STUDIES ON PRODUCTION OF ETHANOL FROM KANS GRASS BIOMASS USING SEQUENTIAL-CO-CULTURE SYSTEM

Ph.D. THESIS

by

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INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE – 247667 (INDIA)
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A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

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CANDIDATE'S DECLERATION

I hereby certify that the work which is being presented in the thesis entitled **"STUDIES ON PRODUCTION OF ETHANOL FROM KANS GRASS BIOMASS USING SEQUENTIAL-CO-CULTURE SYSTEM"**, in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July, 2009 to January, 2014 under the supervision of Dr. Sanjoy Ghosh, Associate Professor, Department of Biotechnology and Dr. Chandrajit Balomajumder, Associate Professor, Department of Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee, India.

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

(LALIT KUMAR SINGH)

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

(CHANDRAJIT BALOMAJUMDER) (SANJOY GHOSH)
Date: Supervisor Supervisor

The Ph.D. Viva-Voice Examination of **Mr. Lalit Kumar Singh**, Research Scholar, has been held on …….

Supervisors Chairman, SRC External Examiner

Head of the Department/Chairman, ODC

DEDICATED TO,

MY PARENTS,

WIFE AND

BELOVED SON AND DAUGHTER

ABSTRACT

The renewable and environment friendly source of energy is now mandatory for the sustainable development of a society. The energy demand is increasing worldwide and the natural fossil fuel resource is depleting. Therefore alternative fuel source is required to bridge the gap between the demand and supply. An unconventional source of energy molecule, ethanol is gaining importance as liquid transportation fuel. It is considered as clean fuel runs in zero carbon cycle. It is produced from sugar via fermentation. The most abundant and cheap source of sugars is lignocellulosic biomass. Ethanol has traditionally been produced from sugar cane and sugar beet juice or from various starch-containing raw materials like corn or wheat. But there is potential conflict between land use for food (and feed) production and energy feedstock production. The utilization of lignocellulosic biomass for ethanol production overcomes this conflict and is less expensive than the conventional agricultural feedstock. Lignocellulosic rawmaterials are grown in all parts of the world.

Among the various lignocellulosic raw materials perennial grasses (C4 plant) are promising because of high yields, low costs, and good suitability for low-quality land with almost no requirement of water supply for its growth, availability throughout the year and no net green house gas production. Kans grass (*Saccharum spontaneum*) possessing all these properties wasused as the lignocellulosic raw material for the present research work. Two main technical problems have been identified from the literature for lignocellulosic biomass to ethanol conversion process: (1) efficient release of soluble sugars from the polymeric structure of lignocellulosic biomass with minimum or no toxics generations and (2) utilization of maximum sugar potential for ethanol production. In the present study both of these problems have been tried to address successfully. Few preliminary experiments were conducted to obtain important parameters (mentioned in Section A of the Results and Discussion) followed by a novel fractionating hydrolysis process for fermentable sugar production (Section B) and finally a sequential-co-culture system was developed to utilize both xylose and glucose sugars in a single reactor (Section C).

Kans grass, a novel raw material was examined for compositional analysis for identification of its sugar potential and found as (% dry weight basis): cellulose 43.78±0.4; hemicellulose 24.22±0.5; acid insoluble lignin 23.45±0.3; acid soluble lignin 2.85±0.4 and ash 4.62±0.2. The total carbohydrate content (i.e cellulose and hemicellulose content) of Kans grass was found as

68 % on dry weight basis. This justified the selection of Kans grass as raw material for ethanol production.

Toinvestigate the effect of acid concentration, biomass loading and reaction time for releasingreducing sugars from Kans grass a 2^3 rotatable central composite design was adopted for designing the experiments and response surface methodology was used to optimize the process.The optimum acid concentration, biomass loading and reaction time were found to be 61.10%(w/w), 10.80% (w/v) and 45 min respectively. The batch processing of all the experiments werecarried out at normal boiling temperature of water under standard atmospheric pressure. Underthese conditions significantly high total reducing sugar yield (83.5 % w/w)was obtained ontotal carbohydrate content basis.

To utilize hemicellulose fraction of Kans grass single step dilute acid treatment was applied and its fermentation to ethanol was carried out by *Pichia stipitis*. 0.204 g/g total reducing sugar was obtained under experimental conditions in which 0.172 g/g was the pentose sugars. After conditioning fermentation was conducted and found that 74% of xylose wasconverted to ethanol with a yield of 0.429 g/g and productivity of 0.231 g/L h.The appropriate mathematical models for cell and ethanol production rate have been identified to explain theoretically the bioconversion of Kans grass hemicellulose acid hydrolysateto ethanol and validated statistically.

During hydrolysis of lignocellulosic biomass various degradation compounds are generated like furfural, hydroxymethyl furfural and phenolic compounds. These compounds are known to be inhibitory and affect the growth of microorganisms. The experiments were designed to identify the tolerable concentration of these compounds. It was found that only 9 % and 7 % reduction in specific growth rate of *P. stipitis* was occurred with 0.4 g/L furfural and 0.5 g/L vanillin concentration respectively. Similarly 8 % and 7 % reduction in specific growth rate of *Z. mobilis* was observed with 1 g/L HmF and 1.0 g/L vanillin concentration respectively. These concentrations of inhibitors were considered as tolerable to the respective microorganisms.

Further experiment was designed to obtain critical level of ethanol concentration for *P. stipitis*and*Z. mobilis*growth. Appropriate model was used to obtain maximum concentration of ethanol over which the growth of microorganisms ceased. It was found that 37 g/L and 104 g/L ethanol concentration was the maximum over which no growth of *P. stipitis* and *Z. mobilis* was observed respectively.

A novel single vessel multi-step fractionating hydrolysis process was developed in the laboratory.The main objective of the investigation was oriented towards the maximum soluble sugar extraction with minimum toxic compounds generation from KGB. The sequential addition of increased sulfuric acid concentration from 1 to 35 % v/v (total nine steps) along with direct steam insertion at 100°C (i.e. at atmospheric pressure) to the reaction system for 30 min each step was successfully used to extract 95.3% of the total reducing sugars (TRS) available in the KGB in the form of carbohydrate polymer. After analyzing the hydrolysate of the entire process steps two main sugar streams were generated as xylose rich fraction (XRF) and glucose rich fraction (GRF). The fermentation media prepared using these sugar streams without any detoxification process contained significantly low concentration of toxics due to moderate temperature and short exposure time. Fermentation of XRF and GRF media were conducted by *P. stipitis* and *Z. mobilis* respectively. The specific growth rate (μ), yield ($Y_{p/s}$) and specific productivity (q_p) of ethanol was found as 0.121 h^{-1}, 0.427 g/g and 0.731 g/g/h in XRF media and 0.173 h^{-1}, 0.443 g/g and 1.179 g/g/h respectively in synthetic media. Similarly μ, $Y_{p/s}$ and $q_p$was found as 0.243 h^{-1}, 0.476 g/g and 3.587 g/g/h in GRF and 0.338 h^{-1}, 0.494 g/g and 4.805g/g/h in synthetic media respectively.

Sequential-co-culture technique was investigated in this experiment for the production of ethanol using XRF and GRF as generated from the aforementioned investigation. The consortium of *P. stipitis* and *Z. mobilis* was used to develop a suitable sequential-co-culture system. The *P. stipitis* cells and respective fermentation media (XRF)were fed to the fermentation vessel, after the set fermentation time *Z. mobilis* cells and respective media (GRF) were fed to the same vessel. Different strategies have been followed and experiments were conductedinitially at flask level. The selected strategy was then applied at bioreactor level using both synthetic fermentationmedia and Kans grass hydrolysate media to compare the kinetic parameters. The sequentialaddition of cultures with their respective media and imposed process conditions, showed better utilizationof total sugars added (>95%). Microaerobic condition for *P. stipitis* and strictly anaerobic conditionfor*Z.mobilis* fermentation were found significant. The average ethanol yield ($Y_{p/s}$) and overall volumetricproductivity (r_{po}) were found as 0.453 g/g and 1.580 g/L/h respectively for Kans grass hydrolysate mediaand 0.474 g/g and 2.901 g/L/h respectively for synthetic fermentation media.

The significance of the present study revealed that the novel process of fractionating hydrolysis of KGB generated toxics in very low quantity and no detoxification process was involved before fermentation. Further the developed sequential-co-culture system of *P. stipitis* and *Z.*

*mobilis*was found suitable and successfully used for bioconversion of xylose and glucose in a single bioreactor system.

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Lalit

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ABBREVIATIONS

ADF:	Acid detergent fiber
ADS:	Acid detergent solution
AFEX:	Ammonia fiber explosion
AIL:	Acid insoluble lignin
ANOVA:	analysis of variance
AR:	Analytical reagent
ASL:	Acid soluble lignin
ATP:	Adenosine triphosphate
CBP:	Consolidated bioprocessing
CCD:	Central composite design
d.w.:	Distilled water
DM:	Dried matter
DMC:	Direct microbial conversion
DNS:	Di-nitro salicylic acid
DP:	Degree of polymerization
FC:	Folin-Ciocalteau
GAE:	Gallic acid equivalents
GC:	Gas chromatography
GHG:	Greenhouse gases
GMO:	Genetically modified organism
GRAS:	Generally recognize as safe
GRF:	Glucose rich fraction
GYP:	Glucose-yeast extract-peptone
HmF:	Hydroxymethyl furfural
KGB:	Kans grass biomass
LAP:	Laboratory analytical procedures
NDF:	Neutral detergent fiber
NDS:	Neutral detergent solution
OD:	Optical density
RMSD:	Root mean square deviation
RSM:	Response surface methodology
SD:	Standard deviation
SEM:	Scanning electron microscopy

SHF:	Separate hydrolysis and fermentation
SSCF:	Simultaneous saccharification and co-fermentation
SSF:	Simultaneous saccharification and fermentation
TCC:	Total carbohydrate content
TRS:	Total reducing sugars
XRF:	Xylose rich fraction
XYP:	Xylose-yeast extract-peptone

CHAPTER 1 INTRODUCTION

1.1 Background

The steadily increased crude oil consumption and depleting oil reserves creates increased concern for the security of the oil supply worldwide. The growing world population, more industrialization, monopolistic nature of producer and geopolitical conflicts in the Middle East with finite supply of petroleum are the main reasons for unstable trading prices [Kurian and Kishore, 2008; Saxena et al., 2009; Singh et al., 2012]. Today the transportation sector is sharing about 60% of the total petroleum based oil consumption worldwide. Further it contributes 70% carbon monoxide and 19% carbon dioxide to the environment globally [Goldemberg, 2008; Balat, 2010]. The negative impact of use of fossil fuels on the environment, particularly greenhouse gas emissions (GHGs), has put pressure on society to search for renewable fuel alternatives. Almost all countries are working on the development and expansion of alternative energy sources (non conventional) which produces no GHGs (solar energy, wind power, tidal power) or runs on zero carbon cycle (ethanol, biodiesel, hydrogen, natural gas) [Singh et al 2012b]. Particularly due to technological developments and cost reductions of the renewable sources of energy especially solar, hydro, wind and biomass energy are gaining momentum most recently [Kataria et al., 2009]. Johansson et al. (1992) reported that by the year 2025, renewable energy sources could be a major contributor to direct fuel use (nearly 30%) and global electricity supplies (60%). Most of the oil fields are geographically located in politically sensitive areas of the world. Oil importing countries are also affected by frequently fluctuating prices of the commodity at the international level. The major benefits of biofuels include sustainability, greenhouse gas reduction, biodegradability, carbon sequestration, higher combustion efficiency, international competitiveness, supply reliability. The developing country like India, use of biofuels may increase number of jobs in rural areas, agricultural development, reduce the dependency on imported petroleum.

The most common renewable fuel today is ethanol and is by far from the other renewable fuels used for transportation. It is an important renewable liquid fuel for motor vehicle [Lewis, 1996; Farrell et al., 2006]. The use of ethanol and ethanol/gasoline blend as fuel is very well recognized as an alternative to petroleum based motor fuel. The mixture of ethanol (10 %) and gasoline (90 %) is called gasohol or E10 [Balat 2010]. Ethanol is an oxygenated fuel contains 35 % oxygen. It is also an octane enhancing additive and removes free water from fuel pipe

lines to prevent plugging [Lang et al., 2001b]. Use of ethanol in fuel reduces particulate and NO_x emission on combustion.

In India, Ethanol is procured mainly from molasses, a by-product of sugar production. The ethanol demand in India is driven mainly by the industrial segment. Currently, 5% blending of ethanol is effective but it is not mandatory and the oil marketing companies decide about the blending considering the logistic costs and the price of ethanol. Also, the blending is effective only in nine states and six union territories. The current production of ethanol in India is pegged at 2.2 billion liters of which fuel grade ethanol stands at approx 200 million liters only. Government is mulling over introducing the 10% blending of ethanol into petrol guideline by October 2008. At 5% blending, the ethanol requirement stands at 560 million liters and a further 10% blending will require a fuel ethanol production of approx 1100 million liters. So the demand for fuel grade ethanol is very high.

Ethanol is produced from grains such as wheat, barley or corn, or from sugar cane as well as sugar beet. However it is feared that there will be a limited supply of such starch and sugar raw materials in the future. Besides this the imminent conflict in the use of agricultural land for the purpose of food and energy production is unavoidable. Hence, future large-scale supply of ethanol will most certainly be based on its production from lignocellulosic biomass as source raw material [Sims et al., 2010]. Global annual production of biomass is 1×10^{11} tons sequestering 2×10^{21}J (annual petroleum production amounts 2×10^{20} J, whereas technically recoverable endowment of conventional crude oil is 2×10^{22} J). Thus energy stored in Earth's plants in one decade is equivalent to the energy stored as conventional crude oil [Smeets et al., 2007; Raines and Binder, 2011]. Lignocellulosic materials are geographically more evenly distributed than the fossil fuels, thus the sources of energy will, to a larger extent, are domestic and provide security of supply. Various lignocellulosic biomaterials have attracted the most interest of research for production of ethanol [Sun and Cheng, 2002; Rass-Hansen et al., 2007; Zhang et al., 2010; Flamos et al. 2011]. These include corn stover, wheat straw, sugar bagasse, rice straw, rice hull, corn cob, oat hull, corn fiber, woodchip, and cotton stalk [Eklund and Zacchi, 1995; Esteghlalian et al., 1997; Moniruzzaman et al., 1997; Chang et al., 2001; Saha, 2003; Saha et al., 2005; Ballesteros et al., 2006; Jeffries, 2006; Sun and Chen, 2007; Balat, 2008; Kumar et al., 2009a]. The lignocellulosic raw material is less expensive than conventional agricultural feedstock and can be produced with lower input of fertilizers, pesticides, and energy. Among the lignocellulosic biomaterials perennial crops (e.g., grasses)

are found promising because of high yields, low costs, ability to grow in marginal lands with almost no requirement of water supply, wide availability throughout the year.

