0.371 g)
Water extractives	121	-	-
Ethanol extractives	29	-	-
Cellulose	363	319	145.15
Hemicellulose	222	12.2*	4.86
Lignin	183	175.4	172

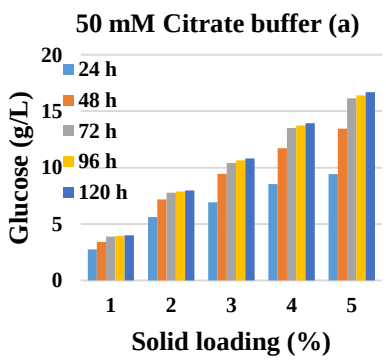
**Xylan, Composition percentages as on oven dry weight basis.*

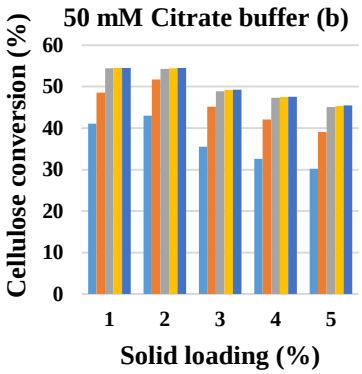
6.3.1.1. *Effect of citrate buffer strength on substrate loading and cellulose conversion efficiency*

A typical heterogeneous structure of hemicellulose of sorghum biomass constitutes xylose, arabinose and organic acids (acetic acid and gluconic acid) [82,115]. Acidic nature of a hydrolysis medium is autogenously induced by hemicellulose derived organic acids. Therefore, a major portion of the hemicellulose presence in the pretreated biomass can potentially alter the pH of enzymatic hydrolysis medium which

optimally proceeds between pH 4.8–5.5. Several reports demonstrated the fact that lignocellulosic biomass containing organic acids shows adverse effect on enzyme action and growth of fermenting organisms [177]. However, in the present study 94.6% of hemicellulose hydrolysis was observed during the dilute sulfuric acid pretreatment. According to the NREL procedure [31], 12.2 mg of de-acetylated xylan was found in the 546 mg of pretreated biomass (Table 6.2). Therefore, considering the aforementioned evidence, enzymatic hydrolysis of pretreated biomass was performed in low buffer strength i.e., 5 mM and 0.5 mM citrate buffer with the optimum protein loading of 60 mg/g of cellulose, and the results were compared with that of 50 mM citrate buffer strength.

The obtained results are found to be promising for the future trend of enzymatic hydrolysis process. Figure 6.4b, 6.4d and 6.4f, represents that, cellulose conversion efficiency and glucose yield are not significantly affected by the buffer strengths (50 mM or 5 mM or 0.5 mM).





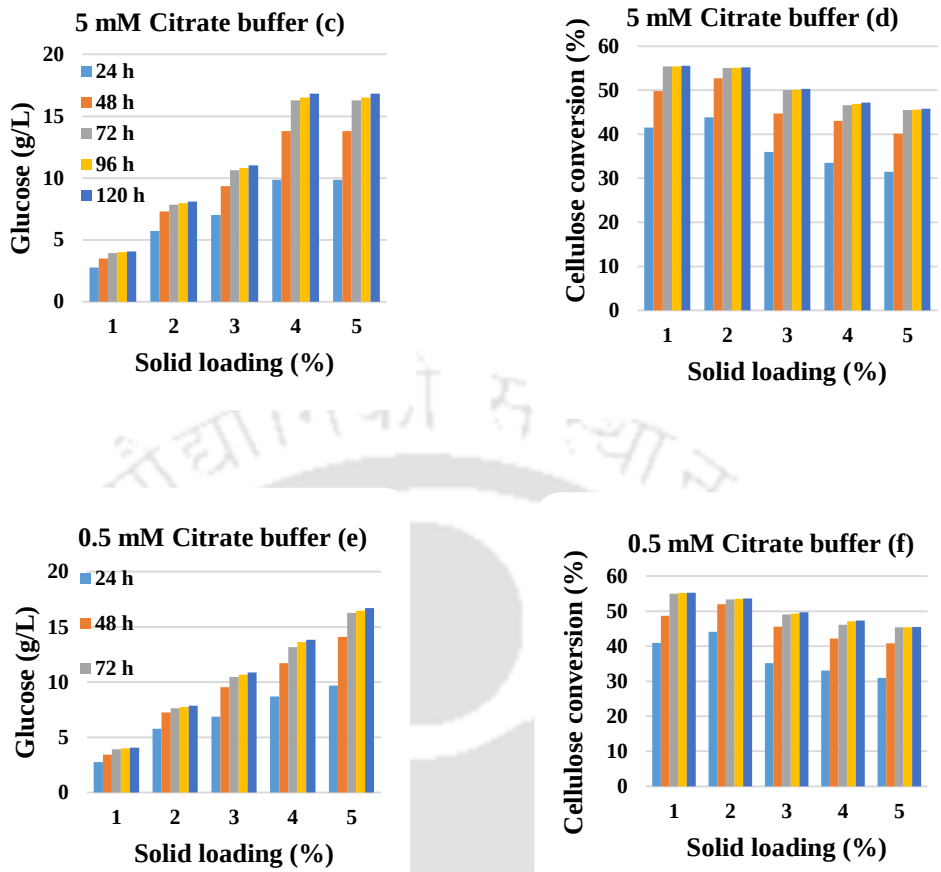


Figure 6.4: Effect of substrate loading on glucose yield and percentage of cellulose conversion at different citrate buffer strengths

As compared to 1% substrate loading, a similar cellulose conversion (i.e., 54.5%) was observed with 2% substrate loading. Further increase in the substrate loading (3–5%) led to reduction of cellulose conversion (Figure 6.4b, 6.4d and 6.4f). Kristensen et al., (2009) observed that decreasing cellulose conversion is an inherent effect of increased substrate loading. This might be due to the non-productive adsorption of enzyme on lignin and other insoluble components are the major cause for reduced enzymatic hydrolysis [178]. However, glucose concentration (in terms of g/L) increased with corresponding substrate loading (Figure 6.4a, 6.4c, 6.4e) (which is contrast to the percentage of cellulose conversion) but, the percentage of cellulose

conversion gradually decreased by increasing the substrate loading (Figure 6.4b, 6.4d and 6.4f). In terms of sustainable utilization of lignocellulosic biomass, it is necessary to choose optimum cellulose conversion parameters instead of high sugar yielding conditions. This is mainly because, reuse (re-enzymatic hydrolysis) of unconverted cellulose might increase the production cost of lignocellulosic bioethanol. Therefore, in the present work optimized cellulose conversion parameters obtained were 2% substrate loading with 60 mg protein for 72 h reaction time.

6.3.2. Conditioning of pre-hydrolysate

Even though the detoxification of pre-hydrolysate through over-liming by $Ca(OH)_2$ is well-known and inexpensive method, but sugar loss is a major problem impended with over-liming process [7]. Moreover, fermentative inhibitors formed in the present study did not exceed beyond the inhibiting limits of *P. stipitis*. According to Delgenes et al, (1996), 2 g/L of furfural, 5 g/L of 5-HMF and 11.9- 15 g/L of acetic acid are found to be inhibiting concentrations of *P. stipitis* and other yeast species. Therefore, instead of performing the detoxification process, pre-hydrolysate was neutralized with $Ca(OH)_2$.

This neutralized hydrolysate was concentrated under reduced pressure for the preparation of hemicellulosic hydrolysate which contains (g/L): glucose- 20.12, xylose- 88.5, and arabinose- 7.2, along with 5-HMF- 2.59, formic acid- 5.73, and acetic acid- 14.16. Remarkable furfural separation was observed while concentrating neutralized hydrolysate by rotary evaporator which was operated at 35 °C (4 L Heating bath, B-491) and 72 milli bar vacuum pressure (Vacuum controller, V-850). Around 80% of the furfural was successfully removed without any sugar loss (HPLC chromatogram shown in Figure 6.5).

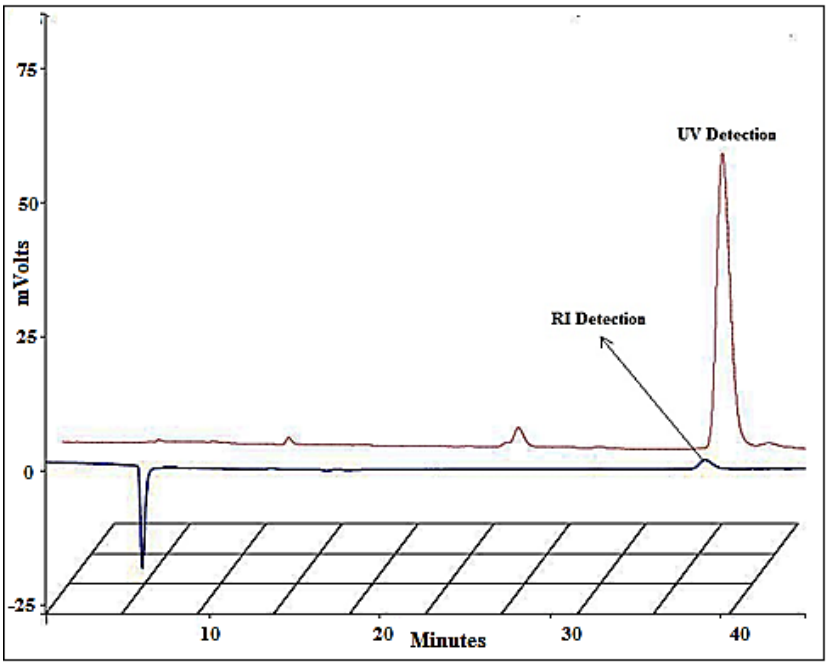


Figure 6.5: Furfural removal from pre-hydrolysate during the rotary evaporation

Moreover, the amount of power (kWh) required to concentrate the neutralized hydrolysates are calculated according to the following equations.

$$kW = \frac{V \times 4.18 \times T}{3142.14 \text{ BTU}} \tag{6.3}$$

Where, V is the water bath volume in liters, 4.18 is the specific heating capacity of water in Joules (the amount of heat required to raise the temperature of 1 gram of water by 1°C), T is the temperature rise from ambient to required temperature in °C and BTU is the British Thermal Unit.

In the current process, around 0.077 liters of water was evaporated from the pre-hydrolysate through the rotary evaporator in 0.7 h. The power rating (kW) specification of rotary evaporator assembly was found to be in (kW) 1.7, water bath (4 L); 0.21, vacuum pump; 0.225, cooling thermostat (Lauda- Alpha- R8) at 20 ºC cooling output.

While substituting the values in Eq. 6.3,

$$kW = \frac{4 \times 4.18 \times 5}{3412.14} = 0.024$$

Therefore, 0.0245 kW power required to raise the temperature by 5 ºC (from ambient to 35 ºC) for 4 L capacity water bath per h. In the present study, water bath having power rating of 1.7 kW can heat 277.5 L of distilled water in one h. Therefore, 1.7 kW can raise the temperature of 5 ºC for 4 L of distilled water in 0.0144 h (or 0.86 min).

Therefore,

$$Units = power(kW) \times time(h) \tag{6.4}$$

$$Units = 1.7 \times 0.0144 = 0.024$$

Around 0.7 h time required to evaporate 0.077 L of hydrolysate, therefore, water bath should be maintained at the set temperature for 0.7 h.

$$Units = 0.024 \times 0.7 = 0.0168 \tag{6.4a}$$

Power consumption of vacuum pump for 0.7 h,

$$Units = 0.21 \times 0.7 = 0.147 \tag{6.4b}$$

Power consumption for the cooling thermostat at 20 ºC for 0.7 h

$$Units = 0.225 \times 0.7 = 0.157 \tag{6.4c}$$

Therefore, power consumption for evaporating the 0.077 L of water from the pre-hydrolysate by rotary evaporator is calculated as below.

$$Units = a + b + c = 0.321$$

Apart from the rotatory evaporator, around 0.077 L of water evaporated within 0.54 h by using traditional distillation method or also called as normal distillation process. Therefore, power consumption for 250 mL heating mantle (having power rating of 0.15 kW) can be calculated according to the following equations Eq. 6.4.

$$Units = 0.15 \times 0.54 = 0.081 \quad (6.4d)$$

Power consumption for the cooling thermostat at 20 ºC for 0.54 h

$$Units = 0.225 \times 0.54 = 0.121 \quad (6.4e)$$

Therefore, power consumption for simple distillation is calculated as below,

$$Units = 6.4d + 6.4e = 0.202$$

Similarly, around 0.4 units and 0.25 units of power was consumed for evaporation of 0.095 L of water from enzymatic hydrolysate by using rotary evaporator and traditional distillation process, respectively. According to the aforementioned findings, power consumption required for traditional distillation process is comparatively lower than that of vacuum distillation. However, while performing the traditional distillation process, around 18% of sugar loss (glucose or xylose) occurred. Generally, temperature range of heating mantle proceeds from ambient to 250 ºC, therefore, sugar decomposition should be initiated during the traditional distillation process. For instance, while evaporating the water from enzymatic hydrolysate containing 648.5 mg of glucose leads to 117.6 mg of sugar decomposition to form 8.9 mg of 5-HMF. It is a known fact that, loss of sugar decreases the overall ethanol production, and the formation of 5-HMF i.e. fermentative inhibitor decreases the fermentation efficiency. Although, concentrating the hydrolysate by rotary evaporator consumed comparatively 0.15 units more than traditional distillation, but the sugar loss

and inhibitors formation was negligible in case of rotary evaporator. However, the cost of hydrolysate evaporation process can be minimized my various types of distillation process. In a sugarcane bioethanol producing industries, the extracted juice containing 12-17.6% of sugar undergoes evaporation process to concentrate the sugar liquor thereby achieved an industrial titer of 8-10% (v/v) [20,179]. Therefore, evaporation cost of hydrolysate can also be minimized by succeeding the sugarcane industry evaporation method.

6.3.3. Fermentation

6.3.3.1. *Fermentation of hemicellulosic hydrolysates and effect of fermentative inhibitors on ethanol yield*

The hemicellulosic hydrolysate (HH) was added to the fermentation medium at different loadings i.e., 25%, 37.5%, 50% and 62.5% (v/v). Chemical compositions of these four fermentation mediums are listed in Table 6.3, and are denoted as HH-A, HH-B, HH-C and HH-D.

Table 6.3: Chemical composition of hemicellulosic hydrolysates used for the xylulosic ethanol production

Compounds (g/L)	HH-A	HH-B	HH-C	HH-D
Glucose	5.02	7.53	10.03	12.58
Xylose	22.14	33.27	44.36	55.48
Arabinose	1.8	2.7	3.6	4.5
Formic acid	1.43	2.11	2.86	3.57
Acetic acid	3.54	5.23	7.08	8.84
HMF	0.64	0.97	1.29	1.6

Fermentation results of HH-A to HH-D (Table 6.4) indicates that ethanol yield, productivity and ethanol conversion efficiency decreased with an increase in the hemicellulosic hydrolysate loading in the fermentation medium. This could be due to rise in the concentration of fermentative inhibitors in the medium. As shown in table 6.3, HH-D contains highest concentration of fermentative inhibitors as compared to all other hemicellulosic hydrolysates.