The production of ethanol from lignocellulosic biomass (composed of carbohydrate polymers like cellulose and hemicellulose, and relatively much lesser quantity of lignin) involves three major steps: (1) hydrolysis, (2) fermentation, and (3) distillation [Demirbas 2004]. Cellulose and hemicellulose fractions of lignocellulose biomass typically comprise two third of the cell mass dry weight. These fractions are polysaccharides and source of sugars which is converted to ethanol. The hydrolysis process step is required to liberate the sugar moieties from their polymeric matrix. Cellulose is a mono-polymer of glucose (C6) and hemicellulose is heteropolymer of pentose and hexose sugars with xylose (C5) as dominating sugar. Three major hydrolysis processes are identified for release of sugars and suitable for ethanol production: dilute acid, concentrated acid, and enzymatic hydrolysis [Broder et al., 1995]. Hemicellulose can be easily hydrolyzed by dilute acids under moderate conditions whereas; more extreme conditions are required for cellulose hydrolysis. The concentrated acid hydrolysis is single step whereas all other methods involve mainly two steps, first step is called pretreatment and second step is the main cellulose hydrolysis process. The efficient release of sugars is considered as the major technical barrier for ethanol conversion process. The pretreatment process is considered as the most expensive step in lignocellulosic to ethanol production process [Zhang et al., 2009].

A number of pretreatment processes have been reported and are under intensive research. A suitable pretreatment process involves (1) disrupting hydrogen bonds in crystalline cellulose, (2) breaking down cross-linked matrix of hemicelluloses and lignin, and finally, (3) raising the porosity and surface area of cellulose for subsequent enzymatic hydrolysis [Li et al., 2010]. The pretreatment processes are broadly categorized as (1) physical, (2) physico-chemical and chemical, and (3) biological pretreatment. The physical pretreatment including grinding, milling, microwave and irradiation [Kumar et al., 2009b; Sun and Cheng, 2002; Leustean, 2009], physic-chemical and chemical pretreatment including alkali, acid, organosolv, ozonolysis, ionic liquid, steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation and CO_2 explosion [Bacovsky et al., 2010; Taherzadeh and Karimi, 2008; Kim and Hong, 2001; Alizadeh et al., 2005; Tomas-Pejo et al., 2008; Garcia-Cubero et al., 2009; Wang and Cheng, 2009; Yang et al., 2008; Silverstein, 2008] and biological pretreatment [Lee et al., 2007].

The existing pretreatment/hydrolysis technologies suffer from low sugar yields, and/or severe reaction conditions, and/or narrow substrate applicability and high capital investment etc. The physical pretreatment processes are highly energy demanding and most of them are unable to remove lignin [Eggeman and Elander, 2005; Zhang et al., 2007]. Further the pretreatment process is followed by enzymatic hydrolysis which is quite slow and cost of enzymes are still high [Kaylen et al., 2000].

After pretreatment/hydrolysis, the liquid hydrolysate contained varying amounts of soluble sugars, both pentose and hexose, and a broad range of substances either derived from raw material or resulting as reaction products from sugar and lignin degradation like furfural, hydroxymetyl furfural and phenolics compounds. Many of these substances may have an inhibitory effect on the microorganisms in subsequent fermentation steps [Nigam 2002]. Thus an additional process step is required known as conditioning or detoxification before actual fermentation is carried out and adds cost to the overall process of ethanol production.

Another main technical constraint is the fermentation of pentose and hexose sugars present in the hydrolysate. It is a prerequisite for the economical production of ethanol that all the sugars must be fermented to ethanol [Penttila et al., 2010; Sims et al., 2010]. No native microorganism is known till date for efficient conversion of both C5 and C6 sugars. The widely accepted ethanol-producing GRAS organisms *Saccharomyces cerevisiae* and *Zymomonas mobilis* are not able to ferment C5 sugars like xylose. Using these organisms to ferment the mixture of sugars, C5 sugars remained unutilized, thus reducing the overall conversion efficiency. Thus a good xylose fermenting microorganism like *Pichia stipitis* is used. For mixed sugar fermentation a co-culture system is recommended [Chen, 2011]. It is further a challenge to choose suitable microorganisms for stable co-culture system development.

There are two types of processes that can be used to convert cellulose and hemicellulose to ethanol (1) separate hydrolysis and fermentation (SHF) and (2) simultaneous saccharification and fermentation (SSF). The SSF may extended to simultaneous saccharification and cofermentation (SSCF) when hemicellulose portion is also hydrolyzed simultaneously with cellulose and fermentation of glucose and xylose as done either by co-culture or by engineered microbe. SSF is found better system than SHF as it overcomes the problem of cellulase enzyme inhibition by released sugars but facing challenge for difference in optimal conditions for hydrolysis and fermentation [Krishna et al., 2001]. For production of ethanol fermentation is carried out mainly in three modes batch, fed-batch and continuous. The choice of fermentation

technique is mainly dependent on the kinetic properties of microorganism and type or composition of lignocellulose hydrolysate.

A perennial grass *Saccharum spontaneum* (Kans grass) used in the present study is native to South Asia and occurs throughout India along the sides of the river was selected as the lignocellulosic raw material for the present work. Kans grass is a self-seeding, resistant to many diseases and pests, tolerant to poor soils, flooding, and drought; improves soil quality and prevents erosion due to its type of root system. It uses less water per gram of biomass produced than other plants [Singh et al. 2012; Chandel et al. 2009]. It is a C4 plant fixes more carbon dioxide than its C3 counterpart. These characteristics make Kans grass biomass (KGB) a novel and potential lignocellulosic candidate for production of fuel ethanol via fermentation.

1.2 Outline

The thesis consists of six chapters. The first two chapters describe the motivation and background of this research. Descriptions of ethanol as a fuel as well as its environmental impact are given in Chapter 2. The pretreatment and hydrolysis of lignocellulosic biomass, inhibitors and inhibition mechanisms of byproducts released during hydrolysis is reviewed. In addition, conditioning processes and fermentation strategies for ethanol production also reviewed in this chapter. Chapter 3 describes materials and methods used in the study including raw material, hydrolysis, microorganisms and fermentation. Chapter 4 consists of results and discussion, the work is presented in three sections A, B and C. Important preliminary findings are given in section A, a novel hydrolysis process is discussed in section B and a suitable fermentation system is presented in section C. Finally, conclusions and suggestions for future studies are presented in Chapter 5 followed by appendixes and list of publications.

CHAPTER 2 LITERATURE REVIEW

2.1 Ethanol

Ethanol or ethyl alcohol, a 2-carbon alcohol has been used by humans since prehistory as the intoxicating ingredient in alcoholic beverages. Ethanol was first prepared synthetically in 1826 by Henry Hennel in Britain and S. G. Serullas in France. Michael Faraday prepared ethanol by acid-catalyzed hydration of ethylene in 1828, a process similar to that used for industrial ethanol synthesis today. With the advent of distillation, which appears to have been discovered first in ancient Arabia, people were able to obtain beverages with higher ethanol content.

Ethanol, CH_3CH_2OH, is a versatile solvent. Its empirical formula is C_2H_6O. It is a volatile, colourless liquid with a slight odour. It burns with a smokeless blue flame. Due to the presence of hydroxyl group and a non-polar end, ethanol is miscible in water, many other organic solvents and also dissolve non-polar substances. It is a monohydric primary alcohol and its boiling point is 78.5°C. It is one of the most exotic oxygen containing organic chemicals because of its unique combination of properties as a solvent, a germicide, a beverage, antifreeze, a fuel, a depressant, and especially because of its versatility as a chemical intermediate for other organic chemicals.

Today, ethanol is the most dominant biofuel and its global production showed an upward trend over the last 25 years with a sharp increase from 2000. Worldwide production capacity in 2005 and 2006 were about 45 and 49 billion liters per year, respectively and total output in 2015 is forecast to reach over 115 billion liters [Licht, 2006].The ethanol produced is mostly used as fuels (92%); industrial solvents and chemicals (4%), and beverages (4%) [Logsdon, 2006]. Ethanol used in the form of gasohol (a mixture of ethanol and gasoline) is considered as an alternative fuel for vehicles and its use as fuel is increasing day by day over the globe. The major advantage of ethanol is its renewability. The petroleum or fossil fuels took thousands of years to generate beneath the earth's surface, whereas ethanol produced from plant biomass that grows only in few months to year. Also we can grow plants according to our need but generation of fossil fuels depends on the natural decaying of the living matters under huge pressure and very long time. The main reasons for high demand of ethanol as fuel includes: the decrease of dependence on foreign crude oil and thus significantly reducing the economy's vulnerability to oil price shocks (very important factor for the country like India), increase the job opportunities in rural areas, decrease in the carbon dioxide buildup in the atmosphere,

reducing air pollution etc. [Demirbas, 2005]. Ethanol contains 35% oxygen and considered as an oxygenated fuel. On combustion it produces carbon dioxide and if derived from a renewable source like biomass no net carbon dioxide buildup takes place in the atmosphere. The additional advantage of ethanol used as fuel is its octane enhancing property and free water removal property which can otherwise plug fuel lines in cold climates [Lang et al., 2001b].

The cost and lesser availability of petroleum and natural gas has generated research interest in bioconversion processes that makes use of renewable biomass resources for the production of fuels and chemical feed stocks. Fermentations under controlled oxygen environment have formed the basis for such microbial chemical and fuel production. By catabolizing organic matter, a variety of reduced organic compounds such as ethanol, methane, acetic acid, lactic acid and others have been produced in lieu of complete aerobic combustion of such organic matters to CO_2 and H_2O[Zeikus et al., 1983].

2.2 Substrates for ethanol production

Ethanol produced from a petrochemical ethylene by acid catalyzed hydration, also called as synthetic ethanol and mainly used as an industrial feedstock or solvent. Approximately 7-9% ethanol is produced via this chemical route, consequently 91-93% ethanol is produced via biochemical route (fermentation) using any plant biomass and thus referred to as bioethanol. Currently the main resources for ethanol production are sugar and starch based materials such as sugarcane and grains [Turhan et al., 2010]. Considering the growing demand for human food and priority for starving human society could make these raw materials potentially less competitive and perhaps expensive feedstock in the near future comparative to lignocellulosic materials [Taherzadeh and Karimi, 2007]. The generalized sources of sugars for bioethanol production are given in figure 2.1 [Zaldivar et al., 2001].

Table 2.1 Comparison of properties of ethanol, ETBE, isooctane and gasoline [Whyman and Hinman, 1990]

Property	**Ethanol**	**ETBE**	**Isooctane**	**Unleaded regular gasoline**
Formula	C_2H_5OH	$(CH_3)_3COC_2H_5$	C_8H_{18}	C_4-C_{12}
Molecular weight	46.07	102.18	114	--
Specific Gravity; 15°C	0.79	0.75	0.69	0.72-0.78
Air/Fuel stoichiometry (mol)	14.32	42.9	59.68	57.28
Lower heating value (kJ/kg)	26860	362800	44380	41800-44000
Energy (kJ/L of standard stoichiometric mixture)	3.53	3.61	3.55	--
Octane number				
RON	106	118	100	91-93
MON	89	102	100	82-84
(RON+MON)/2	98	110	100	88
Latent heat of vaporization (kJ/kg; 15°C)	840	--	328	~335
Reid vapor pressure, kPa				
Pure component	16	30	--	--
Blending	83-186	21-34	--	55-103
Water solubility, %fuel				
Fuel in water	100	2.0	negligible	negligible
Water in fuel	100	0.6	negligible	negligible

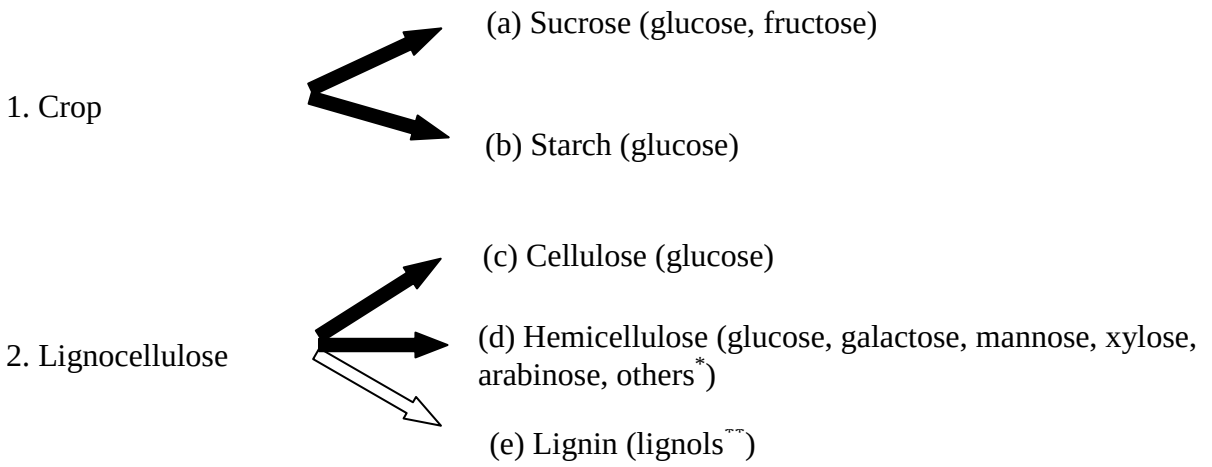


Figure 2.1 Sources of sugars for bioethanol production: (1) Crops and (2) Lignocellulose biomass; (a) to (e) represents the polymers presents in the biomasses and their monomers are mentioned in the brackets respectively; dark coloured arrows represents the sugar moieties in the biomasses that can be fermented to the ethanol.

*L-rhamnose, L-fructose, uronic acid; **coniferyl, sinapyl, vanillyl.

The most important feedstock utilized in tropical and sub-tropical countries for producing ethanol is sugar cane. It is used either in the form of cane juice or cane molasses. Beet molasses is the first choice in European countries and recognized as the most utilized sucrose containing feedstock. Besides these energy crops, sweet sorghum is also a potential raw material for fuel ethanol production. Starch based feedstock like corn and wheat are the most popular in North America and Europe for ethanol production. In tropical countries, other starchy crops as tubers (e.g. cassava) are used for commercial production of fuel ethanol.

The conversion of sucrose into ethanol is easier compared to starchy materials and lignocellulosic biomass because previous hydrolysis of the feedstock is not required since this disaccharide can be broken down by the yeast cells; in addition, the conditioning of the cane juice or molasses favors the hydrolysis of sucrose [Cardona and Sanchez, 2007].

While sugar cane and corn are dominant sources for ethanol production at present, ethanol production from lignocellulosic material has not been proven and is still under development. Even though ethanol has not been largely produced from lignocellulosic material due to its high cost, it is predicted that the use of this feedstock will increase dramatically in the near future and became the main resource for ethanol production, occupying as much as two thirds of total ethanol production in 2050 [Rosillo-Calle and Walter, 2006].

2.3 Lignocellulosic biomass

Currently, a large amount of studies regarding the utilizationof lignocellulosic biomass as a feedstock for producingfuel ethanol is being carried out worldwide. Forcountries where the cultivation of energy crops is difficult,lignocellulosic materials are an attractive option for theproduction of biofuels [Cardona and Sanchez, 2007].

2.3.1 Availability and importance

The annual production of cellulose on earth is estimated to be about 1.5 trillion tons making it an inexhaustible resource for biofuel. The total agro-residue production in India is estimated to be 600 million tons in the year of 2001 out of which 70% is not utilized effectively. Theoretically, these agro-residues are equivalent to 150 billion liters of fuel ethanol assuming stoichiometric conversion. However, unfortunately, research in India on lignocelluloses based ethanol production is in its early phase and for commercial production the suitable technology is still awaiting [Kurian and Kishore, 2008].