Table 6.4: Summary of *P. stipitis* fermentation results of hemicellulosic hydrolysates

Parameters	HH-A	HH-B	HH-C	HH-D
Ethanol concentration (g/L)	12.3	17.8	19.5	19.8
Ethanol yield (g_P/g_S)	0.45	0.43	0.39*	0.35*
Ethanol productivity (g/L/h)	0.51	0.42	0.40	0.36
Cell growth (g/L/h)	0.25	0.17	0.15	0.13
Max. Ethanol production Time (h)	24	42	48	54
Ethanol conversion efficiency (%)	88.7	85.6	76	70

***** *Ethanol yield has been calculated by the amount of sugar consumed by the P. stipitis for the production ethanol.*

From Table 6.4, maximum ethanol yield (0.45 g_p/g_s) and productivity (0.51 g/L/h) was observed in HH-A (5.02 g/L of glucose and 22.14 g/L of xylose) with 88.7% ethanol conversion efficiency. Apart from ethanol yield (0.39 g_p/g_s) and productivity (0.40 g/L/h), the highest ethanol concentration (19.5 g/L) was found in HH-C. An approximately similar ethanol concentration (19.8 g/L) was also observed during the fermentation of HH-D (Table 6.4). The low ethanol yield and productivities of HH-C and HH-D could be due to the presence of higher concentration of fermentation inhibitors which induces chemical stress on *P. stipits* that deters the metabolic growth of *P. stipitis*. Moreover, in case of HH-C and HH-D increase in the fermentation period

was also observed. For instance, maximum ethanol production occurs at 24 h and 54 h for HH-A and HH-D, respectively. Glucose and xylose were completely consumed in HH-A and HH-B (Figure 6.6a and 6.6b), whereas in the case of HH-C and HH-D, glucose was completely utilized, but the presence of 4.3 g/L and 12.18 g/L of residual xylose (Figure 6.6c and 6.6d) was observed at the end of maximum ethanol production time. This residual xylose is probably due to product inhibition. Domínguez et al., (2000) have reported around 20 g/L ethanol as inhibitory concentration for *P. stipitis*. Therefore, in the present study further increase in ethanol concentration was not observed. In addition to this, reassimilation of produced ethanol was also observed before complete utilization of xylose (Figure 6.6c and 6.6d). During the reassimilation, ethanol oxidized to acetaldehyde and then acetate which are accumulated intracellularly, or further oxidized through TCA cycle [180].

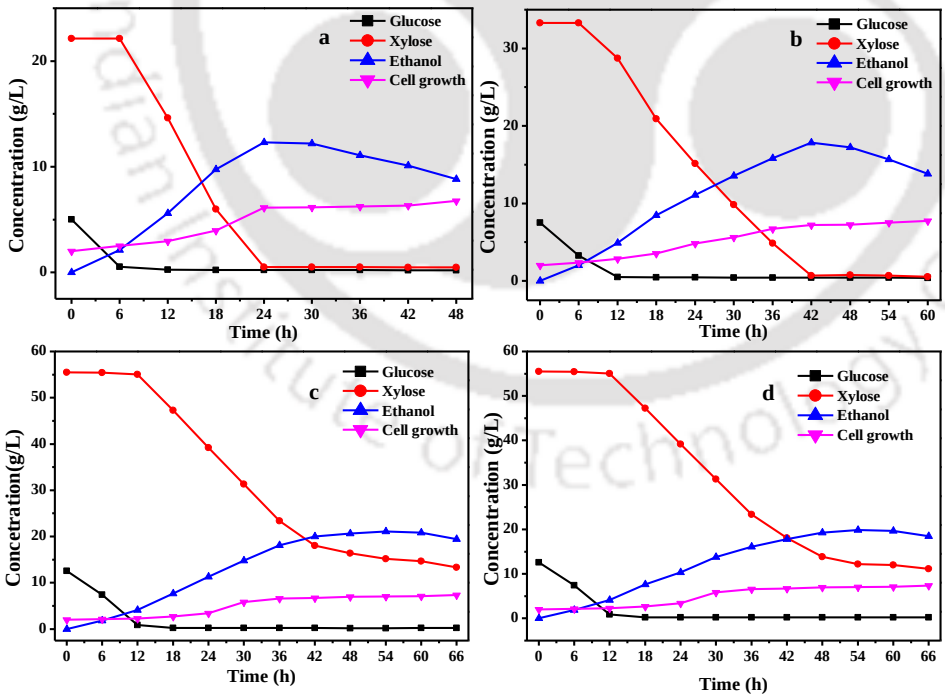


Figure 6.6: Fermentation profiles of different hemicellulosic hydrolysates a) HH-A b) HH-B c) HH-C and d) HH-D by using *P. stipitis*

Moreover, a brief literature reports of *P. stipitis* on fermentation of hemicellulosic hydrolysates (without detoxification) derived from different lignocellulosic sources are listed in Table 6.5. The comparative analysis of the results obtained in the present work with that of the literature data, revels higher ethanol concentration, ethanol yield and productivity.

Table 6.5: Brief literature summaries of different hemicellulosic hydrolysates fermentation using *P. stipitis*

Feed stock	Sugars (g/L)	EY¶ (g_s/g_p)	Ethanol (g/L)	Time (h)	Reference
Rice straw	glu: 3.5–6.4 xyl: 17.5–26.4	0.40 0.44	9.38 10.27	41 41	[161]
Corn stover	glu: 6.3–8.2 xyl: 24.9–33.5	0.40– 0.44	12.3-15.06	48-96	[165]
Paja Brava	glu: 2.5 xyl: 19.8	0.20	4.6	66	[181]
Hardwood	Total sugars: 36.2	0.40	14.5	100	[182]
Wheat straw	glu: 6.4 xyl: 45.0	0.27	7	70	[172]
Sorghum stalks	Glu:5-12.58 Xyl:22.58-55.48	0.45 0.35	12.3 -19.5	24 48	Present study

** Same as NCIM 3498; ¶ Ethanol yield.*

6.3.3.2. *Fermentation of cellulsoic hydrolysates and effect of citrate buffer strength on ethanol yield*

On application of rotary evaporator, 95% of water was successfully removed (without effecting the sugars and citrate buffer) from enzymatic hydrolysate derived from 5 mM citrate buffer strength with cellulosic hydrolysate content of (g/L), glucose- 157.13, xylose- 6.44, cellobiose- 9.04 and 100 mM of citric acid. As shown in Table 6.6, a set of three fermentation mediums were prepared from cellulosic hydrolysate (CH) by increasing the glucose concentrations like, 94.28 g/L (CH-A), 109.99 g/L (CH-B) and 141.42 g/L (CH-C).

Table 6.6: Chemical composition of cellulosic hydrolysates derived from 5 mM citrate buffer

Cellulosic hydrolysates	Glucose (g/L)	Xylose (g/L)	Cellobiose (g/L)	Citric acid (mM)
CH-A	94.28	3.86	5.42	60
CH-B	109.99	4.5	6.32	70
CD-C	141.42	5.79	8.13	90

Table 6.7: Summary of *S. cerevisiae* fermentation results of 5 mM citrate buffer derived cellulosic hydrolysates

Parameters	CH-A	CH-B	CH-C
Ethanol concentration (g/L)	36.5	38.72	43.53
Ethanol yield (g_P/g_S)	0.387	0.352	0.307
Ethanol productivity (g/L/h)	4.05	3.22	2.41
Glucose consumption rate (g/L/h)	10.47	9.16	7.85
Max. Ethanol production Time (h)	9	12	18
Ethanol conversion efficiency (%)	75.9	69	60.3

From the results (Table 6.7), decreasing sugar consumption rate and ethanol productivities were observed by increasing the glucose concentration. Figure 6.7 depicts that, around 51.9 g/L, 44.5 g/L and 35.6 g/L glucose were utilized during the first 6 h of fermentation time and produced 20.02 g/L, 16.58 g/L and 11.88 g/L ethanol from CH-A, CH-B, and CH-C, respectively.

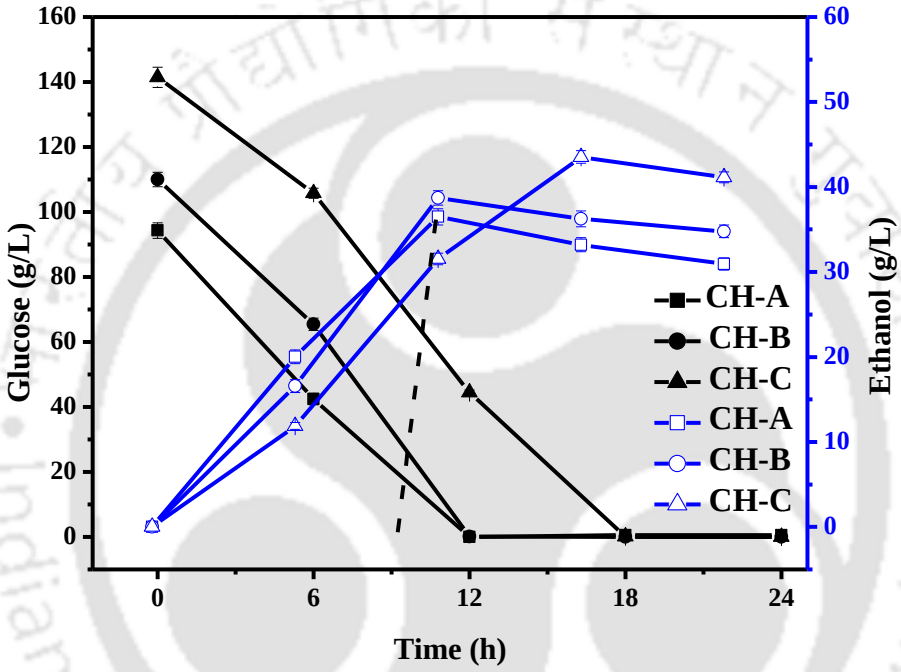


Figure 6.7: Fermentation profiles of cellulosic hydrolysates derived from 5 mM citrate buffer strength by using *S. cerevisiae*

As shown in Table 6.7, citric acid concentration was increased with an increase in the CH loading in the fermentation medium, therefore, due to the inhibition effect of citric acid, reducing sugar consumption rate and ethanol productivity trend was observed in the first 6 h of fermentation time. According to literature reports, 50 mM citric acid deter the metabolic growth of ethanologenic yeast [64,86]. In the present study, concentration of citric acid in CH-C (90 mM) was higher than that of CH-A (60 mM) which eventually lowers the ethanol yield (0.30 g_p/g_s). Moreover, glucose

consumption rate and ethanol productivity were found to be lower during the fermentation of CH-C. Finally, 36.5 g/L, 38.72 g/L and 43.43 g/L of ethanol concentrations were obtained from CH-A, CH-B and CH-D at 9 h, 12 h and 18 h fermentation time, respectively. In general, ethanol concentration above 40 g/L is considered as industrial titer of ethanol which can be produced within 24–36 h of fermentation time [183]. Apart from the ethanol yield (0.30 g_p/g_s) and ethanol conversion efficiency (60.3%), around 43.53 g/L ethanol concentration was successfully produced within 18 h fermentation time.

6.3.3.3. *Fermentation of cellulosic hydrolysates derived from 0.5 mM citrate buffer*

On application of rotary evaporator, around 95% of water was recovered and the resulting cellulosic hydrolysate contains (g/L), glucose- 152.6, cellobiose- 6.4, xylose- 9 and 10 mM citric acid. Fermentation was carried out at a glucose concentration of 122.08 g/L (CH-D) and 137.34 g/L (CH-E) (Table 6.8).

Table 6.8: Summary of *S. cerevisiae* fermentation results of 0.5 mM citrate buffer derived cellulosic hydrolysates

Parameters	CH-D	CH-E
Glucose (g/L)	122.08	137.34
Citrate acid (mM)	8	9
Ethanol Concentration (g/L)	56.4	62.1
Ethanol Yield (g_P/g_S)	0.46	0.45
Ethanol productivity (g/L/h)	3.1	2.5
Glucose consumption rate (g/L/h)	6.7	5.7
Max. Ethanol production Time (h)	18	24
Ethanol conversion efficiency (%)	90.5	88.6

From Figure 6.8, initial concentrations of 122.08 g/L and 137.34 g/L of glucose was completely utilized in 18 h and 24 h fermentation period yielding 56.4 g/L and 62.1 g/L of ethanol, respectively. Citric acid concentration present in the fermentation medium of CH-D and CH-E was found to be 8 mM and 9 mM (Table 6.8), therefore, citric acid inhibition effect would be at negligible level. Consequently, high ethanol yield was attained during the fermentation of 0.5 mM citrate buffer derived cellulosic hydrolysates.

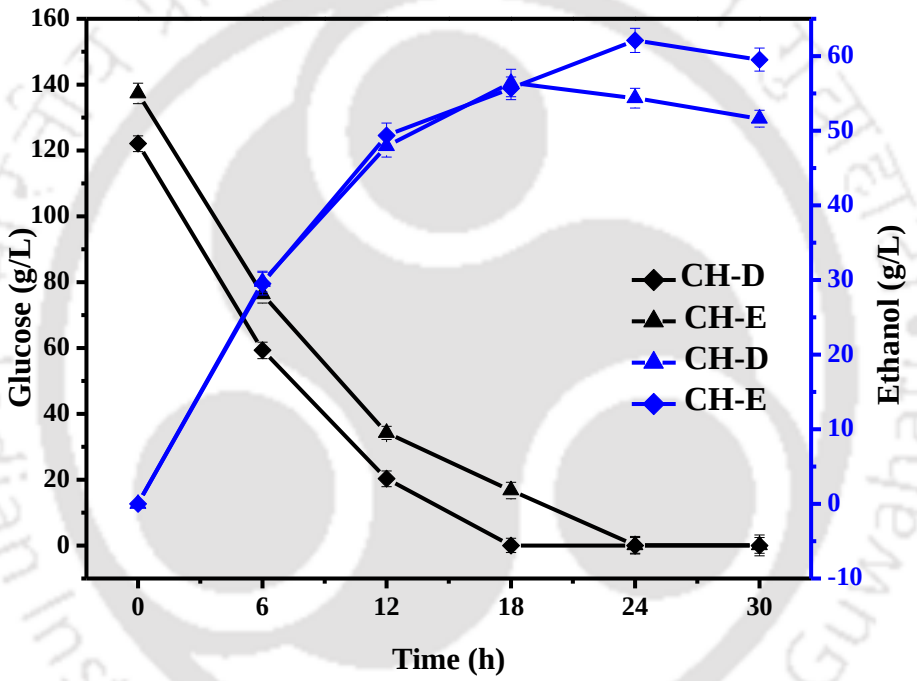


Figure 6.8: Fermentation profiles of different cellulosic hydrolysates derived from 0.5 mM citrate buffer strength by using *S. cerevisiae*

Moreover, ethanol productivity (4.0 g/L/h) and sugar consumption rates (10.4 g/L/h) were higher when fermentation was performed at 60 mM citric acid concentration (derived from 5 mM citrate buffer, Table 6.7). Whereas, 2.58 g/L/h ethanol productivity and 5.7 g/L/h sugar consumption were observed when fermentation medium contained 9 mM citric acid (Table 6.8). This might be due to the

initiation of ethanol inhibition effect on *S. cerevisiae*. According to literature reports, ethanol inhibition effect initiates when the ethanol concentration of fermentation medium reaches 40 g/L and above [90].

Table 6.9: Rate of sugar consumption and ethanol productivities with respect to fermentation time

	CH-D		CH-E	
Time (h)	**R_{SC} (g/L/h)**	**E_{PY} (g/L/h)**	**R_{SC} (g/L/h)**	**E_{PY} (g/L/h)**
6	10.46	4.96	10.16	4.91
12	8.48	3.99	8.59	4.03
18	6.77	3.13	6.7	3.09
24	-	-	5.7	2.58

R_{SC}, rate of sugar consumption; E_{PY}, ethanol productivity;

As evident from Table 6.9, during first 6 h, fermentation of CH-D and CH-E gave the ethanol yield of 29.77 g/L and 29.51 g/L with ethanol productivity of 4.9 g/L/h. Thereafter, ethanol productivities and sugar consumption rates gradually decreased, because the ethanol concentration reached above 40 g/L in the medium (Figure 6.8) which initiated the product (ethanol) inhibition effect on *S. cerevisiae.* However, the highest ethanol concentration (56.4–62.1 g/L), ethanol conversion efficiency (88.6-90.5%) and ethanol yield (0.45-0.46 g_p/g_s) was attained during the fermentation of cellulosic hydrolysates derived from 0.5 mM citrate buffer.