2.3.2 Structure and composition

Lignocellulosic feedstocks are composed of cellulose, hemicellulose, and lignin. The chemical association of these polymers is shown in figure 2.2. Cellulose is a homopolymer of glucose, while hemicellulose is heteropolymer composed of the hexose sugars e.g. glucose, mannose, and galactose, and the pentose sugars e.g. xylose and arabinose [Singh et al., 2011a]. The relative proportion of the individual sugars depends on the raw material e.g. the hemicellulose fraction of hardwoods and agricultural raw materials is rich in pentose sugars, while softwood hemicellulose only contains minor fractions of the pentose sugar D-xylose [Hayn et al., 1993]. The cellulose, hemicellulose, and lignin contents in common agricultural residues and wastes are given in table 2.2 [Sun and Cheng, 2002].

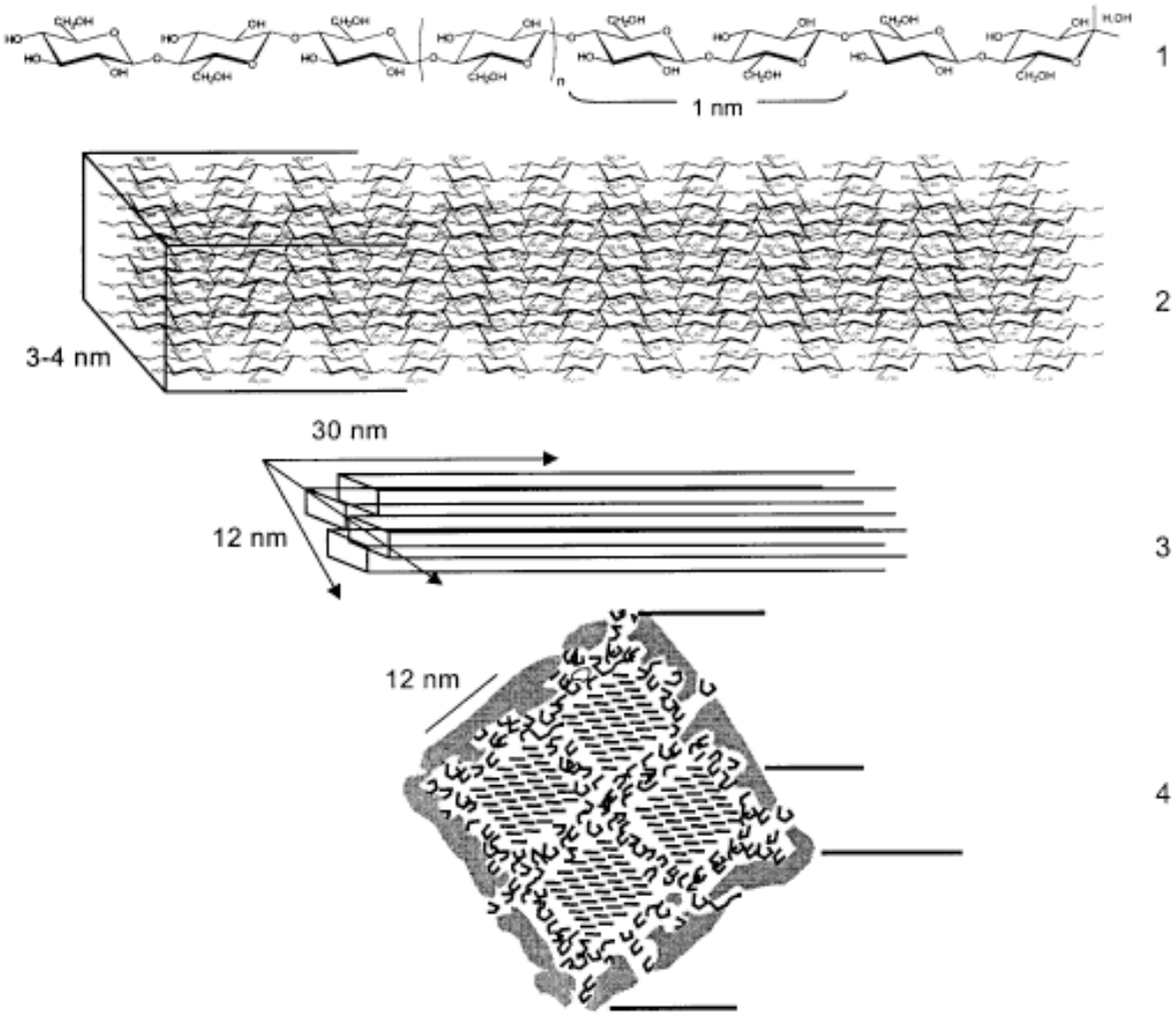


Figure 2.2 Chemical association in lignocellulosic material: (1) the cellulose backbone with length of its basic unit, cellobiose; (2) elementary fibril containing cellulose chains; (3) crystalline cellulose; (4) cross section of microfibril, showing strands of cellulose molecules embedded in a matrix of hemicellulose and lignin. [source: Fengel and Wegener, 1989].

Table 2.2 The contents of cellulose, hemicellulose, and lignin in common lignocellulosic materials. [source: Sun and Cheng, 2002; Jorgensen et al., 2007; Singh et al., 2011a]

Lignocellulosic materials	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hardwoods stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	20
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	85-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0
Water-hyacinth	18.4	49.2	3.55

2.3.2.1 Cellulose

Cellulose is a linear chain polysaccharide with multiple units of glucose. The chemical formula of cellulose is $(C_6H_{10}O_5)n$, contains only carbon, hydrogen and oxygen. The number of repeating sugar units is referred as degree of polymerization (DP) and may be calculated as the ratio of molecular weight of cellulose to the molecular weight of glucose unit. The softwood cellulose and paper manufacturing fibers have average DP values of 3500 and 600-1500 respectively [Sjostrom, 1993]. Cellulose is the main structural constituent of primary cell wall of green plants. The structure of cellulose in the form of recurring units of cellobiose (composed of two consecutive anhydride units of glucose) is shown in the figure 2.3. The straight chain of cellulose is formed by β-1,4-glycosidic bonds which provides cellulose a fibrous, rigid and water insoluble properties. The orientation of glucose residues in this conformation results hydrogen bonding between hydroxyl group and oxygen. This bonding provides high tensile strength to the molecule and further hydrogen bonding between cellulose molecules, build microfibrils (a bundle of 40-70 parallel chains). These microfibrils are arranged in such a manner that provides highly ordered crystalline regions in cellulose molecule. A free aldehyde group is present at the C-1 position (reducing end) and a free hydroxyl group is present at the C-4 position (non-reducing end)[website 1]. Besides the crystalline regions some disordered portions called amorphous regions are also present in the cellulose molecule which is more susceptible to hydrolysis than the crystalline regions. The microfibrils are linked by lignin and hemicellulose moieties [Voet and Voet, 1995].

2.3.2.2 Hemicellulose

Hemicellulose fraction of lignocellulosic material is heterogeneous in nature. It is a complex polymer of pentoses (β-D-xylose, α-L-arabinose) and hexoses (β-D-mannose, β-D-glucose, α-D-galactose). Other sugar moieties like α-L-rhamnose and α-L-fucose may also be present in this polymer matrix. Plant biomass contain about 15-35% of hemicellulose fraction in which the most important and abundant part is xylan. The secondary cell wall of hardwood and herbaceous plants may contain 20-30% xylan fraction [Girio, 2010; Wyman, 1999].Partial chemical structure of hardwood hemicellulose (O-acetyl-4-O-methylglucuronoxylan) is shown in figure 2.4.Unlike cellulose, hemicellulose is a branched polymer of relatively shorter chain length with random, amorphous structure. The hydrolysis of hemicelluloses by mineral acids to their soluble sugars (i.e. monomers like xylose, mannose, glucose, galactose etc.) is relatively easier as compared to the cellulose [Morohoshi, 1991; Sjostrom, 1993].

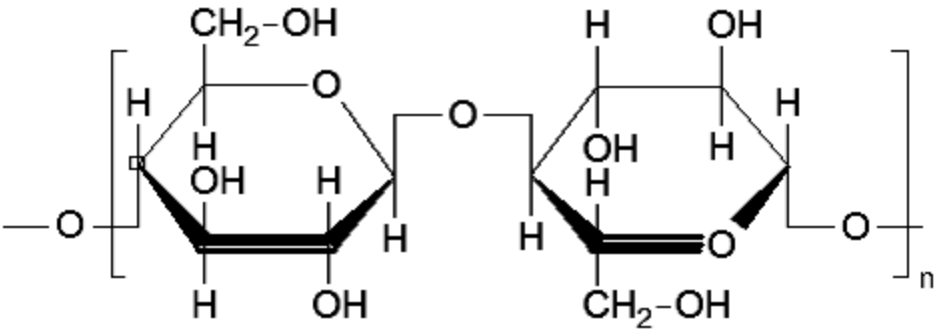


Figure 2.3 Structure of cellulose as recurring units of cellobiose. [source: Voet and Voet, 1995].

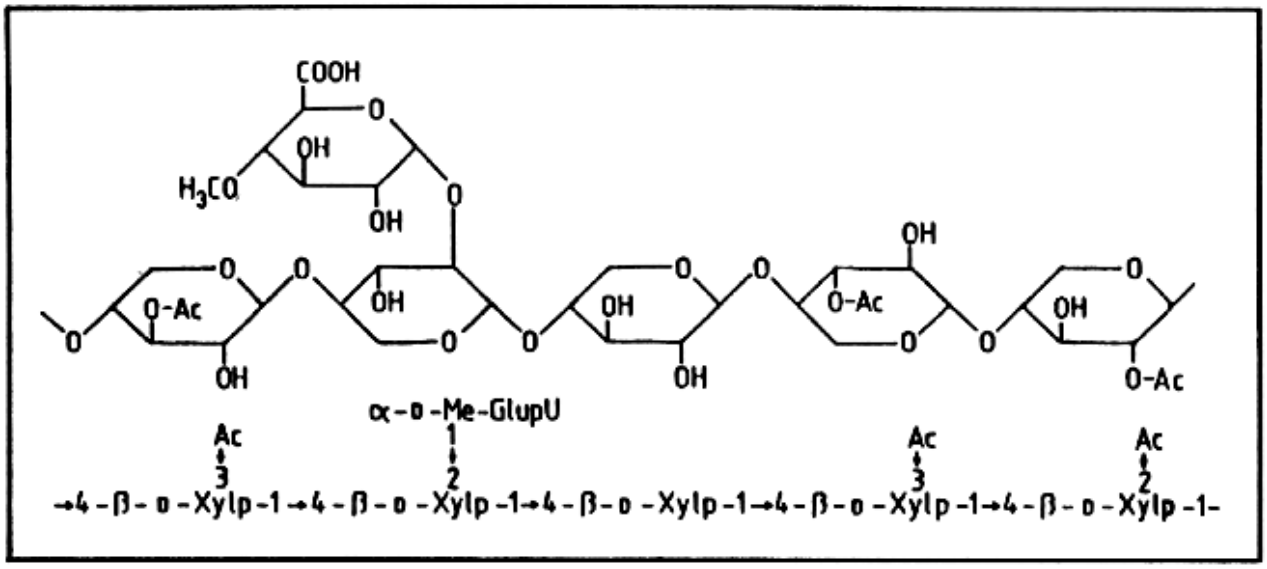
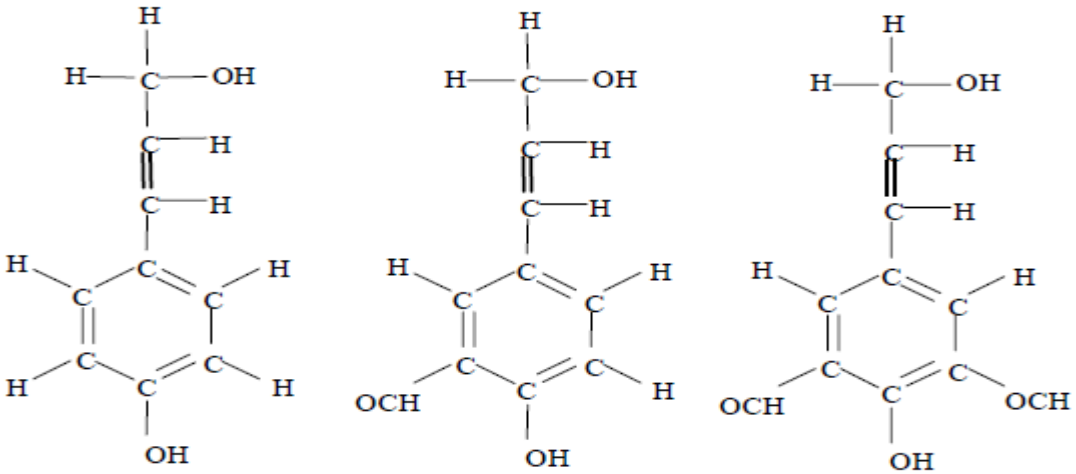


Figure 2.4 Partial chemical structure of hemicellulose of hardwood (O-acetyl-4-O-methylglucuronoxylan). [source: Fengel and Wegener, 1984].

2.3.2.3 Lignin

The other main constituent of cell wall of plant biomass is lignin. It is interspersed between cellulose and hemicellulose, cements fibers together which provides structural strength and defense against physical, chemical and microbial attacks to the plants. Lignin is a three-dimensional, stable, high molecular weight compound consisting thousands of phenyl-propane units. The complex and random nature of lignin is due to the unsystematic polymerization of phenyl-propane units (p-coumaryl, coniferyl and sinapyl alcohols). The quantity of these phenyl-propane units known as lignin precursors, shown in figure 2.5, depends on its origin e.g. coniferyl alcohol is the primary component in softwood lignin whereas; coniferyl alcohol and sinapyl alcohol are found in balanced quantity in hardwood. In grasses, p-coumaryl alcohol is the exclusive constituent [Ralph et al., 2004]. Thus lignin may be classified in three main categories, softwood, hardwood and grass lignin based on chemical structure of the precursor [Takayoshi, 2006].

Lignin is hydrophobic in nature but hydroxyl and methoxyl groups present in precursor of lignin interact with cellulose microfibrils which can hold significant quantity of water. Lignin is highly resistant to chemical and enzymatic degradation provides stiffness to the plants. However, even overcoming to this natural defense of lignin by its degradation, the generated aromatic compounds are known to highly toxic to fermentation organisms involved in the bioconversion of cellulose or hemicellulose to ethanol. A typical structure of lignin is shown in figure 2.6 [Website 2]. The main source of lignin is the black liquor of pulp and paper industry. Majority of lignin is used as fuel and burned in the recovery boilers. It may also used as a source of specialty chemicals to be used in detergent, cosmetic or biosorbent based industries [Gardfeldt, 2008].



(a) (b) (c)

Figure 2.5 Phenyl-propane units as precursors of lignin (a) p-coumaryl, (b) coniferyl and (c) sinapyl alcohols.

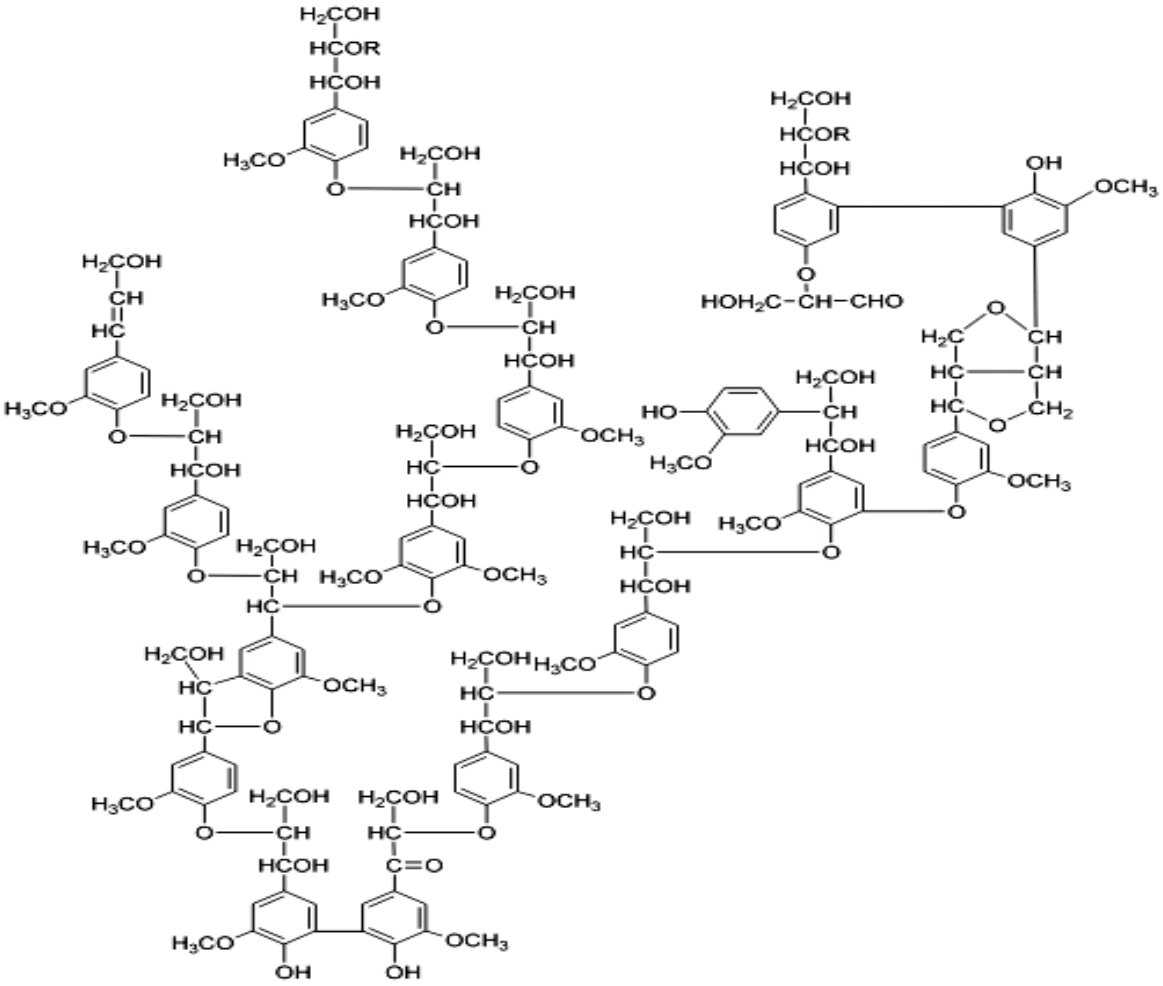


Figure 2.6 A typical lignin structure showing different linkages between precursors. [source: Website 2]

2.3.3 Categories

The lignocellulosic materials may be categorized in four major groups based on the source from nonfood crops as shown in figure 2.7[Chang et al., 2001; Chen and Liu, 2007; Esteghlalian et al., 1997; Saha et al., 2005; Sun and Cheng, 2005; van Walsum and Shi, 2004; Rubio et al., 1998].The first group contains forest biomass like wood (hard wood, soft wood) and residues (saw dust, bark thinning). Soft wood contains more lignin and less hemicellulose (ca. 25% each) in comparison to hard wood (ca. 21% lignin and 30% hemicellulose). The second group contains agricultural residues, and these residues may be sub-grouped to food crops (corn stover, corn cob, fibers, sugar bagasse, wheat straw, rice straw, rice and oat hull etc.) and non-food crops (cotton stalk, cotton gin etc.). The third group is designated as herbaceous grass (switch grass, bermuda grass, alfalfa fiber, kans grass, reed canary grass etc.). Some waste materials also find a place here in the categories of lignocellulosic biomass which have studied by the researchers for ethanol production[Lissens et al., 2004; Li et al., 2007; Hu et al., 2008]. These waste materials may be from residential sources (waste paper, waste food) or non-residential/industrial sources (waste paper/board, paper mill sludge etc.).

Among the various lignocellulosic biomaterials perennial crops (e.g. grasses) are promising feedstock because of high yields, low costs, and good suitability for low-quality land with almost no requirement of water supply for its growth and its availability throughout the year [Kataria et al., 2009]. *Kans* grass (*Saccharum spontaneum*) having all these properties, was selected as the lignocellulosic raw material for the present work. *Kans* grass is a perennial grass, native to South Asia and occurs throughout India along the sides of the river. It grows up to three meters in height, with spreading rhizomatous roots. In the Terai-Duar savanna and grasslands, a lowland eco region at the base of the Himalaya range in Nepal, India, and Bhutan, *Kans* grass quickly colonises exposed silt plains created each year by the retreating monsoon floods, forming almost pure stands on the lowest portions of the floodplain. It is self-seeding, resistant to many diseases and pests, and can produce high yields with low applications of fertilizer and other chemicals. It is also tolerant to poor soils, flooding, and drought; improves soil quality and prevents erosion due to its type of root system. It uses less water per gram of biomass produced than other plants [Singh et al., 2011b; Chandel et al., 2009]. These characteristics makes *Kans* grass biomass (KGB) a novel substrate with great potential for the production valuable products.

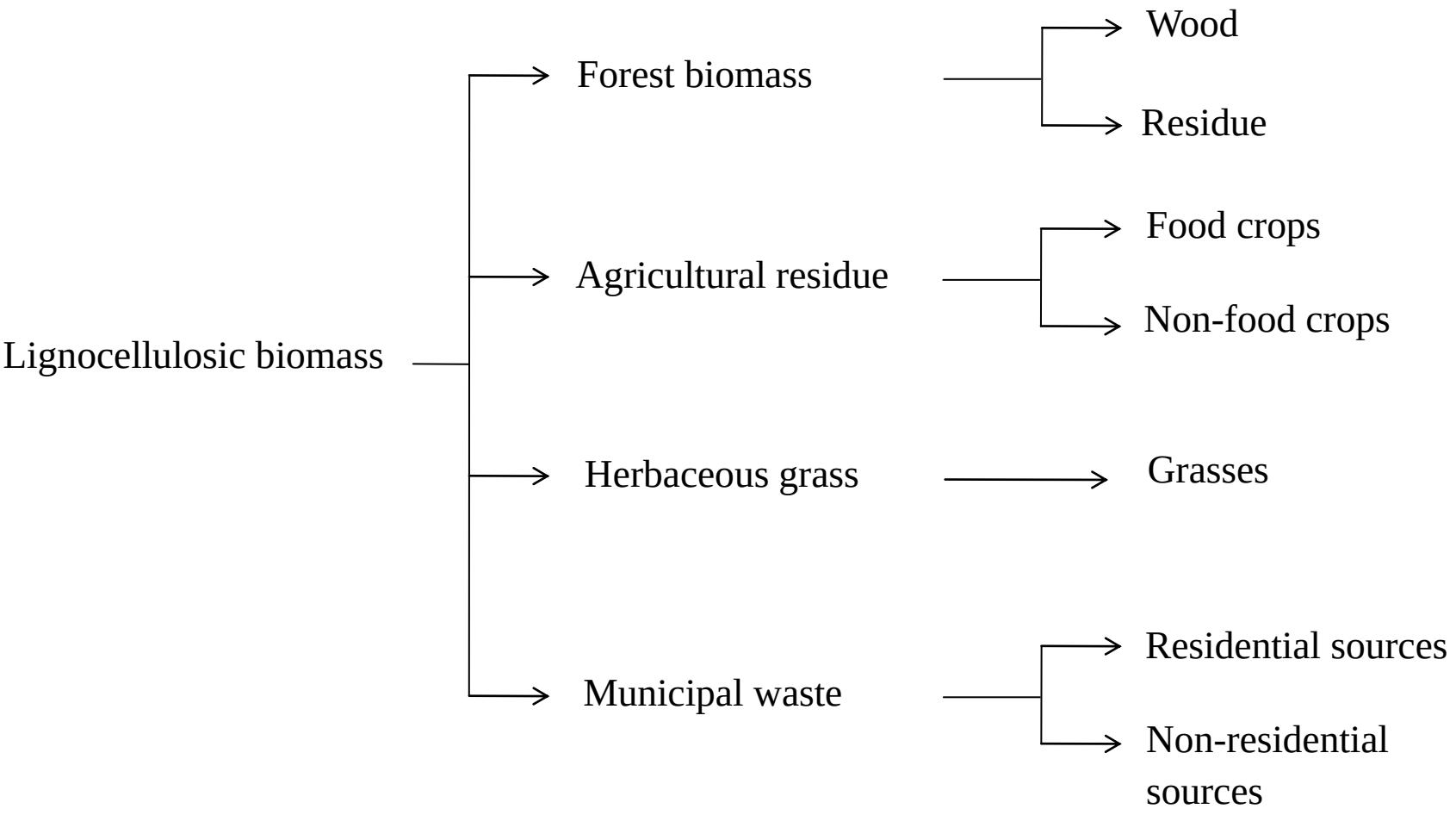


Figure 2.7 Categorization of lignocellulosic biomass.

2.4 Fermentable sugar production from lignocellulosic biomass

The process of bioconversion of ethanol from lignocellulosic biomass includes the liberation of fermentable sugars from biomass followed by fermentation and distillation to recover ethanol from the broth. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds and also by some covalent bonds. The pretreatment/hydrolysis processes are used for delignification to liberate cellulose and hemicellulose from their complexes with lignin and depolymerization of the carbohydrate polymers to produce free sugars. For utilizing fermentable sugar potential of lignocellulosic biomass the first requisite is to produce these sugars in soluble form from their insoluble polymeric structure for easy bioconversion to valuable products using biocatalysts like enzymes, microorganisms or a combination thereof. To achieve this objective two types of processes are available (1) single step hydrolysis process and (2) pretreatment and hydrolysis in two or more steps i.e. multi-step process. The important criteria for a process to be recognized as a good hydrolysis process are as follows: (1) maximize fermentable sugar yields, (2) minimize or avoid degradation of produced soluble sugars, (3) minimize or avoid the generation of microbial growth inhibiting compounds, and (4) be cost effective [Brodeur et al., 2011; Sun and Cheng, 2002].

2.4.1 Single step hydrolysis process

In single step hydrolysis of lignocellulosic biomass comprised only concentrated acid hydrolysis process wherein concentrated mineral acid (in most of the cases it is sulfuric acid) is added to unhydrolyzed, ground biomass and reacted at a relatively reduced temperature of 100°C or less. A mixture of monomeric sugars found in the hydrolysate, primarily glucose and xylose. The concentrated acid process has a very high sugar yield (>90%), can hydrolyze variety of feedstock, is relatively complete and rapid (10-12 h duration), and gives little degradation of soluble sugars [Hamelinck et al., 2005]. Clausen and Gaddy (1993) described in their patent (US5,188,673)a single step concentrated sulfuric acid hydrolysis of corn stover. 70 % acid concentration was able to hydrolyze corn stover 100 % (although not in monomeric form) at 50°C in 5-10 min. After few modifications finally, 70 % sulfuric acid at 50°C followed by dilution to 30-50% at 100°C was found suitable for hydrolysis at 50°C with in 10 min. The critical factors needed to make the process economically viable are to minimize the amount of acid and optimized sugar recovery from acid-sugar stream for acid recycling. Efforts have been made in this direction and membrane separation with 80% acid recovery and continuous ion exchange method with over 97% acid recovery; only 2% loss of sugar is achieved. Further acid is re-concentrated via multiple effect evaporators. Without acid recovery, large quantity of base

preferentially lime or $Ca(OH)_2$ must be used to decrease acidity the sugar solution thus generating large quantity of hydrated gypsum is formed and precipitates. It is easily filtered from the sugar solution, although its inertness would also allow it to pass harmlessly through fermentation and distillation. The gypsum may have some value as an agricultural soil conditioner, but can also mean a waste problem when generated in bulk quantity. The low temperature and pressure employed allow the use of relatively low cost materials such as fiberglass tanks and piping. Although sugar degradation is very low in the case of concentrated acid hydrolysis but detoxification process step is generally required to remove or reduce the concentration of the inhibitory compounds prior to fermentation as discussed later. Since it is a single stage hydrolysis process giving a mixture of pentose and hexose sugars, mainly xylose and glucose which needs to separated before going to ethanol fermentation because no native strain is quite capable to ferment both type of sugars simultaneously to ethanol. The Arkenol Inc. and Masada Resource Group are involved in the concentrated acid hydrolysis of cellulosic to ethanol conversion process [Taherzadeh and Karimi, 2007a].

2.4.2 Multi step hydrolysis process

In all other types of hydrolysis processes of lignocellulosic biomass consists two or more steps, primarily known as pretreatment of biomass followed by main cellulose hydrolysis process. The purpose of pretreatment processes is to remove lignin and hemicellulose, reduce cellulose crystallinity and increase the porosity of the biomass. The pretreatment process is generally followed by enzymatic hydrolysis of cellulose, catalyzed by cellulase enzyme. The pretreatment of lignocellulosic biomass is among the most costly steps and has a major influence on the costs of both prior operation like size reduction of the biomass and subsequent operations like enzymatic hydrolysis and fermentation. A number of pretreatment processes are under intensive research and are broadly categorized as (1) physical, (2) physico-chemical and chemical, and (3) biological. The effects of various pretreatment processes on lignocellulosic biomass are given in table 2.3.

2.4.2.1 Physical pretreatment

Physical pretreatment of lignocellulosic biomass excludes the use of any chemical agent. It includes pyrolysis, liquid hot water treatment, mechanical comminution [McMillan, 1994; Wyman, 1996]. Pretreatment with gamma-ray, electron-beam and microwave irradiation are also under investigation. Feedstocks can be comminuted by an arrangement of chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials reduced to 10–30 mm after chipping and 0.2–2mm after milling or grinding. Vibratory ball milling is also an

effective method to reduce cellulose crystallinity of spruce and aspen chips. The power requirement of mechanical comminution of lignocellulosic materials depends on the final particle size. Mechanical disruption of lignocellulosic material also depolymerizes lignin [Inoeu et al., 2008]. Pyrolysis of lignocellulosic materials, at temperatures greater than 300°C rapidly break down cellulose and produce gaseous products and char [Kilzer and Broido, 1965; Demirbas, 2008; Shafizadeh and Bradbury, 1979].The break down may be much slower and less volatile products are formed at lower temperatures. Zinc chloride or sodium carbonate is used to break down pure cellulose at lowertemperature. Irradiation s are also investigated and found to be an effective means of break down the complex structure of lignocellulosic biomass. Yang et al., (2008) used gamma irradiation at 500 kGy and glucose yield of 13.40% was obtained from wheat straw. Microwave irradiation of 700 W caused significant weight loss in lignocellulosic biomass due to break down of its constituents [Zhu et al., 2005].