6.3.4. Mass Balance Analysis

Mass balance analysis was performed to evaluate the sugar formation and their subsequent conversion into bioethanol. During the pretreatment process, around 205 g of sugar was produced from one kg of sorghum stalks which includes 2.3 g of cellobiose, 3.14 of xylobiose, 34.17 g of glucose 151.74 g of xylose and 12.95 g of arabinose. Among them, *P. stipitis* having capability to convert both glucose and xylose into bioethanol. As shown in table 6.4, the maximum ethanol yield was found to be 0.45 ($g_p/g_{s)}$ during the fermentation of HH-A. Therefore, around 86.3 g of ethanol produced from 186.61 g of sugar (glucose and xylose). Furthermore, residual biomass (546 g) remained after the pretreatment was subjected to enzymatic hydrolysis process which produced 2.69 g of cellobiose, 170.88 g of glucose and 4.94 g of xylose. It is a known fact that, *S. cerevisiae* has the capability to convert glucose into ethanol. As shown in table 6.8, the maximum ethanol yield was found to be 0.46 (g_p/g_s) during the fermentation of CH-D. Therefore, around 78.6 g of ethanol can be produced from 170.8 g of glucose. Finally, around 162.5 g of bioethanol was produced from 1 kg of sorghum biomass (Figure 6.9). According to ethanol density calculations, around 206 mL of bioethanol produced per 1 kg of sorghum biomass. Therefore, 206 L of bioethanol can be produced from one ton of sorghum stalks by using the current process.

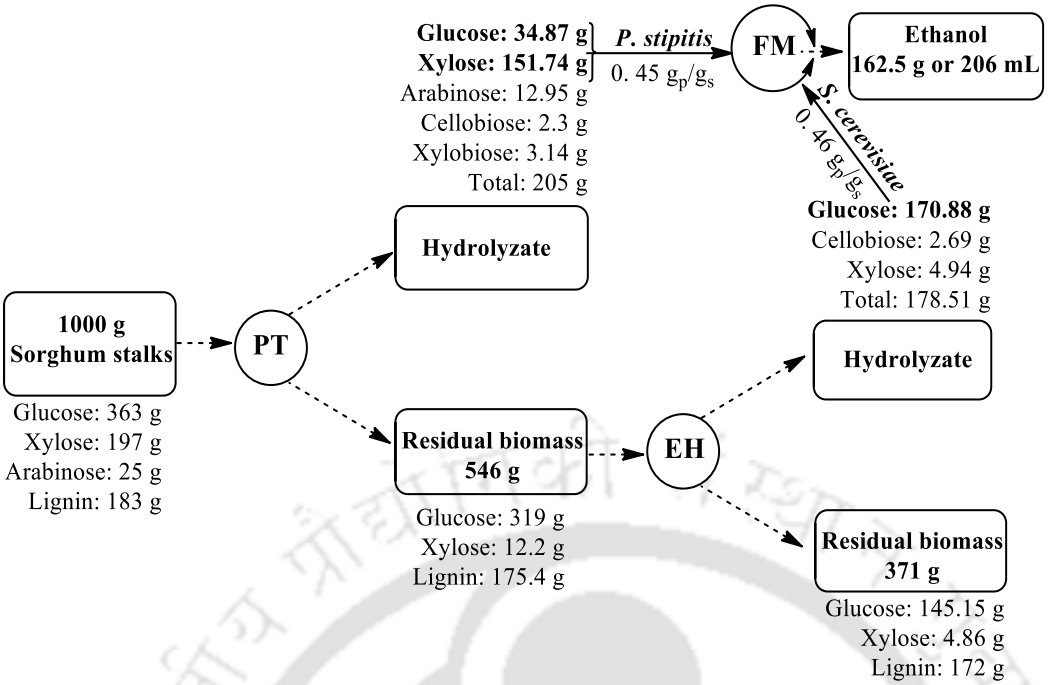


Figure 6.9: Mass balance analysis of current process, where, PT, EH and FM are representing pretreatment, enzymatic hydrolysis and fermentation, respectively.

6.4. Summary

Dilute sulfuric acid pretreatment has shown significant effect on hemicellulose hydrolysis which accounted to be 94.6% at the solid loading of 5% (w/v). During the enzymatic hydrolysis of pretreated biomass in 50 mM, 5 mM and 0.5 mM citrate buffer strengths, around 54.3% of cellulose conversion was attained at 2% (w/v) solid loading by using 60 mg of cellulase protein/g of cellulose. Resulting in about 383 g of sugar yield from 558 g of holocellulose (cellulose and hemicellulose) content in 1 kg sorghum biomass. While concentrating the sugar content in hydrolysates (derived from pretreatment and enzymatic hydrolysis), rotary evaporator was found to be a significant method to recover the water from hydrolysates, whereas around 18% of sugar loss was observed during the traditional distillation method. Finally, the maximum bioethanol concentration of 19.5 g/L and 62.1 g/L was obtained during the fermentation of HH-C and CH-E by using *P. stipitis* and *S. cerevisiae*, respectively. According to the mass

balance analysis with corrseponding to the maximum ethanol yield in terms of g_p/g_s obtained in the present study, i.e., 0.45 (g_p/g_s) and 0.46 (g_p/g_s), around 84 g and 78.6 g of bioethanol can be obatined from HH-A and CH-D, recspectvely, Therefore, around 206 mL of bioethanol can be porduced from 1 kg of sorghum biomass which is equivalnet to 206 L of bieothaol per ton of sorghum biomass.

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Chapter 7:

Liquefaction of lignocellulosic biomass for the production of bioethanol

Abstract

In the previous chapter 6, we used calcium hydroxide for the neutralization of pretreatment derived acid hydrolysates which eventually results in generation of calcium sulfate (gypsum). Even though, calcium sulfate can be used as a soil conditioner, but when conducting the lignocellulosic bio-refinery process at larger scale, the disposal of huge amount of generated calcium sulfate would be a major concern. Moreover, only 54.4% of cellulose hydrolysis was obtained at 2% solid loading by employing 60 mg of cellulase protein/g of cellulose and about 145 mg of cellulose still remained after the enzymatic hydrolysis. Since cost of enzyme found to be a major driving force of lignocellulosic biomass conversion technologies, high enzyme dosage and low substrate loading eventually increases the minimum selling price of ethanol. Furthermore, sorghum traits mentioned in chapter 2 are genetically modified which are still under the breeding conditions and testing phase in the research farms which are not executed at commercial level of cultivation yet. Therefore, in this chapter we used a wild type sorghum biomass (*Sorghum bicolor ssp. Verticilliflorum*) for the production of bioethanol. In this chapter, liquefaction of lignocellulosic biomass has been investigated for the production of fermentable sugars and their subsequent conversion into bioethanol. Pretreatment, delignification and enzymatic hydrolysis are three sequential steps involved in the liquefaction process. About 82% of hemicellulose hydrolysis was attained by the pretreatment of wild type sorghum biomass at 121 ºC with 0. 2 M sulfuric acid for 120 min. Further treating the pre-treated residue with 1–

5% (w/v) sodium hydroxide, 81–98% delignification was achieved. Subsequently, around 65-99.6% of cellulose digestibility was attained during the enzymatic hydrolysis process. However, the optimum liquefaction condition of sorghum stalks was obtained by performing the pre-treatment at 121 ºC with 0. 2M sulfuric acid for 120 min, and then delignification at 121 ºC with 2% NaOH for 20 min followed by enzymatic hydrolysis at 10% (w/v) solid loading with 40 mg protein/g of cellulose for 72 h. Furthermore, 18.4 g/L and 74.7 g/L xylulosic and cellulosic ethanol was produced from the pretreatment and enzymatic derived hydrolysates, respectively. Finally, 97.7% of sorghum stalks liquefaction occurred, in this 84% of holocellulose (cellulose and hemicellulose) converted to yield 494 g of fermentable sugars, which is further converted into 225 g of ethanol which is equivalent to 285 mL ethanol/kg of sorghum biomass.

7.1. Introduction

Unlike the technologies used in bioethanol production from edibles sources, lignocellulosic biomass conversion technologies are not fully developed yet. There are several problems involved in lignocellulosic ethanol production including, 1) sugar decomposition into fermentative inhibitors during the pretreatment and their detrimental effect on xylose fermentability [77], 2) Insufficient cellulose conversion due to the presence of recalcitrant lignin, and the high citrate buffer strength (50 mM) used during the enzymatic hydrolysis affects the glucose fermentability, which lead to low ethanol yield and productivity [86]. Due to these typical reasons, it is difficult to get an industrial titer of ethanol which generally exists at 8–10% (v/v) and above, whereas it is easily produced when starch based (edible) sources are used for the fermentation process [2].

Dilute sulfuric acid pretreatment is the most widely used method, which can hydrolyse most of the hemicellulosic fraction of lignocellulosic biomass and it makes residual biomass amenable for the enzymatic hydrolysis [184]. However, several problems have been principally associated with dilute sulfuric acid pretreatment such as sugar decomposition and the resultant fermentative inhibitors severely hampers the xylose fermentability which ultimately reduces the ethanol yield [126]. However, over-liming with calcium hydroxide is a well-established and inexpensive process to detoxify the hemicellulosic hydrolysate, but the amount of solid waste generated during the detoxification and their disposal would be a major problem [68,162]. In addition, sugar loss found to be one of the potential drawbacks of over-liming process.

Moreover, pretreatment derived residual biomass contains high lignin levels, which eventually inhibit cellulose conversion during enzymatic hydrolysis by the

action of non-specific binding of enzymes to lignin [76]. Moreover, in order to obtain high ethanol titer, high substrate loading (15–30% w/v) is generally used during enzymatic hydrolysis that reduces the rate of cellulose hydrolysis and deter the conversion efficiency. Several factors that are likely to reduce the rate of cellulose hydrolysis during the high substrate loading are product inhibition, thermal instability of enzyme and its inactivation [77]. Apart from these, citrate buffer strength (50 mM) used during the enzymatic hydrolysis process is in itself a fermentative inhibitor, which deters the microbial metabolic growth due to the chelation of trace elements that are essential for optimal growth of ethanologenic yeast [86].

Considering the relevant literature reports, it has been observed that production of bioethanol from lignocellulosic biomass at industrial titer with high ethanol yield is a challenging task. Therefore, in the present study, optimization of lignocellulosic biomass liquefaction has been performed to obtain maximum sugar content by using the sequential steps including pretreatment, delignification and enzymatic hydrolysis for the characteristic bioethanol production.

7.2. Materials and methods

7.2.1. Feed stock preparation

In the present study, a wild type sorghum biomass (*Sorghum bicolor ssp. Verticilliflorum*) has been used as a model lignocellulosic substrate. Preparation of biomass for compositional analysis and determination of structural carbohydrates and lignin contents are performed according to NREL procedures and the results obtained are shown in Table 7.1.

Table 7.1: Compositional analysis of a wild type sorghum biomass before and after pretreatment

Composition	Raw (%)	Pre-treated (%)	Pre-treated 628 (mg)
Water extractives	10.34	-	-
Ethanol extractives	1.97	-	-
Cellulose	38.4	59.88	376.07
Hemicellulose	27.1[a]	8.2[b]	51.94[b]
Lignin	21.3	30.[c]	189[c]

[a]Xylan- 22.2%, Arabinan- 2.1% and Acetic acid- 2.7%; [b] Xylan;[c] Acid insoluble residue.

7.2.2. Pretreatment

According to our earlier published papers [51,82,148], optimum condition for sorghum stalks pretreatment was found to be 121 ºC, 0.2 M sulfuric acid for 120 min with a solid loading of 5% (w/v). Therefore, in the present study, pretreatment was conducted at the solid loading of 15% (w/v) to acquire the fermentable sugars (glucose plus xylose) concentration of 40 g/L and above. After the pretreatment, solid and liquid fractions were separated through 0.2 µm nylon membrane under vacuum condition. Solid fraction was washed with distilled water to attain the neutral pH and then dried at 45 ºC for 48 h. Chemical compositional analysis of pre-treated biomass was carried out according to the NREL Procedure [31] and listed in Table 7.1.

7.2.3. De-lignification of pre-treated biomass

Dilute sodium hydroxide was used as a de-lignifying agent for the pre-treated biomass. Different sodium hydroxide strengths (1–5%) were used to maximize the de-lignification process with minimal cellulose loss. This process was conducted at 121

ºC for 20 min with a solid loading of 10% (w/v). After the delignification process, solid and liquid fractions were separated through 0.2 µm nylon membrane under vacuum condition. The solid fraction was washed with distilled water under vacuum condition using 0.2 µm nylon membrane to attain neutral pH and then dried at 45 ºC for 48 h. Weight of the biomass was noted for mass balance analysis. Chemical compositional analysis of de-lignified biomass was carried out according to the NREL procedure and listed in Table 7.2.

Table 7.2: Compositional analysis of samples after delignification process

Composition	**1% NaOH**		**2% NaOH**		**3% NaOH**		**4% NaOH**		**5% NaOH**	
Measurement		422		355		337		322		311
	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)	(mg)	(%)	(mg)
Cellulose	84	354	91	324	92	311	92	297	92	288
Xyaln	6.7	28.5	5.9	21	58	19.6	5.7	18.4	5.4	16.8
AIR	8.6	36.3	2.3	8.1	1.9	6.4	1.4	4.5	1.2	3.7

Based on the above results, another set of de-lignification process was conducted at similar conditions (like temperature, time and NaOH strength and solid to liquid ratio) and the biomass was washed with distilled water and then directly subjected to enzymatic hydrolysis without drying. In general, drying of either acid or alkali treated biomass prior to the enzymatic hydrolysis shows negative impact on cellulose conversion that could be due to the hornification of biomass components.

7.2.4. Enzymatic hydrolysis

Residual materials that remained after delignification process were hydrolysed with cellulase (Celluclast 1.5L®) at 50 ºC for 24–96 h at 140 rpm. Instead of using 50 mM citrate buffer (pH 4.8), 0.5 mM citrate buffer has been used for conducting the

enzymatic hydrolysis. Optimization of cellulose hydrolysis has been carried out by varying the enzyme concentrations (20–60 mg cellulase protein/g of cellulose) with 1% (w/v) substrate loading. After obtaining the optimum conditions for cellulose hydrolysis, effect of solid loading (2–10% w/v) were also performed for the evaluation of cellulose hydrolysis efficiency. An aliquot of 100 µL was collected from the reaction mixture at every 24 h interval, and after appropriate dilution was boiled for 10 min to inactivate the enzyme activity. Further samples were analysed by HPLC for the determination of glucose yield. After the enzymatic hydrolysis, solid and liquid fractions were separated through 0.2 µm nylon membrane under vacuum condition. Thereafter, weight of the solid fraction after drying was noted for mass balance analysis. Liquid fraction derived from enzymatic hydrolysis was concentrated under the reduced pressure using rotary evaporator to reach desired sugar concentration for industrial titer of ethanol production.

7.2.5. Conditioning of pre-hydrolysates

Pretreatment derived acid hydrolysate (pre-hydrolysate-PH) was divided into 2 equivalent fractions (F) and are further referred as PHF-1 and PHF-2. These two fractions, PHF-1 and PHF-2, were heated up to 50 ºC and then neutralized with calcium hydroxide [$Ca(OH)_2$] and magnesium hydroxide [$Mg(OH)_2$], respectively under continuous stirring condition. The resultant $CaSO_4$ of PHF-1 was separated through centrifugation at 8000 rpm for 10 min. The neutralized fractions (PHF-1 and PHF-2) were further filter sterilized under vacuum condition, and pH of both the fractions (PHF-1 and PHF-2) were readjusted to cultivation pH (6) of *Pichia stipitis* aseptically with 10 N H_2SO_4 and was further used in fermentation studies.