2.4.2.2Physico-chemical and chemical pretreatment

The physico-chemical pretreatment process includes mainly steam explosion (autohydrolysis), ammonia fiber explosion (AFEX), CO_2 explosion, SO_2 explosion [Alizadeh et al., 2005; Ballesteros et al., 2006; Eklund et al., 1995]. The steam explosion is one of the most extensively used pretreatment methods [McMillan, 1994]. Steam at 0.69-4.83MPa used for few second to a few min then material is exposed to the atmospheric pressure. Chip size, moisture content steam pressure and residence time are the key factors to affect steam explosion [Duff and Murray, 1996]. Major drawback of steam explosion includes the destruction of xylan and incomplete lignin transformation, and generation of degradation compounds that are inhibitory to microbial growth, enzymatic hydrolysis, and fermentation needs to be washed by water thus reducing the overall yield of saccharification. Similar to steam explosion ammonia fiber explosion is used for pretreatment of biomass. In an AFEX process, 1-2 kg ammonia per kg dry biomass is used at 90°C for 30 minutes of residence time, and then the pressure is suddenly reduced. This process is effective only for the biomasses of low lignin content [Reshamwala et al., 1995; Holtzapple et al., 1991]. Further ammonia must be recovered and recycled after pretreatment to protect the environment. CO_2 explosion is also used for pretreatment of biomass in similar way to the above mentioned two processes but the sugar yield is low as compared to steam or ammonia explosion pretreatment after enzymatic hydrolysis. CO_2 explosion was found cost effective in comparison to steam explosion and AFEX processes [Zheng et al., 1998].

The chemical pretreatment process includes mainly ozonolysis, dilute acid hydrolysis, alkaline hydrolysis, organosolv[Karimi et al., 2006; Sidiras and Koukios, 2004; Cara et al., 2008; Chaudhary et al., 2012]. The ozonolysis pretreatment process effectively removes lignin and does not produce toxic compounds. Although reaction is carried out at room temperature and pressure [Vidal and Molinier, 1988] but large quantity of ozone requirement makes this process very expensive [Sun and Cheng, 2002].The dilute acid hydrolysis is one of the most extensively studied method of pretreatment [Millati et al., 2005; Sanchez et al., 2004; Sues et al., 2005; Taherzadeh et al., 1997a; Parmar and Rupasinghe, 2012].The dilute acid process for hydrolysis of lignocellulosic biomass consist essentially two stages, whereas, first stage is conducted under mild process conditions (e.g. 0.7% sulfuric acid, 190°C) to produce 5-carbon sugars from hemicellulosic fraction while in second stage remaining solid i.e. more resistant cellulose is hydrolyzed under harsher conditions (215°C, 0.4% acid) to produce 6- carbon sugars. The sugar yield of this process is low as compared to the other processes because of the severe conditions used the produced sugars gets converted to further degradation products like xylose converted to furfural and glucose converted to hydroxymethyl furfural which are also inhibitory to the microorganisms used in the subsequent fermentation process[Larsson et al., 2000]. Therefore, an additional process step known as detoxification of hydrolysate is used to remove or reduce the concentration of these inhibitory compounds prior to fermentation leading to enhance the overall cost of the process. Alkaline pretreatment uses bases like sodium hydroxide or calcium hydroxide. This process is mainly used for delignification but it also solubilizes hemicellulose to some extent and thus reducing the overall fermentable sugar yield. Furthermore, this process is effective only for the low lignin content biomasses like straws, herbaceous biomass but not for high lignin content biomasses like softwood [Bjerre et al., 1996]. Iyer et al. (1996) used ammonia recycled percolation process at 170°C for 1 h for treatment of corn stover and switchgrass. The concentration of ammonia was ranged from 2.5 to 20 %. The delignification of both the substrate was found 60-85 %. The organosolv pretreatment process typically use an organic or aqueous solvent mixture like methanol, ethanol, acetone, ethylene glycol etc in combination with dilute mineral acids like sulfuric acid, hydrochloric acid as catalyst [Chum et al., 1988; Sun and Cheng, 2002]. Some organic acids can also be used as catalysts in like oxalic acid, salicylic acid etc. it is used for fractionation of biomass to hemicellulose, cellulose and lignin leaving a highly digestible substrate. The recovery and recycling of solvents from the process vessel is essentially required as the solvents may be inhibitory to the microorganisms and enzymes which make this process too expensive [Sun and Cheng, 2002].

2.4.2.3 Biological pretreatment

In the biological pretreatment processes, mainly fungi are used to degrade lignin known as biodelignification. Fan et al. (1987) and Akin et al. (1995) reported that white-rot fungi like *Phanerochaetechrysosporium* are the most effective basidiomycetes for biological pretreatment of lignocellulosic biomasses. Although biological pretreatment has advantages of low energy use at mild reaction conditions, however, the very low hydrolysis rate (few days to weeks) impedes its implementation. Other well studied microorganisms are *Aspergillusterreus, Trichoderma spp.,Pleurotus spp., Lentinusedodes, Streptomyces griseus, Ricinuscommunis* and genetically engineered *Escherichia coli* and *Klebsiellaoxytoca*[Emtiazi et al., 2001; Mukherjee and Nandi, 2004; Brienzo et al., 2007; Mukhopadhyay, 2011; Peterson and Ingram, 2008].

2.4.2.4 Enzymatic hydrolysis

The abovementioned pretreatment processes are intended to disturb the crystalline structure of cellulose, remove lignin and in some cases hemicellulose too. All these phenomenon leads to increase the porosity of the lignocellulosic biomass. Thus hydrolytic enzymes are allowed to act into the fibers for efficient hydrolysis of the loosen polymer chains of cellulose and hemicellulose [Galbe and Zacchi, 2007]. Three main groups of cellulases are required to hydrolyze cellulose viz. endoglucanase (act on low crystalline region to release free chain ends), exoglucanase (to produce cellobiose units from the free chain ends), and β-glucosidase (to produce glucose from cellobiose units) [Parmar and Rupasinghe, 2013]. For hydrolysis of hemicellulose a number of enzymes are required viz. xylanase, β-xylosidase, galactomannanase, glucomannanase and others [Roman et al., 2006; Georgieva et al., 2008]. The pretreatment processes in which lignin is not removed ligninolytic enzymes are used for lignin degradation [Parmar and Rupasinghe, 2012; Kuila et al., 2011]. Lignin peroxidase, manganese peroxidase and laccase are the main lignin hydrolyzing enzymes [Mtui and Masalu, 2008].

Table 2.3 Pretreatment processes and their effect on lignocellulosic biomass

Pretreatment category	**Process**	**Mechanism and possible changes in biomass**
Physical pretreatment	**Mechanical comminution** Ball milling Hammer milling Colloid milling **Irradiation** Gamma-ray irradiation Microwave irradiation Electron beam irradiation **Others** Pyrolysis Liquid hot water Extrusion	Decrease particle size Increase accessible surface area Decrease cellulose crystallinity and its DP Partial hydrolysis of hemicellulose Partial depolymerization of lignin
Physico-chemical and chemical pretreatment	**Explosion** Steam explosion Ammonia fiber explosion CO_2 explosion SO_2 explosion **Alkali** Sodium hydroxide Ammonia Lime Ammonium sulfite **Dilute acid** Sulfuric acid Hydrochloric acid Phosphoric acid **Organosolv** Ethanol Benzene Ethylene glycol Butanol **Oxidant** Ozone Hydrogen peroxide Wet oxidation	Decrease cellulose crystallinity and its DP Partial or complete hydrolysis of hemicellulose Delignification
Biological pretreatment	Fungi Actinomycetes	Reduction in DP of cellulose and hemicellulose Delignification

2.4.3 Major constraints of existing technologies

The greatest technical and economic barrier in the lignocellulosic biomass to ethanol production process is believed to be the effective release of protected polysaccharides from the complex lignocellulosic biomaterials to fermentable sugars [Eggeman and Elander, 2005; Zhang et al., 2007]. Currently a broad range of pretreatment technologies are available for improved conversion of sugar moieties of lignocellulosic biomass to ethanol. The existing pretreatment/hydrolysis technologies suffer from low sugar yields, and/or severe reaction conditions, and/or narrow substrate applicability and high capital investment etc. The physical pretreatment processes are highly energy demanding and most of them are unable to remove lignin. These methods are not recommended to use at industrial scale. The limitations of the existing pretreatment technologies which are extensively studied at laboratory level are mentioned in Table 2.4 [Brodeur et al., 2011].

After through literature survey it was found that mainly three pretreatment/hydrolysis technologies for generation of soluble sugars from lignocellulosic biomass are useful and applied for large scale industrial production of ethanol: (1) concentrated sulfuric acid hydrolysis, (2) two stage dilute sulfuric acid hydrolysis, and (3) dilute sulfuric acid pretreatment followed by enzymatic hydrolysis. The concentrated sulfuric acid hydrolysis is one of the oldest process discovered by Braconnot in 1819 and are reported to give higher yield of glucose (90 % of theoretical maximum), consequently higher ethanol yield compared to the other technologies like dilute acid process. The concentrated acid hydrolysis process based Arkenol's technology is used by: Izumi Biorefinery of BlueFire Renewables Inc. and Masada Resource Group. The high concentration of acid (30-70 %) makes the process extremely corrosive and requires expensive alloys or non-metallic material for construction of the reaction vessel. During neutralization production of large amount of gypsum is also problematic. Further acid recovery is energy demanding and add costs to the overall process. Besides these limitations one of the major drawbacks is the generation of mixed sugar by this process. Since no native microorganism is able to ferment both type of sugars efficiently therefore, many time xylose and thus hemicellulose fraction is not utilized for the production of ethanol.

The two stage dilute acid hydrolysis process is used by BC International Corporation US and SEKAB in Sweden. Karimi et al. (2006) reported the conditions of two stages dilute acid hydrolysis process as: stage I (201°C, 10 min, 0.5% acid) and stage II (234°C, 3 min, 0.5% acid). During stage I 80.8 % xylose (theoretical maximum) and 7.3% glucose (theoretical

maximum), whereas in stage II 39.3% glucose (theoretical maximum)was obtained. The major drawbacks of this process include the generation of toxic compounds and low sugar yield.

The third main technology dilute acid treatment followed by enzymatic hydrolysis is used by Abengoa Bioenergy US and Iogen Corporation Canada. Saha et al. (2005) reported that stage I of dilute acid pretreatment (1% acid, 1 h, 121°C) followed by stage II of enzymatic hydrolysis (cocktail of cellulase, β-glucosidase, xylanase, 45°, 72 h), 60% saccharification on total carbohydrate content basis was achieved. The major drawbacks of this process include the use of costly enzyme, longer reaction time and an additional process step of detoxification (overliming).

After comparing all the pretreatment and hydrolysis processes concentrated sulfuric acid hydrolysis of lignocellulosic biomass showed dominance and has the advantages over other methods like (1) no enzymes are required; (2) broad range of biomass types can be used; (3) pretreatment at moderate temperature allows the use of plastic construction materials (this is in contrast with dilute acid pre-treatment carried out at temperatures near 180ºC and high pressure); (4) the low temperature used limits the production of inhibitory compounds such as furfural.

Table 2.4 The drawbacks of various pretreatment technologies.

S. no.	Method	Limitations
1	Steam explosion	Destruction of a portion of xylan; toxics generation; incomplete disruption of lignin-carbohydrate matrix
2	AFEX	Not effective for high lignin content like newspaper, aspen chips; ammonia must be recovered and recycled after the treatment; composition barely changed from original
3	Ozonolysis	Large amount of ozone required; very expensive
4	Acid hydrolysis	High cost; formation of toxics; corrosion of equipment
5	Alkaline hydrolysis	Long residence time; irrecoverable salt formed and incorporated in biomass; dilute NaOH treatment is not effective against the high lignin biomasses
6	Organosolv	Solvents need to be drained from the reactor, evaporated, condensed and recycled; high cost
7	Pyrolysis	High temperature; ash production
8	Biological	Very low rate of hydrolysis
9	Mechanical comminution	Power consumption usually higher than inherent biomass energy

2.5 Inhibitors in hydrolysate

The lignocellulosic hydrolysate contains monomer sugars and a broad range of inhibitory compounds [Almeida et al., 2007]. The type and concentration of inhibitory compounds is dependent on the composition of lignocellulosic biomass and the pretreatment/hydrolysis condition mainly high temperature and acid concentration. These inhibitory compounds are known to be toxic for the microorganisms, affect their growth and thus reduce ethanol yield and productivity. The level of toxicity of these compounds also depends on the cell physiological state, availability of dissolved oxygen concentration and pH of the fermentation medium [Taherzadeh et al., 2000]. These compounds mainly reduce the efficient sugar utilization by stressing the cells thereby reducing the product formation [Mussatto and Roberto, 2004]. The inhibitory compounds as generated during pretreatment/hydrolysis of lignocellulosic biomass may be presented in three main groups as: (1) sugar degradation compounds; (2) lignin degradation compounds and (3) other lignocellulosic structural compounds [Pienkos and Zhang, 2009; Parajo et al., 1998; Olsson and Hahn-Hagerdal, 1996].

2.5.1 Sugar degradation compounds

The two types of sugars pentose and hexose produce different type of inhibitory compounds. Pentose sugar produce furfural upon degradation and hexose sugar degrade to give hydroxymethyl furfural. During fractionation of lignocellulosic biomass hemicellulose polymer produce pentoses like xylose and arabinose which gets converted to furfural if the reaction duration is more than 1 h due to dehydration of these sugar moieties [Cruz et al., 2002]. Furfural as toxic compound affects the specific growth rate of microorganisms by reducing cell biomass yield per unit of ATP (Adenosine-5’-triphosphate, energy carrier within cell) [Palmqvist and Hahn-Hagerdal, 2000]. The inhibition of microorganisms by furfural is dependent on the concentration of this compound in the media. Roberto et al. (1991) reported that above 2 g/L furfural concentration inhibited *Pichia stipitis* cell completely whereas, Delgenes et al. (1996) observed that 1.0 g/L and 2.0 g/L furfural concentration reduced the growth of *P. stipitis* by 47 % and 99 % respectively. Another study [Nigam, 2001] reported that ethanol yield was reduced by 90.4 % in the presence of 1.5 g/L furfural by interfering in respiration and growth of *P. stipitis*. Hydroxymethyl furfural (HmF) is produced from the hexose sugars like glucose under acidic conditions due to dehydration. Its effect on the growth of microorganisms is found similar to that of furfural. Like furfural it reduces the growth rate and cell yield by interfering in the activity of dehydrogenases and thus glycolysis [Banerjee et al., 1981].It was found by Liu et al. (2004) that HmF is less toxic than furfural on molar basis

in case of *P. stipitis* NRRL Y-7124 and *S. cerevisiae* NRRL Y-12632. A prolonged lag phase was observed at an intermediate concentration of HmF. *Z. mobilis* was found relatively resistant to furfural and HmF than these yeast cells.