7.2.6. Fermentation

7.2.6.1. *Microorganisms*

The actively growing cultures, *Pichia stipitis* NCIM 3498 and *Saccharomyces cerevisiae* NCIM 3090 were procured from NCIM Pune, India, which are supplied on MGYP agar slants. The composition of MGYP agar medium contains (g/L) 3, Malt extract; 10, Glucose; 3, Yeast extract; 5, peptone; 20, agar; Temperature and pH of the MGYP agar medium maintained at 30° C and 6.4–6.8, respectively.

7.2.6.2. *Sub-culturing and seed culture preparation*

Pichia stipitis 3498 and *Saccharomyces cerevisiae* NCIM 3090 have been used in the present study. Sub-culturing and seed culture preparation was performed according to the method described in section 6.2.4.1.

7.2.6.3. *Xylulosic ethanol production from pretreatment derived hydrolysate*

To evaluate the ethanol conversion efficiency from the filter sterilized hydrolysates of PHF-1 and PHF-2, fermentation experiments were conducted in batch mode for the production of xylulosic ethanol. Each fermentation medium contains, 2% (v/v) of sterilized 50X concentrated nutrient solution (1.7 g of yeast nitrogen base, 1 g of urea and 6.56 g of peptone in 20 mL of distilled water), and 5% (v/v) of inoculums (which gives an initial cell concentration of 2 g/L). Initial pH of the media was adjusted to 5.5 with 10N H_2SO_4 and incubated at 30 °C and 140 rpm. All fermentation samples were taken periodically for HPLC analysis.

7.2.6.4. *Cellulosic ethanol production from enzymatic hydrolysis derived hydrolysate*

The fermentation experiments were also conducted in batch mode with varying enzymatic hydrolysate loading with respect to glucose concentration i.e., 96 g/L and

170 g/L. Along with this, both the fermentation media contain, 4% (v/v) of 25X YP nutrient solution (10 g of yeast extract and 20 g of peptone in 40 mL of distilled water), and 6% (v/v) of seed culture (which provides an initial concentration of 1.6 g/L on cell dry weight basis). Initial pH of the fermentation broth was maintained at 5.5 and incubated at 30 °C and 150 rpm for 48 h.

7.2.7. X-Ray Diffractometer (XRD) Analysis

The X-Ray diffractometer (Bruker, D8- Advanced XRD measurement systems, Japan) analysis of untreated, pretreated and delignified biomass samples was performed to evalute the crystalline nature of cellulose present in the biomass samples. The XRD was equipped with Cu Kα (λ=1.541 Å) radiation settled at 40 KW voltage and 40 mA current. The diffraction angle (2θ) was 8 to 40° at a step size of 0.05° and scan speed 1°/min. The following equation (Eq. 7.1) was used for the determination of crystallinity index (CI) which was based on the diffraction intensities of crystalline and amorphous regions [185].

$$\text{CrI (\%)} = \frac{I_{002} - I_{amp}}{I_{002}} \times 100 \qquad (7.1)$$

Where, I_{002} = the peak corresponding to 002 lattice plane of a cellulose at 2θ = 22.4°, and I_{amp} = amorphous region peak intensity at 2θ = 15.6° [186].

7.3. Results and Discussion

7.3.1. Pretreatment

Pretreatment of sorghum stalk yields around 0.66 g/L of cellobiose, 0.84 g/L of xylobiose, 2.7 g/L of glucose, 38.15 g/L of xylose, 4.08 g/L of arabinose, 5.7 g/L of acetic acid, 0.54 g/L of formic acid, 0.21 g/L of levulinic acid, 0.88 g/L of furfural and

0.91 g/L 5-HMF. Apart from the sugars, the amount of compounds that are formed during the pretreatment process are characterized as fermentative inhibitors. The amount of sugars and fermentative inhibitors formed per gram sorghum stalks during the pretreatment are calculated by the following equations (Eq. 7.2 and Eq. 7.3).

$$\text{Sugars or Inhibitors (mg/mL)} = \text{Conc. detected by HPLC} \times \text{Dilution factor} \quad (7.2)$$

$$\text{Sugars or Inhibitors } (mg/g) = \left(\frac{Sugars\ (mg) \times Volume\ of\ hydrolyzate\ (mL)}{Intital\ weight\ of\ biomass\ (g)} \right) \quad (7.3)$$

Xylobiose, xylose, arabinose and acetic acid are the hydrolysis products of hemicellulose, whereas cellobiose and glucose are the hydrolysis products of cellulose. In addition to this, furfural and 5-HMF are the decomposition products of pentose and hexose sugars, receptively [51]. Further degradation of 5-HMF yields levulinic acid and formic acid, whereas, furfural yields only formic acid [33]. Generally, dilute sulfuric acid pretreatment of lignocellulosic biomass intends to hydrolyse most of the hemicellulosic fraction [82,148]. Therefore, in this study, xylose is found to be a predominant sugar present in the pre-hydrolysate. Apart from this, Dominguex et al., (2000), have suggested that the maximum ethanol producing limits of *Pichia stipitis* (wild type) is 20 g/L, which is technically referred as product inhibition concentration of *Pichia stipites* [91]. Therefore, the amount of fermentable sugars (glucose- 2.87 g/L and xylose- 38.15 g/L) present in the pre-hydrolysate were found be to 41.02 g/L. Since, *Pichia stipitis* having a capability to produce ethanol from glucose and xylose, the expected theoretical ethanol yield could be 20.9 g/L. Therefore, conducting the pretreatment of sorghum stalks at 15% (w/v) solid loading yields sufficient amount of fermentable sugars for xylulosic ethanol production.

However, the amount of cellulose, hemicellulose and lignin remaining after the pretreatment have been listed in Table 7.1 and the percentage of hemicellulose conversion has been calculated according to equation 7.4. From table 7.1, it can be seen that around 82% hemicellulose conversion was achieved during the pretreatment of sorghum biomass.

$$\text{Hemicellulose conversion (\%)} = \left(\frac{I_{Hm} - R_{Hm}}{I_{Hm}} \right) \times 100 \qquad (7.4)$$

Where, I_{Hm} is the initial amount of hemicellulose present in the raw biomass and R_{Hm} stands for residual amount of hemicellulose present in the pretreated biomass.

7.3.2. De-lignification

Although dilute sulfuric acid pretreatment was able to slightly reduce the lignin content of lignocellulosic biomass yet an additional treatment is required to improve the cellulose digestibility during the enzymatic hydrolysis. An alkali treatment can effectively solubilize the lignin components of lignocellulosic biomass [187]. Therefore, in the present study, different NaOH strengths are used as a de-lignifying agent and it has shown synergetic effect on de-lignification of pre-treated biomass. The amount of biomass remaining after the de-lignification process and their composition are listed in Table 7.2. Moreover, percentage of lignin removal, loss of cellulose and xylan are calculated according to Eq.7.5 and are shown in (Table 7.3).

$$\text{Loss of cellulose or xylan or lignin (\%)} = \left(\frac{m_{pr} - m_{ar}}{m_{pr}} \right) \times 100 \qquad (7.5)$$

Where, m_{pr} is the mass of polymer (cellulose/xylan/lignin) present in the pretreated residue and m_{ar} stands for residual amount of polymer present in the alkali treated residue.

Table 7.3: Loss of cellulose, xylan and lignin during the delignification process

Composition (%)	1% NaOH	2% NaOH	3% NaOH	4% NaOH	5% NaOH
Cellulose	5.6	13.6	17.2	20.8	23.2
Xyaln	45.0	59.4	62.2	64.4	67.5
AIR	80.7	95.6	96.6	97.8	98.0

Around 32% to 51% of pre-treated biomass solubility was attained during the delignification process. With an increase of NaOH strength from 1–5% (w/v), a subsequent increase of around 81–98% of lignin solubility was observed with the cellulose loss of 5% to 23% (Table 7.3). Several interesting results were observed during delignification process, i.e., around 80.7% and 95% of lignin solubility was attained at 1% and 2% NaOH, respectively. An improvement of lignin solubility between 1–2% (w/v) NaOH was found to be 14.3%. Whereas, 96.7%, 97.8% and 98% of lignin solubility was attained at 3%, 4% and 5% of NaOH, respectively, and the enhancement of lignin solubility was found to be only ≤ 1.1% at each increment of NaOH concentration. This could be due to the fact that, the major portion of cellulose covered lignin was easily solubilized at lower NaOH strength, whereas a tiny portion of lignin was also covered by cellulose polymer which can only eradicate after gradual solubilization of cellulose [32,64]. In addition to this, around 6.4% to 5.4% of xylan was still found in the de-lignified biomass, which could also be covered by cellulose polymer. Generally, solubility of lignin and hemicellulose/xylan is comparatively higher than that of cellulose. According to Zhang et al., (2010), low concentrations of

NaOH may not be sufficient enough to penetrate into the crystalline region of cellulose because crystalline natured cellulose chains are very densely packed with an inter sheet distance of about 10 Å with a crystalline diameter of only 10 nm [188]. Therefore, presence of a little amount of lignin and xylan after the delignification could be under the protection of cellulose polymer. An evident from Table 7.3, increment in cellulose loss was found to be 10% on increasing the NaOH concentration from 2% to 5% whereas, lignin solubility was improved only 3%. However, based on these results, maximum lignin removal (95%) with minimal cellulose loss (13%) was obtained at 2% NaOH.

7.3.3. Enzymatic hydrolysis

Significance of delignification process can be affirmed by cellulose conversion efficiency during the enzymatic hydrolysis. Therefore, all de-lignified materials (derived from various NaOH concentrations) were subjected to enzymatic hydrolysis for further validation of delignification process. Different enzyme loadings such as 20, 40 and 60 mg of cellulase protein/gram of cellulose (hereafter enzyme loadings are referred as 20 mg/g, 40mg/g and 60 mg/g) were used for the optimization of enzymatic hydrolysis process. During the enzymatic hydrolysis of 1% NaOH treated substrate, around 55.2% and 65.7% (10% increment only) of cellulose hydrolysis was attained at 72 h of reaction time by implementing the enzyme loading of 20 and 40 mg/g, respectively (Figure 7.1a and 7.1b).

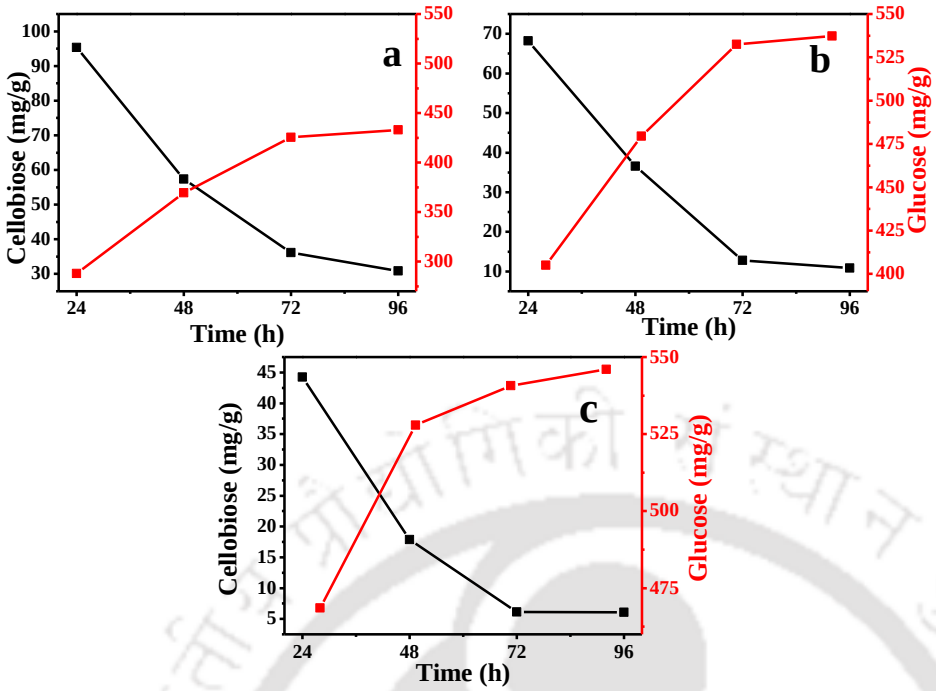

Figure 7.1: Effect of different enzyme loadings (a) 20 mg/g (b) 40 mg/g and (c) 60 mg/g (1% NaOH treatment derived residue) on cellobiose and glucose release.

Percentage of cellulose conversion can be calculated by the following equation (Eq.7.6). However, no further increment in cellulose hydrolysis was observed by increasing the enzyme loading to 60 mg/g (Figure 7.1c). This could be due to the fact that, around 8.6% of lignin was still present in the 1% NaOH treated substrate (Table 7.2). Therefore, recalcitrant nature of lignin hampers further hydrolysis of cellulose via restricting the cellulose accessibility, non-specific binding of enzyme and small molecule inhibition [76,77,86].

$$\text{Cellulose conversion } (\%) = \left[1 - \left(\frac{I_{cc} - Y_{cb+glu}}{I_{cc}}\right)\right] \times 100 \qquad (7.6)$$

Where, I_{cc} is the amount of cellulose before the enzymatic hydrolysis process, Y_{cb+glu} is represents yield of cellobiose and glucose during the enzymatic hydrolysis.

Apart from 1% NaOH treated substrate, a significant cellulose hydrolysis was observed in all the de-lignified substrates i.e., 2% to 5% NaOH treated samples (Figure 7.2). Even at a cellulase loading of 20 mg/g, cellulose conversion about 75–89.5% was attained during the enzymatic hydrolysis of 2% to 5% NaOH treated substrates (Figure 7.2). There are two important factors that plays a vital role in cellulose hydrolysis during the enzymatic process i.e., biomass delignification and its crystallinity (Figure 7.3).