2.5.2Lignin degradation compounds

Lignin is a very complex polymer of phenolics, aromatic and polyaromatic compounds and produces many of these compounds in soluble form during hydrolysis of lignocellulosic biomass. According to the Mussatto and Roberto (2004) low molecular weight phenolic compounds exhibit more toxicity towards fermentation. Heipieper et al. (1994) and Palmqvist and Hahn-Hagerdal (2000) have reported the effect of phenolics on the biological membrane. Phenolics cause partition into membranes due to which integrity lost and thus the ability of the cell membrane to work as selective barrier affected. The consequences of this phenomenon are the reduced sugar transport and growth yield of the cells. The degradation of guaiacylpropane unit of lignin produced vanillic acid and vanillin. These compounds were observed in the hydrolysate of red oak, poplar and pine [Tran and Chambers, 1985; Clark and Mackei, 1987]. Syringyl propane units produce syringaldehyde and syringic acid upon hydrolysis and found in hardwood hydrolysate [Tran and Chambers, 1985; Jonsson et al., 1998].

2.5.3 Other compounds derived from lignocellulosic structure

Some compounds like resins, taninic and terpene acid, acetic acid and other weak acids are also found in the hydrolysate of lignocellulosic biomass [Mussatto and Roberto, 2004]. These compounds may also exhibit toxicity towards microorganisms during fermentation. These are basically raw material extractives but produce less inhibition to microbial growth than lignin derived compounds [McMillan, 1994].Van Zyl et al. (1991) have reported that toxicity of acetic acid depends on the fermentation conditions like pH and dissolved oxygen. Some authors have suggested that low concentration (<100 mmol/L) of acetic acid (weak acid) may increase ethanol yield whereas, yield decreased at higher concentrations [Larsson et al., 1998].The mechanism of growth inhibition due to weak acids is proposed to be due to transport of these acids in to cytosol and then dissociation caused due to neutral intracellular pH therefore, decreasing cytosolic pH [Axe and Bailey, 1995; Pampulha and Loureiro-Dias, 1989].

It is clear from the literature that the inhibitory effect of these toxics varied according to the concentration level and the type of microorganism. Thus it is important to analyze the effect of these compounds on the selected microorganism before conducting the actual fermentation process.

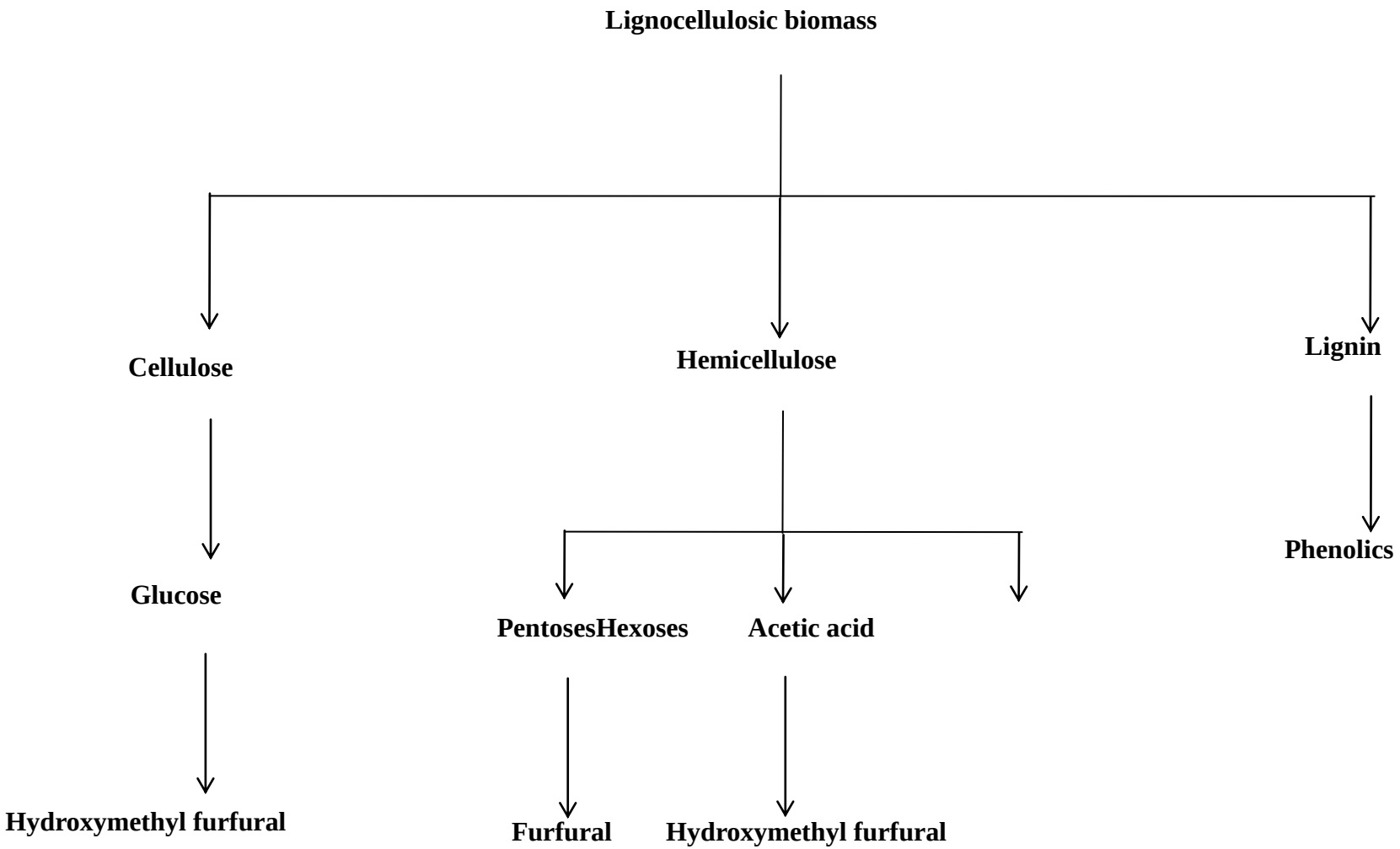


Figure 2.8(a) Generalized view of inhibitors generation from lignocellulosic biomass.

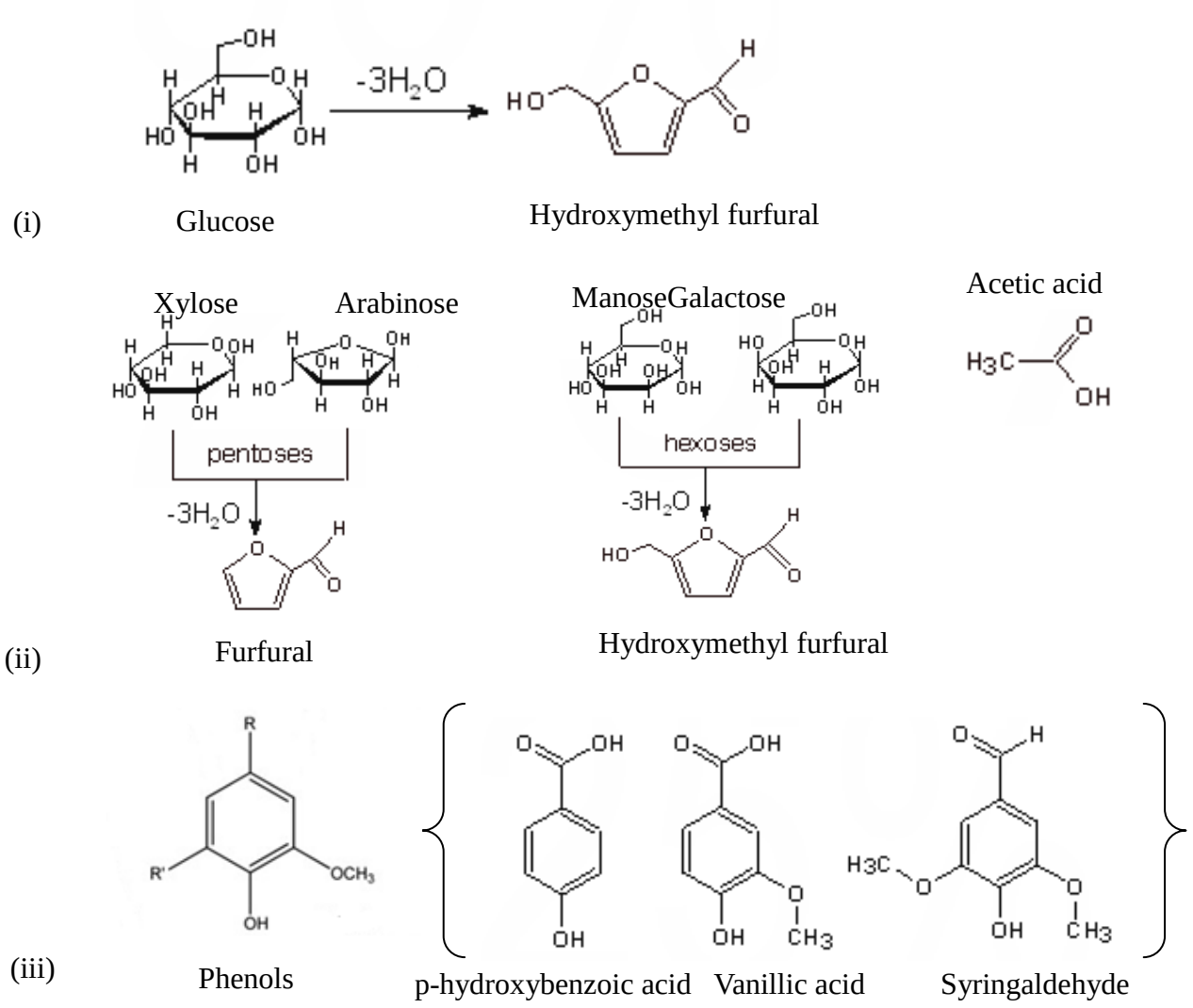


Figure 2.8(b) structures of various inhibitors (i) dehydration reaction of glucose; (ii) degradation reaction of pentose and hexose to generate inhibitors; (iii) structures of various phenolics compounds generated from lignin degradation. [sourec: Marton et al., 2006].

2.6 Conditioning of lignocellulosic hydrolysate

The term conditioning in respect to liquid lignocellulosic hydrolysate refers the methods to treat the hydrolysate to reduce the effect of toxics and prepare the media suitable for fermentation [Pienkos and Zhang, 2009]. Many authors mention this method as detoxification [Taherzadeh et al., 2000; Mussatto and Roberto, 2004]. The efficiency of conditioning or detoxification depends mainly on the type of hydrolysate and the microorganism. The toxicity level in each hydrolysate is different due to varied hydrolysis conditions and the raw material. Also microorganisms have different degree of tolerance [Larsson et al., 1999] therefore, the

choice of conditioning method should consider the degree of toxicity and the characteristics of microorganism. The conditioning process may be categorized in three main groups: (1) biological, (2) chemical, and (3) physical. In biological and chemical conditioning methods the toxic compounds are converted to less toxic compounds and in physical method these compounds are removed from the hydrolysate.

2.6.1 Biological conditioning

The use of enzymes or microorganisms for bioconversion of the toxics present in the hydrolysate to less inhibitory compounds comes under the category of biological conditioning of hydrolysate. Laccase and peroxidase enzymes originated from *Triametesversicolor* used by Jonsson et al. (1998) to detoxify wood hydrolysate. They obtained an enhanced sugar consumption and ethanol productivity. The probable mechanism for this was oxidative polymerization of low molecular phenolics. Schneider (1996) used a mutant species of *S. cerevisiae* and observed that acetic acid concentration was reduced from 6.8 g/L to 0.4 g/L in the hydrolysate medium. Another biological approach to enhance the fermentability of hydrolysate is known as adaptation of microorganism to the hydrolysate [Parajo et al., 1998; Silva and Roberto, 2001]. In this method the cells of one fermentation media is used as inoculum for the next fermentation. Thus after successive fermentations the microorganism may become adaptive to the inhibitory compounds.

2.6.2 Chemical conditioning

The chemical conditioning method is used when acid was utilized to hydrolyze lignocellulosic biomass. Since pH of the acid hydrolysate reached to very low value and it must be raised to the appropriate level of fermentation for efficient conversion sugars to ethanol by the microorganisms. The mechanism of chemical conditioning is based on the precipitation of the toxic compounds and change in the degree of toxicity of the some inhibitors by ionization at particular pH value [Van Zyl et al., 1998; Martinez et al., 2000]. A very old process known as overliming, is very popular and used by many authors for conditioning acid hydrolysate [Roberto et al., 1991a; Van Zyl et al., 1998; Martinez et al., 2001; Mohagheghi et al., 2006]. In this process the pH of the hydrolysate is raised to a value of 9-10 with $Ca(OH)_2$ resulting formation of insoluble gypsum. The pH should be raised to appropriate fermentation pH by acid and gypsum as precipitate must be removed by centrifugation (a costly processing step) before conducting actual fermentation. Furthermore this process leads significant sugar loss and consequently lowers ethanol yield [Pienkos and Zhang, 2009]. Other bases like NaOH, KOH, and NH_4OHwere also used in conditioning process. It was reported by Mohagheghi et al.

(2006) that a significant loss in sugars (35 % xylose, 15 % glucose, 20 % arabinose) was occurred at pH 11. The use of activated charcoal (powdered and granulated) is also very well studied. It is a low cost material with high absorbance capacity [Mussatto and Roberto, 2001]. It is reported by Lee et al. (1999) that the adsorption by using ion exchange resins is very effective method of conditioning but its cost is very high as compared to the other methods.

2.6.3 Physical conditioning

In physical conditioning approach the toxic compounds are physically removed from the hydrolysate. One of the well studied physical conditioning methods is vacuum evaporation [Parajo et al., 1997; Larsson et al., 1999; Rodrigues et al., 2001]. In this method the concentration of volatile toxic compounds like acetic acid, furfural and vanillin reduced to a significant level. The major drawback of this method is the increase in concentration of non-volatile inhibitors and consequently reduced degree of fermentation. Some other physical methods like electrodialysis[Sreenath and Jaffries, 2000], liquid-liquid extraction [Cantarella et al., 2004] and supercritical fluid extraction Persson et al., 2002) were also studied but these methods are not found suitable for large scale processing of hydrolysate.

2.7 Fermentation of hydrolysate for ethanol production

The industrial production of ethanol from lignocellulosic hydrolysate requires such microorganisms which have capacity of producing ethanol with high yield and productivity, and able to ferment broad range of substrates. The lignocellulosic biomass is a source of both pentose and hexose sugars and it is now established that all the sugars liberated from the biomass and present in the hydrolysate should be converted to ethanol for making the process economically viable [Sims et al., 2010]. Fermentation conditions also play crucial role due to specificity of microorganisms. Since the ethanol production is directly linked with the growth of microorganism and thus the factors affecting growth should be considered during the development of a suitable bioprocess for ethanol production.

2.7.1 Microorganisms

The best suited microorganism for ethanol production from lignocellulosic hydrolysate can be identified on the basis of performance parameters like growth rate, yield, productivity, ethanol tolerance, inhibitor tolerance, genetic stability etc. Dien et al. (2003) and Balat (2010) have mentioned the important characteristics required for microorganisms to be suitable for industrial production of ethanol. These includes: ethanol yield > 90 % of theoretical; ethanol tolerance > 40 g/L; productivity > 1 g/L/h; require inexpensive medium; resistant to inhibitors; able to grow at higher temperature and acidic pH to prevent contamination. A wide variety of

microorganisms are investigated for ethanol production but these may be categorized in two main groups as: (1) yeast and (2) bacteria. Some selected microorganisms and important features are given in table 2.5.

2.7.1.1 Ethanol producing yeast

Saccharomyces cerevisiae is the most commonly used yeast known as Baker's yeast. Ohgren et al. (2005) reported that it can also ferment lignocellulose hydrolysate efficiently. The major drawback associated with this strain that it cannot utilize xylose for its growth and ethanol production because it lacks the enzyme to convert xylose to xylulose [Keshwani and Cheng, 2009]. Thus the pentose sugars present in the hydrolysate remain unutilized and therefore, it is not possible to use full sugar potential of the hydrolysate. To utilize the xylose sugar of hydrolysate and significant ethanol production some natural xylose fermenting yeasts like *Candida shehatae, Candida parapsilosis* and *Pichia stipitis*are reported. The xylose is metabolize in these strains because of the presence of xylose reductase (XR) to convert xylose to xylitol and xylitol dehydrogenase (XDH) to convert xylitol to xylulose. Among these xylose fermenting yeasts *P. stipitis* is the most efficient, highly productive and shown promise for industrial applications [Nigam, 2001; Chandrakant and Bisaria, 1998]. It can ferment xylose rapidly with almost no xylitol production with no absolute vitamin requirement and can utilize wider range of sugars [Preez et al., 1986].

2.7.1.2 Ethanol producing bacteria

Among the bacteria *Zymomonas mobilis* is well recognized for its ability to produce ethanol at very high rate. *Z. mobilis* and *S. cerevisiae* have been reported for glucose fermentation and are the most commonly used GRAS (generally recognized as safe) microbes for ethanol production. Among these two microorganisms *Zymomonas mobilis* gives higher ethanol yield (5-10%) and is 2.5 times faster in terms of ethanol productivity than *Saccharomyces cerevisiae*[Sprenger, 1996]. Also *Zymomonas mobilis* is a Gram-negative bacterium follows homoethanolfermentation pathway (shown in Figure 2.9) and tolerates up to 120 g/L. Despite the robustness of *Z. mobilis* for ethanol production at industrial level, it is not well suited for fermenting sugars obtained from lignocellulosic biomass to ethanol production as it is unable to ferment xylose, which contributes up to 30-40% of the total available fermentable sugar in the hydrolysate. Other bacteria such as *Escherichia coli* and *Klebsiellaoxytoca* also attracted interest by showing rapid fermentation, which can be minutes compared to hours for yeast [Hayes, 2009]. The major disadvantage associated with *E. coli* is its narrow pH growth range (6.0-8.0). It is less hardy culture than yeast and lack of data on the use of residual cell mass as

feed supplement are the main obstacle in its use [Dien et al., 2003; Lin and Tanaka, 2006; Balat, 2010]. Some thermophilic anaerobic bacteria have also been reported for ethanol production with the advantage to withstand extreme temperatures but very low ethanol tolerance (<2 % v/v) prevents their use at industrial scale [Georgieva et al., 2007].The metabolic pathway for xylose utilization in bacteria and yeast is shown in figure 2.10. The xylulose is produced from xylose and it enters into the central metabolic pathway.

Mainly two approaches have been identified from literature for bioconversion of both type of sugars (pentose and hexose) to ethanol. First approach relied on the use of recombinant microorganism developed with the aim to ferment both xylose and glucose to ethanol. These include genetically modified strains of *S. cerevisiae*, *Z. mobilis*, *E. coli* and *K. oxytoca*[Brooks and Ingram, 1995; Chu and Lee, 2007; Doran et al. 1994; Ingram et al. 1997; Okuda et al. 2008; Zhang and Lynd, 2010]. Though the high yield could be achieved from these genetically modified organisms (GMO's), their developmental cost, narrow and neutral pH range, long term stability and the utilization of residual cell mass as animal feed like other GRAS organisms are the main disadvantages which restrict the use of GMO's at industrial level. The other approach lies in the use of two microorganisms simultaneously in a single fermentation system, defined as "co-culture". The ideal co-culture system involves such combination of microorganisms that do not affect each other's metabolic activities like their growth and, competitiveness towards substrate or nutrients should be minimal to maximize the production efficiency.

Table 2.5 Some selected microorganisms and their features for ethanol production [Limayem and Ricke, 2012].

Microorganism	**Comments**
Saccharomyces cerevisiae	High ethanol yield (90-97% theoretical), high ethanol tolerance (ca. 10% w/v), unable to utilize xylose (C5 sugar).
Zymomonas mobilis	5-10% more ethanol per glucose, tolerates ca.12 % w/v ethanol, unable to utilize xylose (C5 sugar).
Pichia stipitis	Naturally ferment xylose to ethanol efficiently, conversion efficiency 80-85 %, lower ethanol tolerance 4 % w/v, reassimilate formed ethanol.
Candida shehatae	Ferment xylose, Low ethanol tolerance, low ethanol yield, require micro-aerophilic conditions.
Kluveromycesmarxianus	Able to grow at high temperature, used in SSF/CBP, low ethanol tolerance, low ethanol yield from xylose, lower ethanol yield.
Esherichia coli	Able to ferment pentose and hexose sugars, low ethanol tolerance, narrow pH and temperature range, low tolerance to inhibitors.
Recombinant strains *E. coli FBR3, KO11* *S. cerevisiae CPB.CP4* *Z. mobilis CP4* *K. oxytoca M5A1 (pLOI555)*	Less hardy cultures, low productivity, plasmids carrying required genes are often rejected by host, use of residual GMO's as an ingredient in animal feed is still not established.

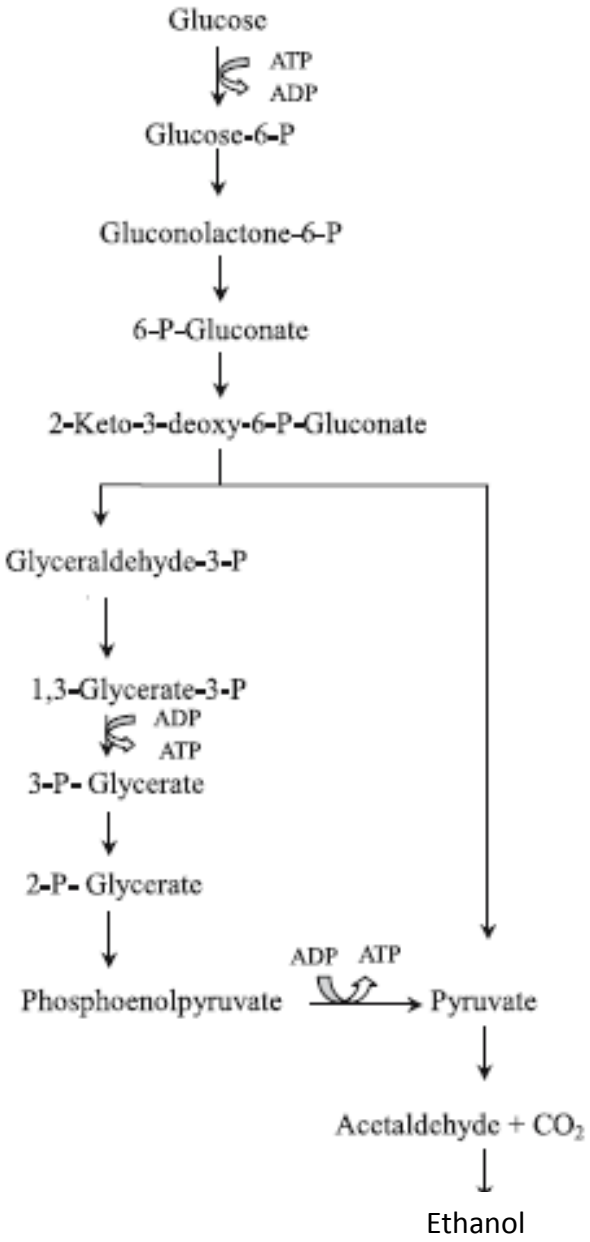


Figure 2.9 The Enter-Doudoroff pathway in *Z. mobilis*. [source: Zhang, 2003]

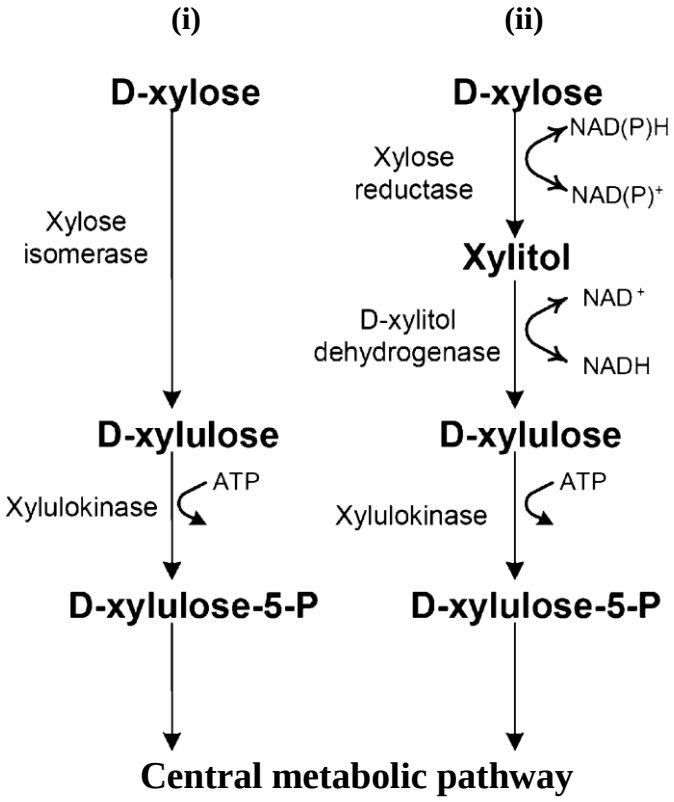


Figure 2.10 The metabolic pathway to utilize xylose in (i) bacteria and (ii) fungi. [source: Karhumaa et al., 2007]

2.7.2 Fermentation of hydrolysate

In many pretreatment/hydrolysis processes of lignocellulosic biomass where lignin is removed first and then hemicellulose and cellulose fractions were hydrolyze to release soluble sugars like ozonolysis, alkaline pretreatment, organosolv, biological methods or concentrated acid treatment, a mixed sugar stream is generated in which both pentose and hexose sugars were present. To utilize all sugar potential of lignocellulose hydrolysate for ethanol production no native microorganism is available till date and if GMO's are ruled out to use at industrial level the only way remained is the use of a co-culture system to ferment the mixed sugars. In other cases where hemicellulose and cellulose are hydrolyzed separately, mono-culture systems may be used to ferment xylose and glucose in separate reactor system. It was mentioned in a workshop by US Department of Energy (DOE), 2005 that prospects of co-culture fermentation is very credible and high-payoff for lignocellulosic to ethanol production. A large number of data is available on mono-culture systems whereas limited information is available on the development of co-culture systems [Singh et al., 2014]. In either case the fermentation can be performed in three modes: (1) batch, (2) fed-batch, and (3) continuous. The kinetic properties of microorganisms and type of hydrolysate are the key factors for choosing a suitable mode of fermentation along with the process economics [Olsson and Hahn-Hagerdal, 1996; Chandel et al., 2007].

Batch fermentation is a closed system in which the microorganism encounters a high sugar concentration at the beginning and high ethanol concentration at the final stage. Batch systems are found labour intensive with low productivity [Shama, 1988]. Batch systems are found very simple. Except acid or base for pH control and air for aerobic fermentation is added to the system after inoculation [Balat, 2010].

In fed-batch operations microorganisms encounters a low substrate concentration and increasing amount of ethanol as time of fermentation progresses. Woods and Mills (1985) reported that a yield of 0.41 g/g was achieved in fed-batch mode in comparison to the bath mode with only 0.29 g/g yield when xylose was fermented by *P. tannophilus* RL171. The ability to retain maximum viable cell concentration with prolongs life time and high ethanol concentrations are the main advantages of fed-batch system over batch cultivation.

In continuous mode of fermentation, substrate and other nutrients are fed continuously to the fermentation system and a product stream containing residual sugar, biomass and ethanol is continuously withdrawn from the system. The microorganism encounters a low substrate and high ethanol concentration during the fermentation. Continuous cultivation often gives higher

productivity than batch system but substrate utilization is not complete and consequently the yield is lower. Lawford (1988) reported a threefold increase in productivity of ethanol in continuous operation than that in batch mode. For achieving high ethanol productivity along with high yield, increased cell density culture or in situ ethanol removal is recommended [Ghosh and Ramachandran, 2007]. The high cell density may be achieved in two ways: by cell recycling or use of immobilized cell [Singh et al., 2009; Yu et al., 2007]. Using high cell density means more carbon is diverted for production of cell mass than ethanol initially. Use of immobilized cell adds extra cost to ethanol production due to immobilization procedure [Olsson and Hahn-Hagerdal, 1996].

In co-culture systems the choice of mode of fermentation depends on the type of microbial system used in co-culture. Chen (2011) reported that most of the existing co-culture systems were operated in batch mode and some in continuous mode. The major drawback of batch mode is that the glucose can suppress xylose fermentation at initial stage as xylose utilization is completely inhibited at 2.3 g/L glucose concentration and microorganisms have greater affinity towards glucose than that for xylose [Grootjen et al., 1991]. This limitation may be overcome by using high fermentative potential microorganism like *S. cerevisiae* which can convert glucose rapidly and lower down its concentration to 2.3 g/L and allowed to ferment xylose. Again the high ethanol concentration at the end of glucose fermentation may lead to inhibition of xylose fermenting microorganism.

2.8 Strategies for ethanol production from pretreated lignocellulosic biomass

For bioconversion of pretreated biomass (primarily composed of hemicellulose and cellulose fractions) to ethanol the main strategies are (1) separate hydrolysis and fermentation (SHF) and (2) simultaneous saccharification and fermentation (SSF). The SHF consisted of mainly two steps: (a) hydrolysis of pretreated biomass using cocktail of enzymes to produce sugar syrup and (b) fermentation of the produced sugars to ethanol using microorganisms. Lau and Dale, (2009) used SHF and achieved only 40 g/L ethanol concentration from AFEX treated corn stover. Shen et al. (2011) reported 15.3 g/g ethanol production from sweet sorghum bagasse using SHF. The advantage associated with this strategy is that hydrolysis and fermentation can be carried out at their optimal conditions. The inhibition of hydrolyzing enzymes (cellulase and β-glucosidase) by the presence of cellobios and glucose above the inhibitory levels and low biomass to enzyme ratio are the main disadvantages of SHF [Balat, 2010].