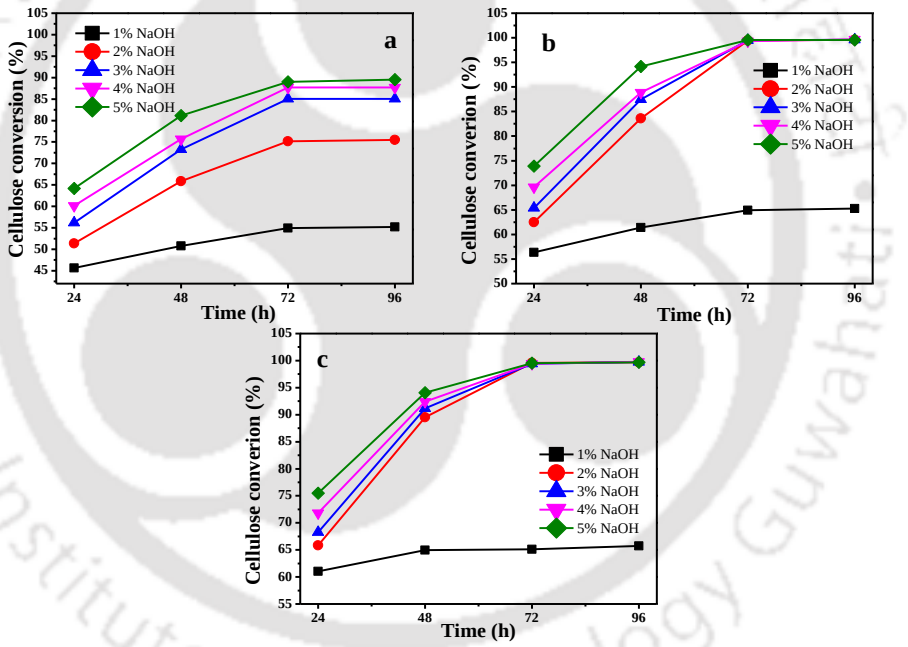

Figure 7.2: Effect of different enzyme loadings on different substrates derived from various strength of NaOH treatment (a) 20 mg/g (b) 40 mg/g (c) 60 mg/g

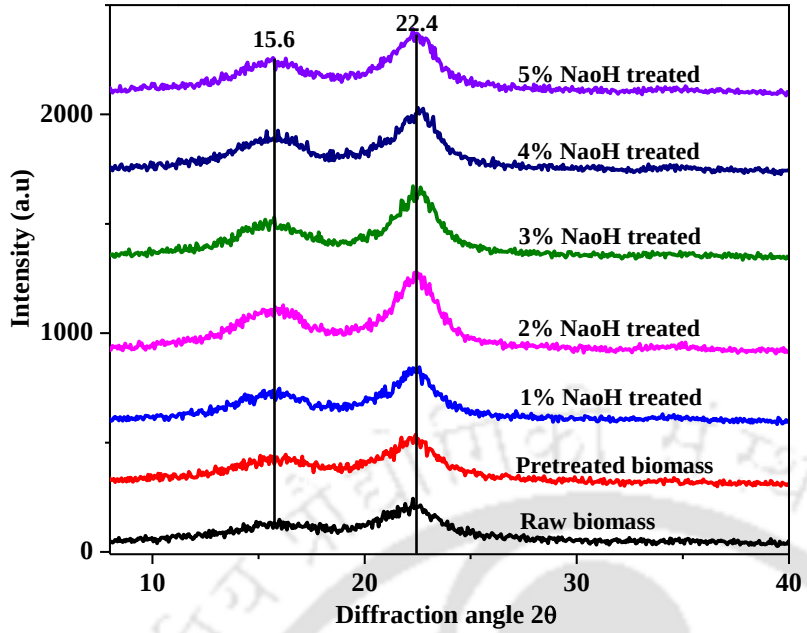

Figure 7.3: Crystalline index of biomass sample derived from different NaOH strengths

As shown in Table 7.2, around 95.6% of delignification was observed in 2% NaOH treatment and the crystalline index was found to be 56%, whereas crystalline nature of 1% NaOH treated biomass was found to be 64%. Due to these reasons, around 20% of increment in cellulose hydrolysis was attained at 2% NaOH treated substrate. While performing the enzymatic hydrolysis of 3%, 4% and 5% NaOH treated substrates, cellulose hydrolysis of around 85%, 87% and 89.5%, respectively was attained at similar enzyme loading (20 mg/g). However, increment in the cellulose hydrolysis between 3–5% NaOH treated sample was found to be less than 5% (Figure 7.2a). A study conducted by National Renewable Energy Laboratory, Department of Energy, USA (NREL/TP-5100-61563) was also obtained 80-86.5% of cellulose hydrolysis during the enzymatic hydrolysis process with the enzyme loading of 19-33 mg/g [66]. Therefore, complete cellulose hydrolysis was not attained at enzyme loading

of 20 mg/g. This could be due to the fact that the amount of enzyme is insufficient for complete cellulose hydrolysis.

However, almost complete cellulose hydrolysis was achieved with a cellulase loading of 40 mg/g at 72 h reaction time (Figure 7.2b), and further increasing the cellulase loading to 60 mg/g slightly increases the percentage of cellulose hydrolysis at 24 h and 48 h, but when it comes to optimum enzymatic hydrolysis time (72 h), the percentage of cellulose hydrolysis was found to be similar in both the cellulase loadings (40 and 60 mg/g) (Figure 7.2c). Since, enzyme cost is considered to be a major driving force of lignocellulosic biomass conversion into bioethanol, the optimum enzyme loading for the present study was considered to be 40 mg/g.

7.3.3.1. *Optimization of substrate loading for enzymatic hydrolysis*

The optimum enzymatic hydrolysis condition for all the de-lignified biomass at 72 h of reaction time was found to be 40 mg/g of cellulase loading. The optimum de-lignified condition (NaOH concentration) has been characterised based on the cellulose loss during the de-lignification process and the amount of cellulose being hydrolysed during the enzymatic hydrolysis process. As evident from Table 7.3 and Figure 7.2b pre-treated biomass de-lignified with 2% NaOH concentration showed 13.5% of cellulose loss, and the cellulose conversion was found to be 99.4%. Even though lower cellulose loss was found at 1% NaOH concentration but the amount of unconverted cellulose present after the enzymatic hydrolysis was found to be 35% (Figure 7.1b). Therefore, sample derived from 2% NaOH delignification was further used in substrate loading studies of enzymatic hydrolysis process.

In the present study, substrate loading of enzymatic reaction increased till high viscous slurry was obtained. In general, mixing of a reaction medium is significantly

hampered when it remains in a high viscous slurry state [189–191]. Consequently, uniform distribution of enzyme and its binding on active sites of cellulose component get affected which eventually reduces the efficiency of cellulose hydrolysis. Therefore, due to mass transfer limitations, 10% (w/v) was considered as the optimum loading for the experimentation. Since, mixing of enzymatic reaction was done in an orbital shaker hence in the present work substrate loading beyond 10% (w/v) was not studied. Generally, enzymatic hydrolysis at high substrate loading (15–30%) has been performed in well-organized reactors, which contains specially designed impellers (Rushton impellers and Helical impeller) that provides intensive mixing regime to facilitate better mass transfer within the reaction mixture [190]. Moreover, Zhang et al., (2010) performed the enzymatic hydrolysis at high solid loading (15–30% (w/v)) in a reactors containing Rushton impellers and helical impeller. The glucose released during the study was found to be 77.3 g/L and 77.7 g/L, respectively. Therefore, performing the enzymatic hydrolysis in well-organized reactors (containing Rushton impellers and Helical impeller) at high solid loadings (15–30%) reduction in cellulose conversion efficiency was observed [190]. This could be due to the non-specific binding of enzyme on non-cellulosic components (lignin and hemicellulose) and end product inhibition [76,77,86]. However, in the present study, performing the enzymatic hydrolysis in a conical flask with an agitation speed of 140 rpm (through the orbital shaker) cellulose conversion efficiency was not affected by increase in the substrate loading (from 1–10% w/v) (Figure 7.4).

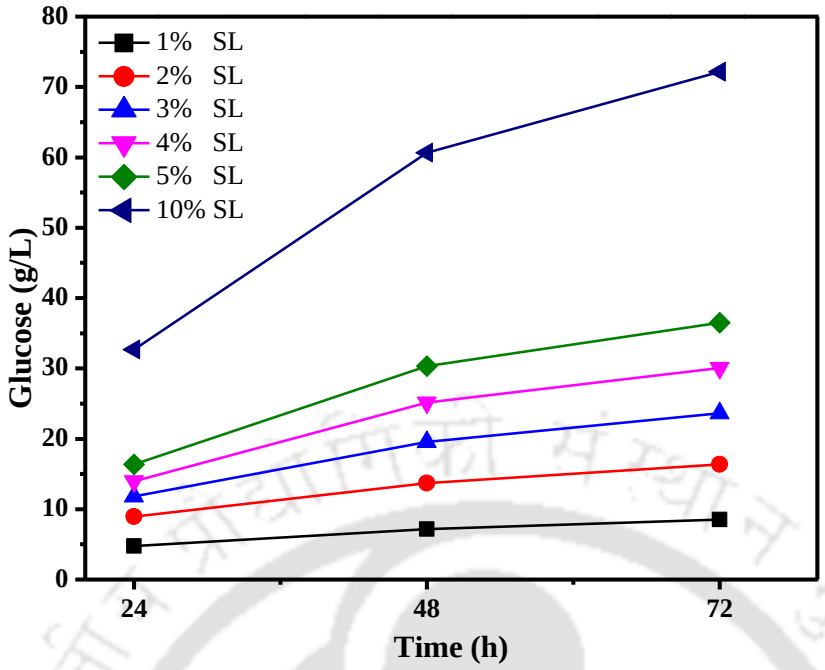

Figure 7.4: Effect of solid loading (SL) on enzymatic hydrolysis efficiency and glucose release at 40 mg/g

The cellulose conversion efficiency remains constant at all solid loadings (1-10% w/v). Due to the presence of minor amount of lignin and xylan, which are covered by cellulose, effect of non-productive absorption of enzyme will not occur as enzyme may not be able to reach xylan or lignin prior to the complete hydrolysis of cellulose. Performance of enzymatic hydrolysis at higher solid loadings results into the end product inhibition which could be due to the high concentration of glucose and cellobiose in the reaction mixture. However, enzymatic hydrolysate derived from 10% (w/v) solid loadings contained 72.1 g/L of glucose, 1.2 g/L of cellobiose and 0.8 g/L of xylose. According to the literature reports, end product inhibition occurs when the enzymatic hydrolysate contains glucose above 76 g/L and cellobiose 10 g/L [190,191]. However, to meet the industrial titer of ethanol production, a high initial glucose concentration is required, thus, with the help of rotary evaporator, around 63.5% of

water had been evaporated from the enzymatic hydrolysate (derived from 10% solid loading) without any sugars loss. The concentrated solution obtained after evaporation contains 198 g/L of glucose, 10 mg of cellobiose and 3 mg of xylose.

7.3.4. Fermentation

7.3.4.1. *Xylulosic ethanol production from pre-hydrolysate*

Detoxification of hydrolysate is necessary process to enhance the xylose fermentability, which eventually increases the ethanol yield and productivity. Several detoxification methods are established to reduce the toxicity of pre-hydrolysate such as stream stripping, over-liming, tri-alkyl-amine extraction, ion exchange resins, activated charcoal absorption, and over-liming coupled with activated charcoal absorption [82]. Among them, over-liming with calcium hydroxide is the most reliable and cost effective method to detoxify the pre-hydrolysate [68,162]. However, various problem impended with over-liming process like sugar loss are found to be the major problem when pH of pre-hydrolysate increased to elevated levels. In addition, solid waste ($CaSO_4$) generated during the $Ca(OH)_2$ treatment for over-liming or neutralization creates a huge disposal problem. Therefore, $Mg(OH)_2$ was used to neutralize the acid hydrolysate. In this study, 0.2 M H_2SO_4 was used for the pretreatment of sorghum biomass, hence, the amount of either $Ca(OH)_2$ or $Mg(OH)_2$ required for the neutralization process are almost same. Around 15 g of $Ca(OH)_2$ was used for neutralization of 1 L acid hydrolysate which generates 30 g of $CaSO_4$. Whereas, no solid waste was generated with the use of $Mg(OH)_2$ for the neutralization. Moreover, no sugar loss or removal fermentative inhibitors were observed during the neutralization of acid-hydrolysates. But due to exothermic reaction the pre-hydrolysate volume decreased, which gives rise to increased sugar and fermentative inhibitors

concentration in terms of mg/mL, but their overall concentration per total volume of hydrolysate remains constant. Prior to fermentation studies, pH of the pre-hydrolysate was adjusted to the cultivation pH of *Pichia stipites* (i.e.,5.5).

Table 7.4: Fermentation summary of pretreatment and enzymatic hydrolysis derived hydrolysates by *P. stipitis* and *S. cerevisiae*, respectively.

Parameters	$Ca(OH)_2$	$Mg(OH)_2$	EDH-1	EDH-2
Ethanol concentration (g/L)	18.4	18.4	44.8	74.7
Ethanol yield (g_P/g_S)	0.45	0.45	0.47	0.46
Ethanol productivity (g/L/h)	0.25	0.25	3.7	2.9
Sugar consumption rate (g/L/h)	0.56	0.56	7.9	5.33
Max. Ethanol production Time (h)	72	72	12	30
Ethanol conversion efficiency (%)	88.3	88.3	92.4	91.5

A batch cultivation was carried out to evaluate the fermentation efficiency of *Pichia stipitis* on both pre-hydrolysates (PHF-1 and PHF-2), which are further referred as Mg-fermentation medium and Ca-fermentation medium. Summary of fermentation results are presented in Table 7.4. Even though without performing the over-liming process, a significant xylulosic ethanol conversion efficiency (88.3%) was attained during the fermentation of both pre-hydrolysates. Almost similar concentration (18.4 g/L and 18.4 g/L) of ethanol was produced with the ethanol yield of 0.45 g_p/g_s and productivity of 0.25 g/l/h (Figure 7.5a and 7.5b).

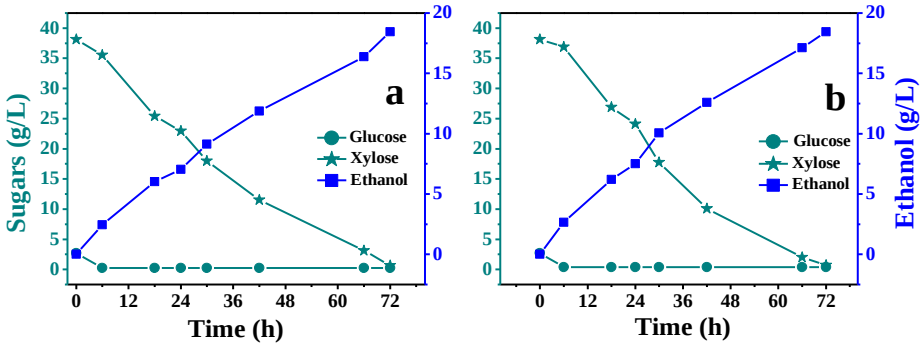

Figure 7.5: Sugar consumption and ethanol production profiles of *P. stipitis* during the fermentation of pretreatment derived hydrolysate (a) PHF-1 and (b) PHF-2

According to ion exchange chromatography (IC) analysis, the concentration of SO_4^{2-} ions present in the Mg fermentation medium is 5 times higher than that of Ca-fermentation medium. Even the presence of high concentration of SO_4^{2-} ions did not affect the ethanol yield, productivity and sugar consumption rate. This could be attributed to the availability of Mg ions in the fermentation medium, which significantly influences the metabolic growth of a microbe. Typically, the efficiency of microbial conversion of substrate to product may be improved by altering Mg ions concentration so that more magnesium ions are available to the cells [192]. Especially in yeast based ethanolic fermentation, magnesium availability is important in governing the central pathways of carbohydrate catabolism. The previous literature reports ensure that the Mg ions influence the activation of key enzymes for the optimum flow of substrate to ethanol and it (Mg ions) stabilizes the cell membrane to protect yeast from chemical and physical stress [192].

In addition to this, the amount of fermentative inhibitors such as furfural, 5-HMF, acetic acid and formic acid present in both pre-hydrolysates did not exhibit detrimental effect on ethanol producing *Pichia stipitis*. Therefore, no lag phase was

observed during the fermentation of both the pre-hydrolysates. According to the Delgenes et al., (1996), 11.9- 15 g/L of acetic acid, 5 g/L of 5-HMF and 2 g/L of furfural are the inhibitory concentrations of *P. stipitis* and other yeast species [183] . In the present study, the amount of furfural, 5-HMF and acetic acid derived from pretreatment process is 3 times lower than that reported by Delgenes et al., (1996). This signifies the viability of current pretreatment process. Generally, when the acid-hydrolysate contains elevated levels of fermentative inhibitors then over-liming ($Ca(OH)_2$) step can be performed to partially convert the toxic components into non-toxic forms for the enhancement of hydrolysate fermentability. The potential draw backs of over-liming process are sugar degradation due to hydroxide-catalysed degradation reactions, and formation of solid waste (calcium sulfate). Generally, solubility of calcium sulfate is very low at neutral and alkaline pH levels, therefore it forms solid waste ($CaSO_4$) even during neutralization of acid hydrolysate. However, in the present study, over-liming process and solid waste formation are terminated, and $Mg(OH)_2$ is suggested as a significant neutralizing agent for the acid hydrolysate and it also enhances the hydrolysate fermentability.

7.3.4.2. *Cellulosic ethanol production from enzymatic hydrolysate*

Generally, lignocellulosic biomass contain different types of organic acids which are structurally incorporated within their respective polymers. For example, hemicellulose contains acetylated xylan back bone and three-dimensional methoxylated lignin contains various types of organic acids such as vanillic acid, *p*-coumaric acid, ferulic acid and cinnamic acid [34]. These organic acids in lignocellulosic biomass can alter the pH of the enzymatic hydrolysis medium (which optimally proceed at 4.8–5.5) and creates unfavourable conditions for enzyme action. Thus, enzymatic hydrolysis was conducted at high citrate buffer strength (50 mM) to

restrict the pH change [86]. In addition, use of 50 mM citrate buffer fundamentally hampered the growth metabolism of glucose fermenting microorganism [86]. This resulted in low ethanol yield and productivity. Moreover, enzymatic hydrolysis in 50 mM citrate buffer strength is commercially not feasible. However, in the present study, significant percent of acetic acid and lignin were removed successfully during dilute acid pretreatment and delignification step, respectively.

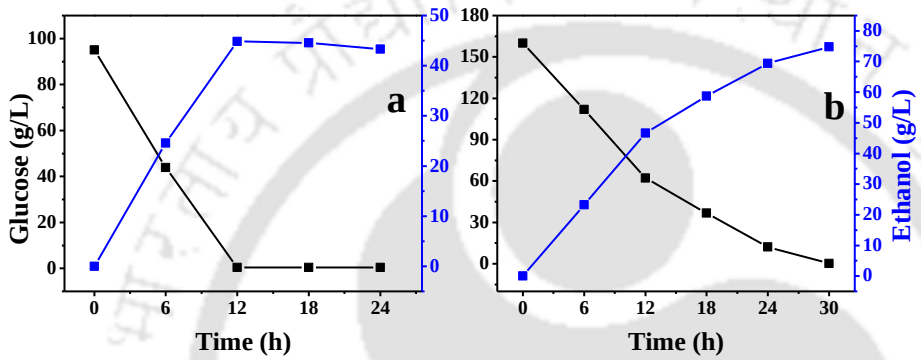

Figure 7.6: Glucose consumption and ethanol production profiles of *S. cerevisiae* during the fermentation of enzymatic hydrolysis derived hydrolysate (a) CDH-1 and (b) CDH-2

Thus, by considering the aforementioned facts, enzymatic hydrolysis was conducted at low citrate buffer strength i. e., 0.5 mM, which is 100 times lower than that of standard citrate buffer strength. As expected, significant ethanol production of 44.8 g/L and 74.7g/L of was achieved by utilizing the glucose concentration of 95 g/L and 160 g/L, respectively (Figure 7.6a and 7.6b). Thus, the glucose to ethanol conversion efficiency and ethanol yield were found to be above 91% and 0.46 g_p/g_s, respectively. As compared to EDH-1 fermentation (95 g/L glucose), sugars consumption rate and ethanol productivity was found to be lower during the fermentation of EDH-2. During the fermentation of EDH-1 sugars consumption rate

and ethanol productivity was observed to be 7.9 g/L/h and 3.7 g/L/h, respectively. Whereas, 5.3 g/L/h sugar consumption rate and 2.5 g/l/h ethanol productivity were attained during the EDH-2 fermentation (Table 7.4). Reduction of sugar consumption rate and ethanol productivity could be due to high concentration of ethanol production (74.7 g/L) during the EDH-2 fermentation. According to the literature reports, when the ethanol concentration of fermentation medium reached above 55 g/L, it initiates the product inhibition effect [90]. As evident from Table 7.4, until the ethanol concentration reaches 58.7 g/L in 18 h, sugar consumption rate and ethanol productivity was found to be 6.8 g/L/h and 3.2 g/L/h, respectively. Thereafter, sugar consumption rate and ethanol productivity deceased to 5.3 g/L/h and 2.4 g/L/h, respectively, which could be due to ethanol inhibition effect. However, in the present study, 74.7 g/L ethanol production was attained, which is equivalent to 9.4% (v/v) (based on ethanol density). Usually, a typical range of industrial titer of bioethanol production from edible sources such as corn (USA), sugarcane (Brazil) and sugar beet (EU) have been found to be 8-12% [2,12]. However, in the present study, around 9.1% (v/v) of bioethanol production was achieved by using inedible agriculture waste.

7.3.5. Determination of optimum liquefaction process for sorghum biomass

During the dilute sulfuric acid pretreatment process, around 37.2% of sorghum biomass solubilisation was attained (Based on the HPLC analysis and NREL procedures) which yields 11.7 mg of glucose, 165.3 mg of xylose and 7.7 mg of arabinose. Subsequently, while performing the delignification process of pre-treated biomass, around 32.7%, 43.4%, 46.2%, 48.6% and 50.39% of biomass solubilities were observed at alkali (NaOH) concentration of 1%, 2%, 3%, 4% and 5%, respectively (Figure 7.7). The amount of cellulose, hemicellulose loss and lignin solubility are calculated according to the Eq. 7.4 and clearly depicted in Table 7.3.

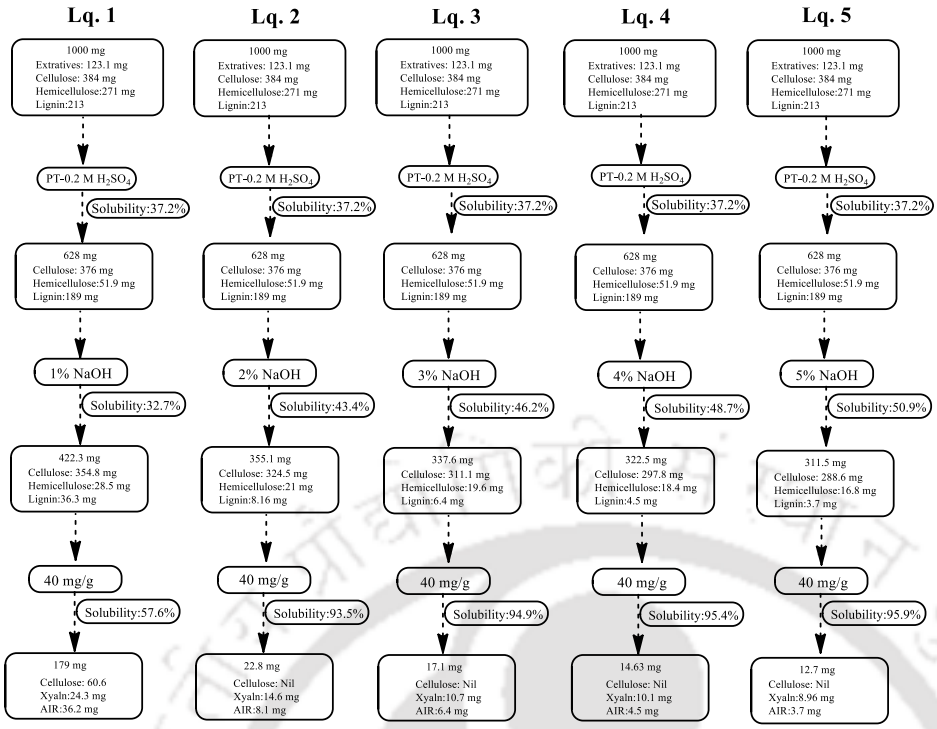

Figure 7.7: Liquefaction of sorghum biomass at various parameter

Finally, around 179.5 mg, 22.8 mg, 17.15 mg, 14.6 mg and 12.7 mg of residual biomass was remained after the enzymatic hydrolysis of de-lignified substrates (derived from 1%, 2%, 3%, 4%, 5% NaOH strengths), and these liquefaction processes (Lq. P) are further denoted as Lq. P_1, Lq. P_2, Lq. P_3, Lq. P_4 and Lq. P_5, respectively (Figure 7.7). In addition, the amount of lignin, cellulose and xylan remains after enzymatic hydrolysis was shown in Figure 7.7. Therefore, solubility of sorghum stalks after the sequential steps (dilute acid pretreatment, delignification and enzymatic hydrolysis) were found to be 82%, 97.7%, 98.2%, 98.5% and 98.7% at Lq. P_1, Lq. P_2, Lq. P_3, Lq. P_4 and Lq. P_5, respectively. From Table 7.3 and Figure 7.7, along with 5% loss of cellulose, 25% of unconverted cellulose was observed at Lq. P_1. Almost a complete cellulose hydrolysis was observed at Lq. P_2 with the cellulose loss of 13.5%. Even though the highest liquefaction was attained at Lq. P_3, Lq. P_4 and Lq. P_5, but the amount

of cellulose loss during the delignification was found to be higher than that of Lq. P_2. However, no significant increment in biomass liquefaction was observed from Lq. P_2 to Lq. P_5 which accounted to be 1% increment only. Therefore, based on the maximum sugar yield (cellulose hydrolysis) with minimal sugar loss, Lq. P_2 is considered to be optimum process for sorghum biomass hydrolysis.

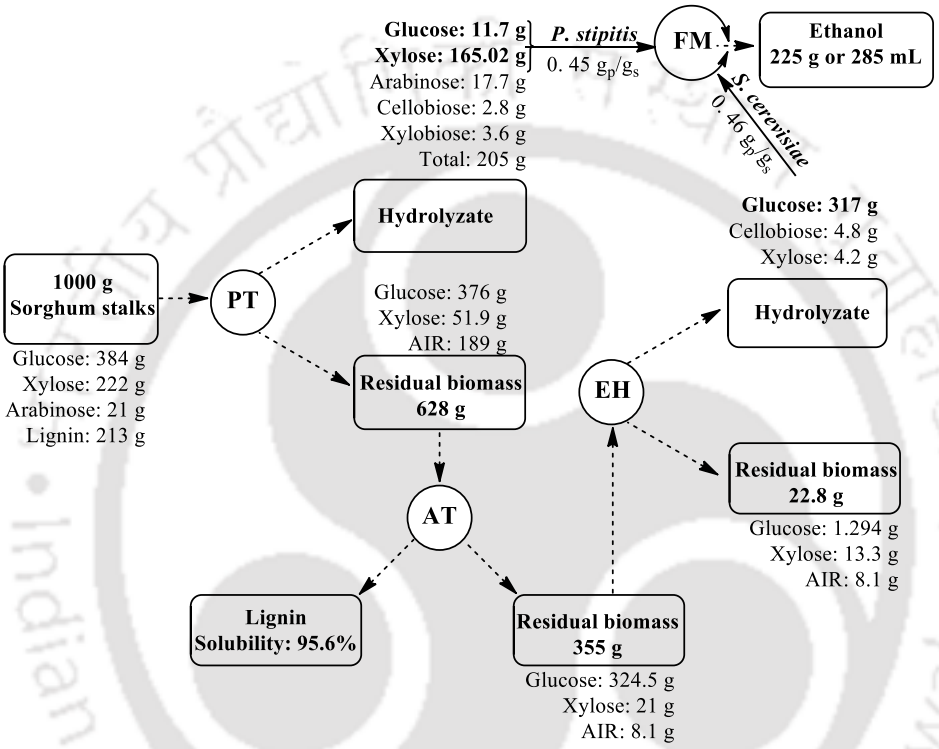

Figure 7.8: Mass balance analysis of Liquefaction process 2. Where, PT, AT, EH and FM are representing pretreatment, alkali treatment, enzymatic hydrolysis and fermentation, respectively.

The complete mass balance analysis of liquefaction process 2 is represented in the Figure 7.8. During the pretreatment process, around 165.3 g of xylose and 17.7 g of arabinose and 11.7 g of glucose (per kg of sorghum stalks) was attained. Among them, *Pichia stipitis* can ferment both glucose and xylose into ethanol [82,172]. As shown in the Table 7.4, during the fermentation of pretreatment derived hydrolysate, about and the found to be 0.45 g_p/g_s ethanol yield was observed. Eventually, around

90.12 g of ethanol has been produced from 176.72 g of fermentable sugars (glucose- 11.7g and xylose- 165 g) by *Pichia stipitis*. Moreover, 317 g of glucose, 4.2g of xylose and 4.8 g of cellobiose was attained at enzymatic hydrolysis process. Furthermore, aound 0.46 g_p/g_s ethanol yield was achieved during the fermentation of enzymatic hydrolysate by *S. cerevisiae* (Table 7.4). This eventually yields 161.67 g of ethanol from 317 g of glucose. Therefore, summation of ethanol produced from pretreatment and enzymatic hydrolysis derived hydrolysate is accounted to be 225 g. Based on the ethanol density (0.789 g/mL), around 285 mL of ethanol has been produced per kg of sorghum stalks.

7.3.6. Cost analysis of developed process

Although the process is developed in laboratory scale, yet we have made an attempt to estimate the production cost of bioethanol at industrial scale. The bioethanol production cost has been estimated on the basis of monomeric sugars yield (especially, glucose and xylose) and ethanol conversion efficiency during the lignocellulosic biomass hydrolysis process (includes pretreatment, delignification and enzymatic hydrolysis) and fermentation process, respectively. Apart from the monomeric sugars yield and their ethanol conversion efficiency, several factors are need to be considered for the cost analysis of developed bioethanol production process such as,

Plant construction: Capital investment required for the construction of bioethanol plant is generally depends on the production capacity of bioethanol in terms of L/day. There are several factors such as size of the crusher for the particle size reduction of lignocellulosic biomass, size of reactors that required for lignocellulosic biomass conversion into bioethanol which includes pretreatment reactor, neutralization rector, delignification reactor, enzymatic hydrolysis reactor, fermentation reactor and size of the distillation columns and molecular sieve columns for the preparation of fuel grade

(anhydrous ethanol) bioethanol also influence the capital investment of bioethanol plant. Moreover, power requirement to run the bioethanol pant also influence the overall estimation of bioethanol production.

Separation process: In the laboratory process we used 0.2 µm nylon membrane filter for the solid liquid separation under vacuum condition but in the large scale studies continuous screw press used for the solid-liquid separation.

Chemicals: There is huge amount of price difference have been observed while purchasing of chemicals that required for lignocellulosic biomass conversion process. For example, 2.5 L of 98% H_2SO_4 cost is 980 rupees, whereas when it purchasing it in large quantity like 1 metric ton, the price of 98% H_2SO_4 is found to be 6000 rupees. Therefore, a similar difference has could be there for cellulase, citric acid, magnesium hydroxide, sodium hydroxide, yeast extract and peptone.

Value added Byproducts: i) Non-hydrolyzed biomass has capability to generate steam and power, ii) CO_2 that generated during the fermentation process and it conversion into dry ice, iii) preparation of bio-fertilizer after the distillation of ethanol from fermented broth. These by-products can be credited to reduce the further cost of bioethanol.

Therefore, performing the cost analysis must require aforementioned details which clearly elucidate the perfect cost analysis of developed bioethanol production process. However, the process developed in laboratory scale, we performed cost analysis based on the materials that required for the lignocellulosic biomass into bioethanol production. The optimum process parameters of lignocellulosic biomass hydrolysis which includes temperature, biomass solid loading, catalyst loading and reaction times has been shown in Table 7.5.

Table 7.5: Optimum process parameters for lignocellulosic biomass conversion process

Lignocellulosic biomass conversion steps	Solid loading (w/v)	Catalyst loading	Temperature (ºC)	Residence time (h)	Biomass Solubility (%)
Pretreatment	15%	0.2 M H_2SO_4 [a]	121	2	37.2
Delignification	10%	2% NaOH [b]	121	0.3	64.5
Enzymatic hydrolysis	10%	40 mg protein/g cellulose [c]	50	72	97.2

[a] *0.2 M H_2SO_4 is equivalent to 1.12 mL, Therefore, 1.12 mL of H_2SO_4 required for the pretreatment of 15 g raw biomass.*
[b] *2% NaOH is equivalent to 2 g NaOH, Therefore, 2 g NaOH required for the delignification of 10 g pretreated biomass.*
[c] *1 mL of cellulase contains 160 mg of protein, therefore, 0.25 mL of enzyme provides 40 mg of cellulase protein that has been used per gram of cellulose.*

The initial polymeric carbohydrates content of sorghum biomass and their monomeric sugars yield during the pretreatment and enzymatic hydrolysis and their subsequent conversion into bioethanol has been calculated per ton of sorghum biomass and the results are listed in Table. 7.6. Moreover, cost of the material that required for the bioethanol production per ton basis has been shown in Table. 7.7 and availability of materials source and their cost (per ton basis) is shown in the Table. 7.8.

Table 7.6: Monomeric sugar yield and ethanol production from lignocellulosic biomass: Based on developed process

Structural Carbohydrates	I_{sc} (kg/ton)	S_Y (%)	Monomeric sugar yield (kg)	F_{efi} (%)	Ethanol (kg/ton)	Ethanol (L/ton)
Cellulose	384	85	328.7	90	150.8	191.1
Xylan	222	76	169.2	88	75.9	96.2
Arabinan	21	84	17.7	NA	NA	NA
Total	627	NA	515.6	NA	226.7	287.3

Isc, initial sugar content present in the raw biomass; S_Y, sugar yield; F_{efi}, fermentation efficiency.

Table 7.7: Cost analysis of lignocellulosic biomass to bioethanol: On material basis

	Biomass	Chemical	Rupees
Pretreatment	1000 kg	74.6 L H_2SO_4	823
Delignification	628 kg	125.6 kg NaOH	2638
Enzymatic hydrolysis	324.5 kg	81.12 L Cellulase	3916*
Citrate buffer preparation	3555 L	0.34 kg Citric acid	5
Fermentation			
Pre-hydrolyzate neutralization	4333 L	64.5 kg $Mg(OH)_2$	451
Enzymatic hydrolyzate	4400 L	-	-
Yeast extract		87.3 kg	1833
Peptone		174.6 kg	3317
Biomass Cost	1000 kg	-	2000
Total			14983
Production cost of bioethanol/L			52.15

**According to NREL report (NREL/TP-5100-61563 April 2014) [66], contribution cost of cellulase enzyme for 1-gallon bioethanol production was 0.36 dollars, consequently, cellulase contribution is 28 dollars for 75 gallons of bioethanol production/ton of cornstover and they were used 20 mg of cellulase protein concentration per gram of cellulose. In the present study, we used 40 mg of cellulase protein concentration per gram of cellulose and obtained 287 L of bioethanol which is equivalent to 75 gallons. Therefore, cellulase contribution could be 56 dollars which is equivalent 3916 rupees.*

Table 7.8: Material cost/ton

Chemical	Purity (%)	Cost/ton (rupees)	Source
H_2SO_4	98	6000	India Mart
NaOH	99	21000	Alibaba.com
$Mg(OH)_2$	99	7000	Alibaba.com
$C_6H_8O_7.2H_2O$	99	14000	Alibaba.com
Yeast Extract	Microbial grade	21000	Alibaba.com
Peptone	Microbial grade	19000	Alibaba.com

7.4. Summary

During the pretreatment of sorghum stalks, hemicellulose conversion of 82% was attained and the resulted pre-hydrolysate fermented without detoxification, which has produced 18.4 g/L of xylulosic ethanol by *P. stipitis*. Moreover, around 81–98% of delignification was achieved during the alkaline treatment of pre-treated residue. Subsequently, 81–99.6% cellulose hydrolysis was achieved by employing 40 mg of protein per gram of cellulose. Moreover, due to the presence of lower citrate buffer strength, an industrial titer of 74.7 g/L cellulosic ethanol was produced within 30 h. Eventually, the optimum liquefaction process solubilizes 97.7% of sorghum biomass, in this about 84% of holocellulose hydrolysed to yields 526 g of sugars per kg of sorghum stalks in which 494 g of fermentable sugars (glucose and xylose) yields 225 g of ethanol and the ethanol yield found to be 0.45 g_p/g_s. Therefore, the current process can be able to produce 285 L (225 g/0.789 g/L ethanol density) of bioethanol per ton of sorghum biomass. Moreover, cost analysis of the developed process has been performed based on the materials basis and the production cost of the bioethanol was estimated to be 52.15 rupees/L.

Chapter 8:

Conclusions and Future cope of the work

8.1. Conclusions

In the present study, an attempt has been made to obtained physical insight into sulfuric acid pretreatment and enhanced ethanol fermentation using *P. stipitis.* Around 82–97% hemicellulose hydrolysis with optimum pentose sugar released was achieved at 121 ºC, 0.2 M H_2SO_4 for 120 min. Further, due to the presence of minimal fermentative inhibitors, pre-hydrolysate was subjected to fermentation without detoxification. Moreover, in order to avoid the disposal of gypsum ($CaSO_4$), magnesium hydroxide ($Mg(OH)_2$) has been used for neutralization of pre-hydrolysate. Anaerobic fermentation of pre-hydrolysate neutralized with $Mg(OH)_2$ gives better fermentability during the cultivation of *P. stipitis* which results into ethanol productivity 0.25 g/L/h and ethanol yield of 0.45 g_p/g_s which are found to be similar with that of $Ca(OH)_2$ treated hydrolysate.

Moreover, enzymatic hydrolysis of pre-treated biomass was carried out at lower citrate buffer strengths such as 5 mM and 0.5 mM and the cellulose conversion efficiency results were found to be similar with that of standard citrate buffer strength (50 Mm). Due to the presence of significant amount of lignin in the pre-treated biomass, around 54.4% cellulose conversion efficiency was observed at 60 mg of cellulase protein per gram of cellulose with 2% (w/v) solid loading. During the enzymatic hydrolysis process, to decrease the cellulase loading and increase the cellulose conversion efficiency and solid loading, subsequent alkaline treatment of pre-treated biomass was performed for maximum de-lignification with minimal cellulose loss. As

a result, round 99.6% cellulose conversion efficiency was achieved by using 40 mg of cellulase protein per gram of cellulose at 10% (w/v) solid loading in 0.5 mM citrate buffer strength. Furthermore, an industrial titer of 74.7 g/L ethanol production was attained with higher ethanol yield of 0.46 g_p/g_s.

8.2. Future scope of the work

All the experiments conducted in the thesis were at laboratory scale. These studies can also be scaled up by using large scale reactors. However, in accordance to the chapter 7, we proposed a process which has significant features towards the industrialization. A schematic diagram of the proposed process is shown in Figure I. During the process of lignocellulosic biomass liquefaction, one dry ton of sorghum biomass containing 627 kg of holocellulose converted into 7.6 kg of cellobiose, 3.6 kg of xylobiose, 328.7 kg of glucose, 169.2 kg of xylose and 17.7 kg of arabinose. Moreover, instead of using naturally occurring strains such as *Pichia stipitis* and *Saccharomyces cerevisiae*, genetically modified microbial strains can be used to ferment cellobiose and arabinose along with glucose and xylose. Therefore, around 300 L of bioethanol (based on ethanol density of 0.789 kg/L) can be produced per dry ton of sorghum biomass.

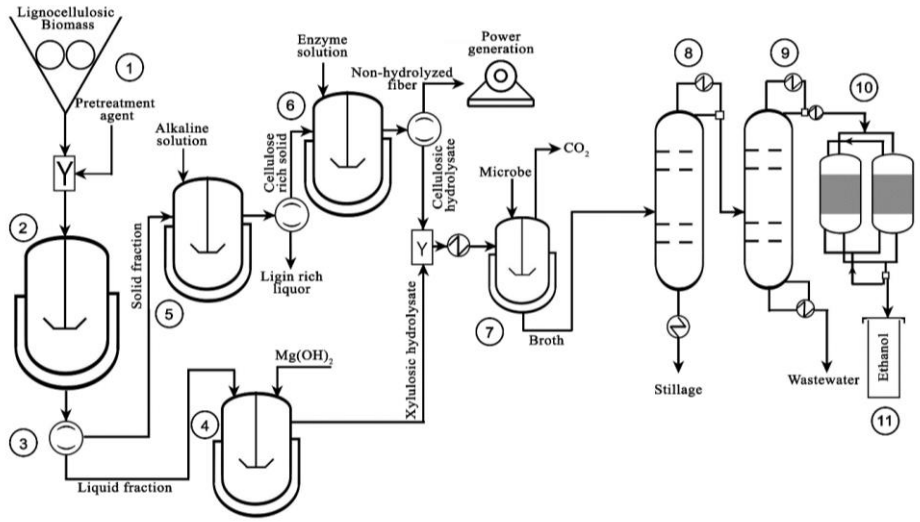

Figure I Proposed bioethanol production from lignocellulosic biomass

1) Crusher 2) Pretreatment reactor 3) Solid and liquid separation 4) Neutralization reactor 5) Delignification reactor 6) Enzymatic hydrolysis reactor 7) Fermenter 8) Concentration column 9) Rectification column 10) Molecular sieves for ethanol dehydration 11) storage tank.

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Indian Institute of Technology Guwahati

LIST OF PUBLICATIONS

Published research articles

1. Narendra Naik Deshavath, Mood Mohan, V. Venkata Dasu, Vaibhav V. Goud, P. Srinivasa Rao, Tamal Benarjee, Dilute acid pretreatment of sorghum biomass to maximize the hemicellulose hydrolysis with minimized levels of fermentative inhibitors for bioethanol production, 3 Biotech (2017) 7:139.doi: 10.1007/s 13205-017-0752-3.

2. Narendra Naik Deshavath, V. Venkata Dasu, V. V. Goud, P. Srinivasa Rao, Development of dilute sulfuric acid pretreatment method for the enhancement of xylose fermentability,Biocatalysis and Agricultural Biotechnology 11 (2017), 224–230.

3. Narendra Naik Deshavath, Mahanta S, Goud V V., Dasu VV, P. SR. Chemical composition analysis of various genetically modified sorghum traits: Pretreatment process optimization and bioethanol production from hemicellulosic hydrolyzates without detoxification. Journal of Environmental and Chemical Engineering 2018. doi:10.1016/J.JECE.2018.08.002.

4. Mohan M, Narendra Naik Deshavath, Banerjee T, Goud V V., Dasu V V. Ionic liquid and sulphuric acid based pretreatment of bamboo: Biomass delignification and enzymatic hydrolysis for the production of reducing sugars. Industrial Eng Chem Res 2018;57:10105–17. doi:10.1021/acs.iecr.8b00914.

5. Mood Mohan, Robinson Timung, Narendra Naik Deshavath, Tamal Banerjee, Vaibhav V. Goud, Venkata V. Dasu, Optimization and hydrolysis of cellulose under subcritical water treatment for the production of total reducing sugars. RSC Adv., 5(2015),103265-103275. DOI: 10.1039/c5ra20319h.

6. Robinson Timung, Narendra Naik Deshavath, Vaibhav V. Goud, and Venkata V. Dasu, “Effect of Subsequent Dilute Acid and Enzymatic Hydrolysis on Reducing Sugar Production from Sugarcane Bagasse and Spent Citronella Biomass,” Journal of Energy, vol. 2016, doi:10.1155/2016/8506214.

Research articles are Under Review

1. Narendra Naik Deshavath, Gourab Mukherjee, Vaibhav V. Goud, V. Venkata Dasu, Chivukula V. Sastri, Influence of Furfural and 5-Hydroxymethylfurfual in Reliable Estimation of Reducing Sugars.

2. Narendra Naik Deshavath, Veeranki Venkata Dasu, Vaibhav V. Goud, Sustainable strategies for the production of an industrial titer of bioethanol from sorghum stalks.

3. Narendra Naik Deshavath, Veeranki Venkata Dasu, Vaibhav V. Goud, Liquefaction of lignocellulosic biomass for the production of bioethanol: An industrially feasible approach.

Book chapters

1. Narendra Naik Deshavath, S. K. Sahoo, M. M. Panda, S. Mahanta, D. S. N. Goutham, V. V. Goud, V. V. Dasu, Annapurna jetty, The Cost Effective Stirred Tank Reactor for Cellulase Production from Alkaline Pretreated Agriculture Waste Biomass. In: Ghosh S. (eds) Utilization and Management of Bioresources. Springer, Singapore 2018, 25-35. doi.org/10.1007/978-981-10-5349-8_3

2. Narendra Naik Deshavath, Bijayeeni Singh Deo, Jyothika Boddu, Komali vykuntam, Vaibhav. V Goud, V. Venkata Dasu, Dilute acid pretreatment efficiency on various solid loadings and effect of different neutralizing agents on xylulosic ethanol production. In: Rita Kundu and Rajiv Narula (eds) Advances in Plant & Microbial Biotechnology Springer, Singapore 2019 (Accepted in Springer).

3. Narendra Naik Deshavath, Veeranki Venkata Dasu, Vaibhav V. Goud, Lignocellulosic feedstocks for the production of bioethanol: Availability, structure and composition. In: Mahendra Rai and Avinash P. Ingle (eds) Sustainable Bioenergy, Elsevier (Accepted).

National and International conferences

1. N.Naik, R.Timung, V.V.Goud, V.V.Dasu, (2014). Dilute acid pretreatment of bamboo for the production of fermentable sugars, National Conference on "Sustainable Development of Environmental Systems" (NCOSDOES- 2014), June 20-21, Indian Institute of Technology (IIT), Guwahati, India.

2. R.Timung, N.Naik, V.V.Goud, V.V.Dasu, (2014). Hydrolysis of sugarcane bagasse to produce reducing sugar for bioethanol production, Indo-US Conference on "Advanced Lignocellulosic Biofuels" (Indo-US CALB- 2014), November 10-11, CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad, India.

3. N. N. Deshavath, S. K. Sahoo, M. M. Panda, S. Mahanta, D. S. N. Goutham, V. V. Goud, V. V. Dasu, Annapurna jetty, The Cost Effective Stirred Tank Reactor for Cellulase Production from Alkaline Pretreated Agriculture Waste Biomass, Utilization and Management of Bioresources, Springer (In press). 6th International Conference on Solid Waste Management, Nov 24-26, 2016.

4. Narendra Naik Deshavath, Bijayeeni Singh Deo, Jyothika Boddu, Komali vykuntam, Vaibhav. V Goud, V. Venkata Dasu, Dilute acid pretreatment efficiency on various solid loadings andeffect of different neutralizing agents on xylulosic ethanol production (Accepted in Springer). Biospectrum- 2017, International conference on Biotechnology and Biological science, Aug 24-25, 2017.

भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

A1. Preparation of standard curve of sugars, organic acids, furans and ethanol

High performance liquid chromatography has been used for preparation of standard plot of cellobiose, glucose, xylose, arabinose, acetic acid, formic acid, levulinic acid, 5-hydroxymethyfurfural, furfural and ethanol to determine the unknown concentration of corresponding component. The stock solution about 10 g/L of each component prepared separately and made an appropriate dilution to attain concentration range between 0.2–1 g/L. The standard plots are drawn with respect to the known concentration of a compound *vs* peak area (derived from HPLC). The points were fitted with a linear regression model using Origin software. The unknown concentration of compounds in the test sample was determined based on a standard plot. The standard plot for glucose, xylose, arabinose, cellobiose, acetic acid, formic acid, levulinic acid 5-hydroxymethyfurfural, furfural and ethanol is depicted in Figure A1.1, A1.2, A1.3 and A1.4, A1.5, A1.6, A1.7, A1.8, A1.9 and A1.10, respectively. All the experiments such as compositional analysis of biomass, pretreatment, enzymatic hydrolysis and fermentation were performed in triplicate and the average values reported in the thesis.

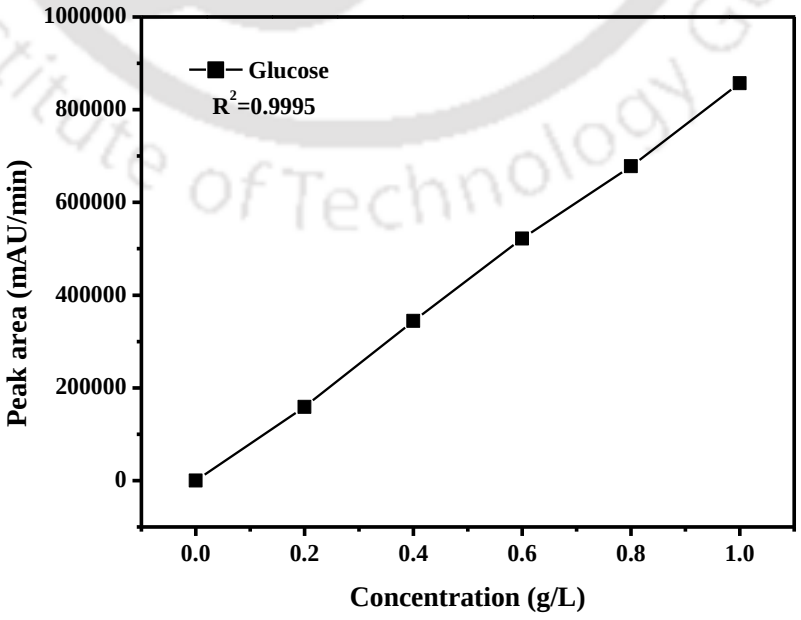

Figure A1.1: Standard curve drawn between known glucose (g/L) *vs* peak area.

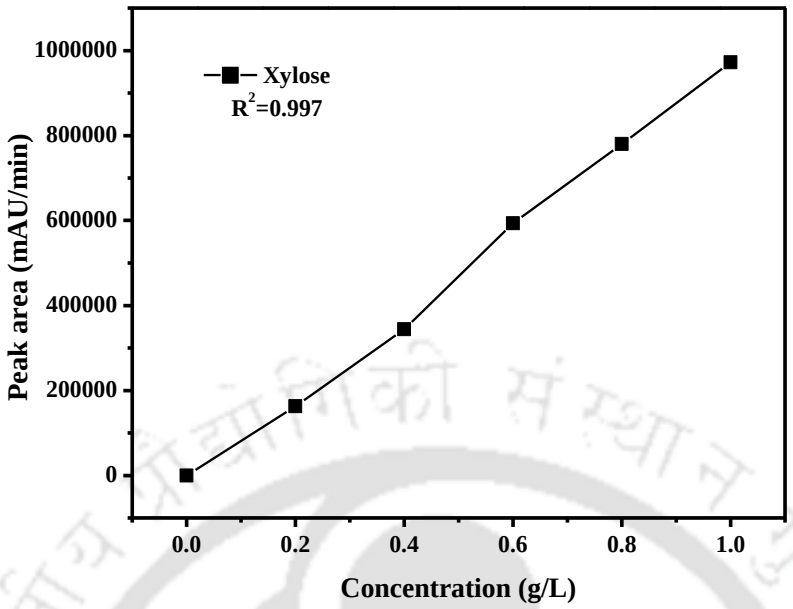

Figure A1.2: Standard curve drawn between known xylose (g/L) *vs* peak area.

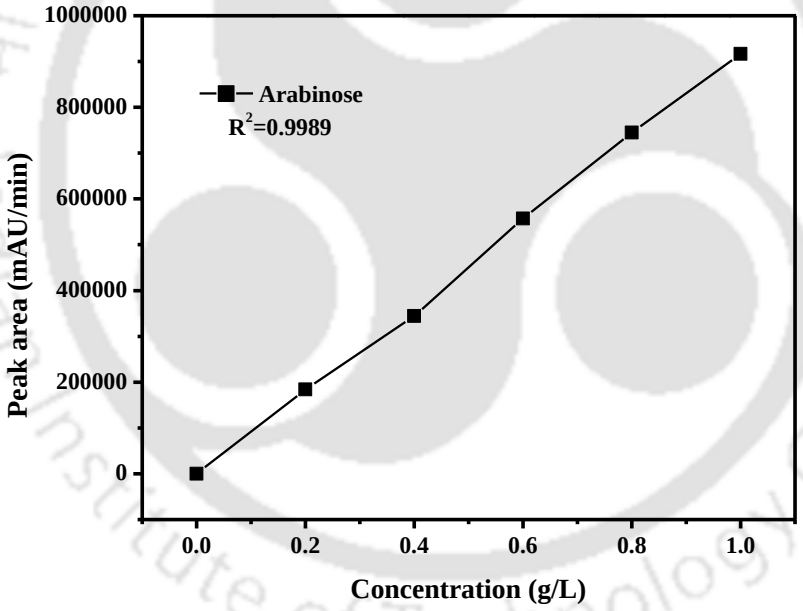

Figure A1.3: Standard curve drawn between known arabinose (g/L) *vs* peak area

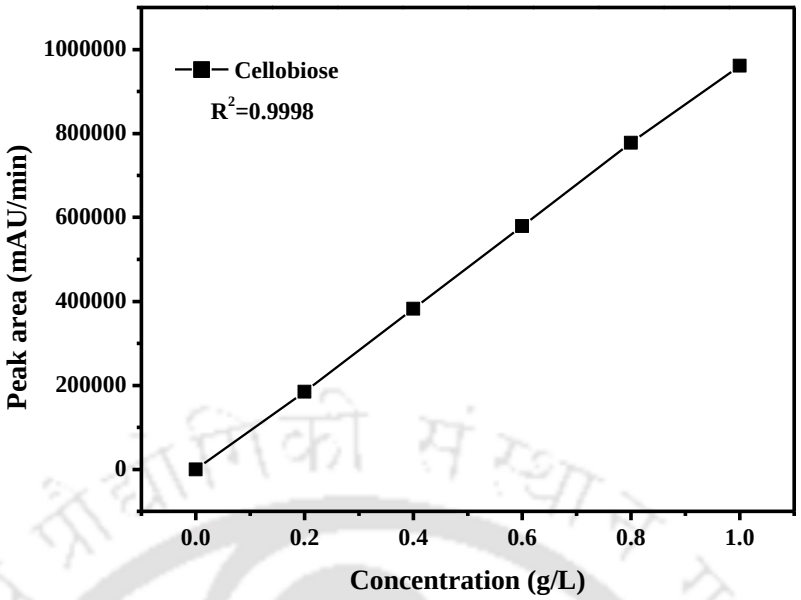

Figure A1.4: Standard curve drawn between known cellobiose (g/L) *vs* peak area.

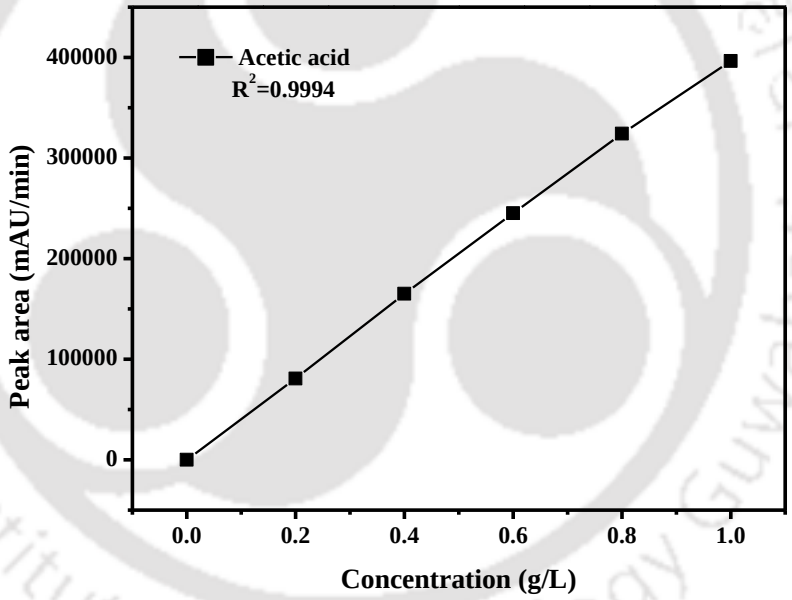

Figure A1.5: Standard curve drawn between known acetic acid (g/L) *vs* peak area.

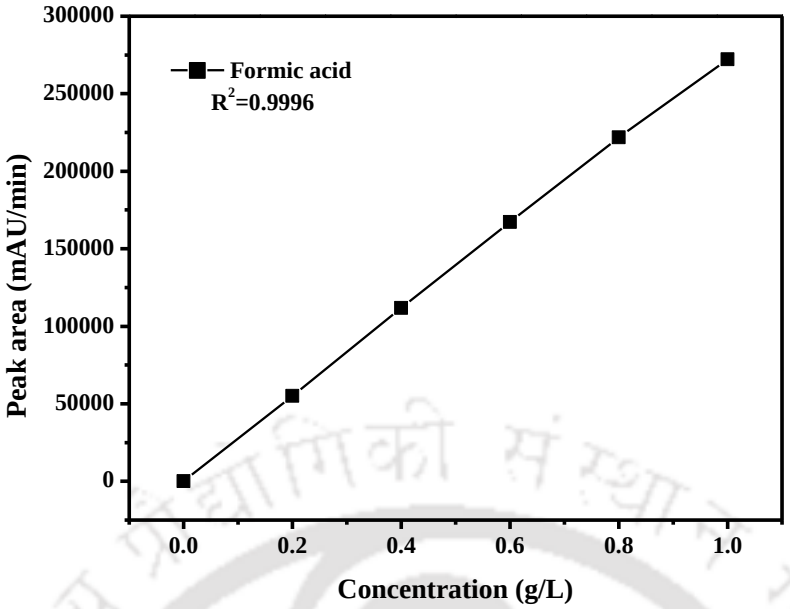

Figure A1.6: Standard curve drawn between known formic acid (g/L) *vs* peak area

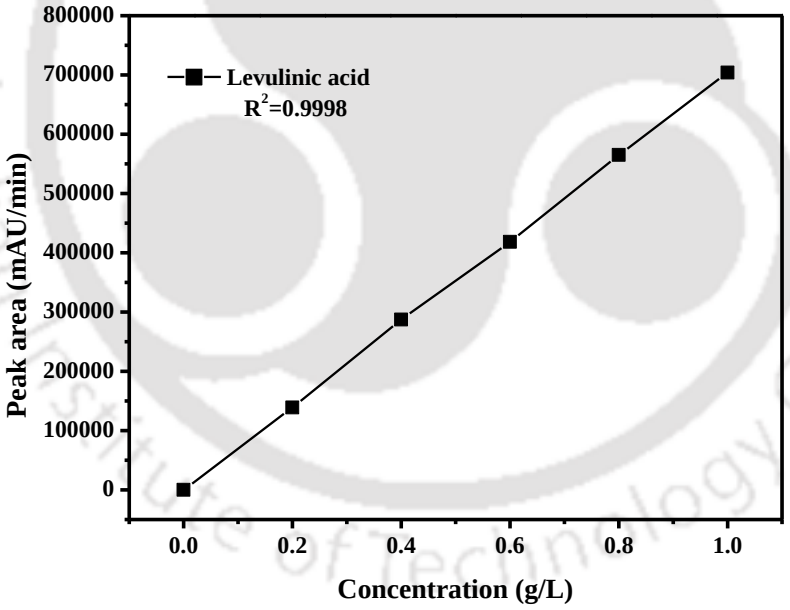

Figure A1.7: Standard curve drawn between known levulinic acid (g/L) *vs* peak area

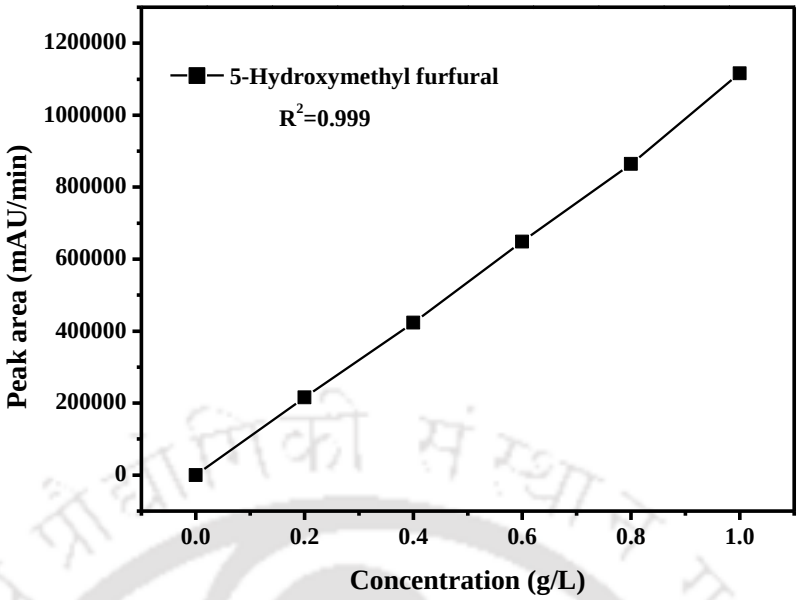

Figure A1.8: Standard curve drawn between known 5-HMF (g/L) *vs* peak area

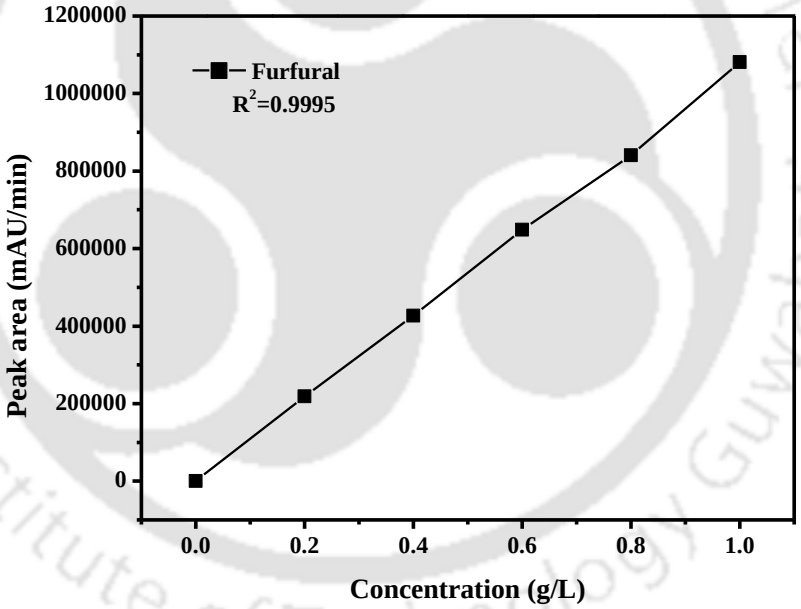

Figure A1.9: Standard curve drawn between known furfural (g/L) *vs* peak area

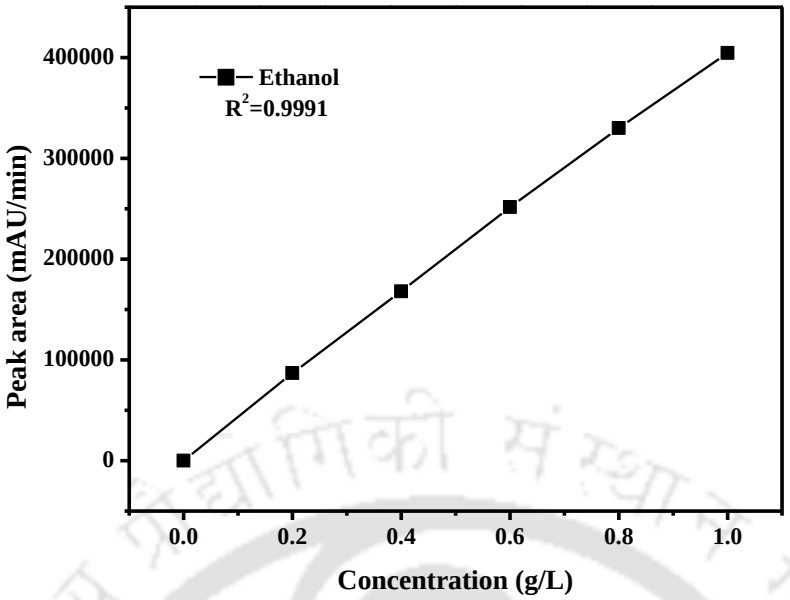

Figure A1.10: Standard curve drawn between known ethanol (g/L) *vs* peak area