When the enzyme hydrolysis and fermentation processes are carried out in single reactor vessel at a time then it is called simultaneous saccharification and fermentation (SSF). The glucose produced from cellulose hydrolysis is immediately consumed by the microorganisms and favorable fermentation conditions lead to ethanol production [Kumar et al., 2009b]. Zhang et al. (2010) achieved 64.6 g/L ethanol concentration from steam explosion treated corn stover with 30 FPU/g dry mass enzyme loading. The important aspect of SSF is the enhanced enzyme hydrolysis due to utilization of sugars by the microorganisms thus prevents the enzyme inhibition by sugar concentration [Ramachandran and Hashim, 1990]. The bottleneck of SSF is the different optimal conditions (like pH, temperature, substrate concentration) for enzymatic hydrolysis and fermentation [Menon and Rao., 2012]. SSF and SHF was compared by Shen et al. (2012) for ethanol production from sweet sorghum bagasse.

To utilize hemicellulose potential of lignocellulosic biomass the SSF is modified to simultaneous saccharification and co-fermentation (SSCF) [Hamelinck et al., 2005]. The cellulose and hemicellulose fractions are hydrolyzed by cellulase and xylanase respectively and giving a mixture of xylose and glucose. Zhang and Lynd (2010) reported ethanol yield of 0.39 g/g using engineered microorganisms from waste paper sludge from SSCF process. In most of the SSCF processes genetically modified strains have been used to ferment xylose and glucose in a single reactor system. Whereas, another option, co-culture technique can also be used in SSCF.

The most advance technique in which enzyme production is coupled with cellulose hydrolysis and ethanol fermentation is called as consolidated bioprocessing (CBP) or direct microbial conversion (DMC) [Balat, 2010]. A modified microorganism is the prime requirement for this technology which possesses the characteristics of producing cellulase and ethanol at high yield and productivity [Hamelinck et al., 2005]. The challenge is still open to develop a suitable engineered microbial strain to be used in CBP. Very low ethanol yields are reported due to susceptibility of modified strains towards the ethanol [Zaldivar et al., 2001].

2.9 Ethanol recovery

Ethanol is recovered from the fermentation broth by distillation followed by dehydration. The ethanol is concentrated in a rectifying column of distillation unit. From this column ethanol is recovered just below the azeotrope (95 %) and called as hydrated ethanol [Hamelinck et al., 2005]. The solids are removed by centrifugation followed by drying in a rotary drier. The hydrated ethanol can be used in high ethanol content fuel like E95, however if ethanol is used

to mix with gasoline, the water content should not be more than 1 %. The use of molecular sieve (synthetic zeolite adsorbents) is a common method to produce anhydrous ethanol [Taherzadeh and Karimi, 2007].

2.10 Composite discussion on challenges in production of ethanol from lignocellulosic biomass

- Effectively releasing the locked polysaccharides from recalcitrant lignocellulose to fermentable sugars is among the greatest technical and economic barriers.
- The leading lignocellulose pretreatment/hydrolysis technologies suffer from low sugar yield, and/or severe reaction conditions, and/or costlier enzymes use, narrow substrate applicability, and high capital investment etc.
- Enzyme hydrolysis process is very slow and time consuming as compared to the acid hydrolysis. Further, efficient enzyme recovery and reuse is not addressed well.
- Hemicellulosic fraction of lignocellulosic biomass comprises about one quarter of the total carbohydrate content and must be used for economical production of ethanol like commodity product, whereas it is lost in many cases like steam explosion, alkaline pretreatment etc.
- In most of the pretreatment/hydrolysis processes due to severe reaction conditions, the soluble sugars liberated further converted to their respective dehydration products like furfural (from xylose) and hydroxymethyl furfural (from glucose), thus reducing the overall availability of these sugars for bioconversion to valuable products.
- Since these sugar dehydration products and phenolic compounds generated during the hydrolysis process steps are known to be inhibitory to the microorganisms in further fermentation thus essentially required to remove or reduce in acceptable concentration. Detoxification, an additional process step is used to remove or reduce the concentration of these inhibitory compounds. Also 8-10% sugar loss is reported during overliming.
- In some cases like concentrated acid treatment, mixture of soluble sugars (C5 and C6) obtained and no native microorganism is available for efficient bioconversion of these sugars to ethanol.
- The widely accepted ethanol-producing GRAS organisms *Saccharomyces cerevisiae* and *Zymomonas mobilis* are not able to ferment C5 sugars like xylose. Using these organisms alone to ferment the mixture of sugars, C5 sugars remained unutilized, thus reducing the overall conversion efficiency.

- Though *P. stipitis* utilize both type of sugars but alone cannot be used because of its low ethanol tolerance and productivity. A co-culture system of suitable microorganisms may be useful to utilize both types of sugars.

2.11 The overall process

Extensive literature survey suggested that the ethanol production from lignocellulosic biomass consisted of mainly three steps namely: (1) hydrolysis, (2) fermentation and (3) recovery. Furthermore these steps may be divided in various subunits depending on the type of processes used. A generalized view in accordance with the existing technologies is given in the figure 2.11.

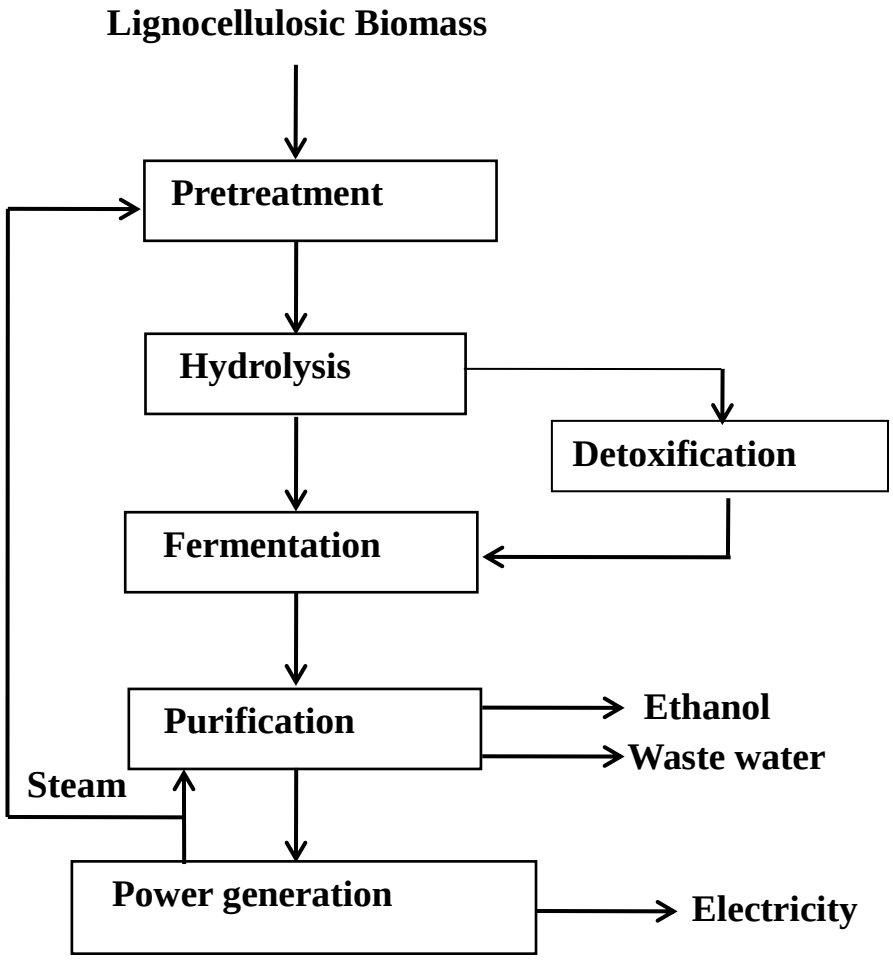


Figure 2.11 A generalized view of ethanol production from lignocellulosic biomass via existing technologies.

2.12 Objectives

After going through the literature thoroughly and considering the bottlenecks of the problem the following objectives for the present investigation has been fixed. Looking at the advantages of using perennial grass as raw material Kans grass (*Saccharum spontaneum*) was thought to be proper for the study.

(1) To characterization the Kans grass biomass (*Saccharum spontaneum*).
(2) To optimizethe process parameters for total reducing sugars.
(3) To study the effect of toxics including ethanol on growth and performance of microorganisms.
(4) To develop a suitable method that produces maximum TRS (both C5 and C6 sugars) with least amount of toxics generation. It was given due importance to get the C5 and C6 sugars separately.
(5) To develop a suitable co-fermentation technique to convert the TRS to ethanol in a single stage using two organisms (*Z. mobilis* and *P. stipitis*).

CHAPTER 3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

The sample of Kans grass biomass (*Saccharum spontaneum*) (KGB)was collected from the side of the Gang Nahar in Jwalapur (about 20 km from Roorkee), Haridwar (Uttarakhand), India. The photograph of the KGB is shown in figure 3.1(a). The sample was chopped in small pieces, washed and air dried. After six days of air drying sample was screened to select the fraction of particles with the size between 2.36 to 5.60 mm and homogenized in a single lot. The homogenized KGB was then oven dried at 70°C for overnight and was analyzed by using standard methods for determination of its main composition. Photograph of ready to analyze KGB is shown in figure 3.1(b).

3.1.2 Microorganisms and culture media

*Pichiastipitis*NCIM 3498 used in the present study was procured from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The culture was grown at 30 ± 0.2°C and stored at 4°C on agar slants of malt-glucose-yeast extract-peptone (MGYP) medium contained (g/L): malt extract 3; glucose 10; yeast extract 3; peptone 5 and agar 20, pH 6.0 ± 0.2. The medium used for inoculum preparation contained the same composition as used for storing except glucose which was replaced by xylose with same concentration. The media was sterilized by autoclaving at 121°C for 15 min.

Zymomonas mobilis MTCC 91 was procured from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMT) Chandigarh, India. The growth media of *Z. mobilis*consisted (g/L): glucose 20; yeast extract 5; $MgCl_2$ 0.1; $(NH_4)_2SO_4$ 0.1; KH_2PO_4 0.1. pH was adjusted to 5.0 ± 0.2. The media was sterilized by autoclaving at 121°C for 15 min wherein glucose was sterilized separately and mixed with other media components in laminar flow chamber under sterilized conditions.

All chemicals used in the study were of analytical grade and procured from HIMEDIA Laboratories, Mumbai and S D Fine-Chem Limited, (SDFCL) Mumbai. The chemicals were used as received.

Figure 3.1(a) Photograph of KGBas collected from the side of the Gang Nahar in Jwalapur (about 20 km from Roorkee), Haridwar (Uttarakhand), India in the month of September 2009.

Figure 3.1(b) Photograph of ready to analyze KGB sample.

3.1.3 Fermentation media

(a) *P. stipitis* fermentation

The synthetic fermentation media of *P. stipitis* was composed of (g/L): yeast extract 1.0; $(NH_4)_2HPO_4$ 2.0; $(NH_4)_2SO_4$ 1.0; $MgSO_4 \cdot 7H_2O$ 0.25; and trace element solution of 1 ml/L. The trace element solution contained (g/L): $CuSO_4 \cdot H_2O$ 2.5; $FeCl_3 \cdot 6H_2O$ 2.7; $MnSO_4 \cdot H_2O$ 1.7; $Na_2Mo_2O_4 \cdot 2H_2O$ 2.42; $ZnSO_4 \cdot 7H_2O$ 2.87; $CaCl_2 \cdot 6H_2O$ 2.4 and medium pH was adjusted to 6.0 ± 0.2 with concentrated H_2SO_4 (98%) of 0.5 ml/L. The media was sterilized by autoclaving at 121°C for 15 min. The sugar solution contained glucose 6.0 g/L and xylose 60.0 g/L was sterilized separately and mixed with other media components under aseptic conditions. The hydrolysate fermentation media was prepared by replacing sugar solution from KGB hydrolysate. Other constituents were same as present in synthetic media. The synthetic medium was used to compare the fermentation parameters.

(b) *Z. mobilis* fermentation

The synthetic fermentation media of *Z. mobilis* was composed of (g/L): glucose 100; $(NH_4)_2SO_4$ 1.0; KH_2PO_4 0.02; $MgSO_4$ 0.5; Yeast extract 6.5 and media pH was adjusted to 5.5 ± 0.2. The media was sterilized by autoclaving at 121°C for 15 min wherein glucose was sterilized separately and mixed with other media components in laminar flow chamber under sterilized conditions. The hydrolysate fermentation media was prepared by replacing sugar solution from KGB hydrolysate. Other constituents were same as present in synthetic media. The synthetic medium was used to compare the fermentation parameters.

3.1.4 Compositional analysis of KGB

Cellulose estimation: (a) acetic/nitric reagent: prepared by mixing 150 ml of 80% acetic acid 15 ml concentrated nitric acid; (b) anthrone reagent: prepared freshly by dissolving 0.2 g anthrone in 100 ml concentrated H_2SO_4 and chilled in ice cold water for 2 h prior to use and (c) cellulose; (d) H_2SO_4 (67 % v/v).

Hemicellulose estimation: (a) Neutral detergent solution (NDS): 18.61 g disodium ethylenediaminetetraacetate and 6.81 g sodium borate decahydrate were dissolved in 200 ml distilled water in 1 L beaker. 200 ml solution of 30 g sodium lauryl sulfate and 10 ml 2-ethoxy ethanol was added to it. 4.5 g disodium hydrogen phosphate was dissolved in 100 ml distilled water and added to above said solution. Finally volume was made up to 1 L with distilled water and pH was adjusted to 7.0; (b) decahydronaphthalene; (c) sodium sulphite; (d) acetone; (e) acid detergent solution (ADS): 20 g cetyltrimethyl ammonium bromide (CTAB) was mixed with 1 L, 1N H_2SO_4.

Lignin estimation: (a) H_2SO_4: 72 % w/w (ca. 12M H_2SO_4); (b) Deionized water.

3.1.5 Total reducing sugar (TRS) estimation

(a) sodium potassium tartrate solution: 200 g Rochelle salt was dissolved in 500 ml distilled water; (b) dinitrosalicylic acid reagent (DNS reagent): 1 g dinitrosalicylic acid, 0.2g crystalline phenol and 0.05 g sodium sulphite were dissolved by stirring in 100 ml 1% NaOH; (c) Stock solution: 0.1 g glucose was dissolved in 100 ml distilled water.

3.1.6 Xylose estimation

(a) thiourea (ca. 4 g) was mixed in 100 ml of glacial acetic acid; (b) 2 g p-bromoaniline was dissolved in 100 ml of acetic acid saturated with thiourea solution; (c) Stock solution: 0.1 g D-xylose was dissolved in 100 ml distilled water.

3.1.7 Ethanol estimation

(a) $K_2Cr_2O_7$ reagent: 34 g of $K_2Cr_2O_7$ was dissolved in 500 ml distilled water and 325 ml concentrated sulfuric acid; (b) 2 M NaOH solution; (c) stock solution of ethanol: 1 g/L stock solution of ethanol (AR) was prepared carefully. Deionized water was used for serial dilution to obtain various concentrations of ethanol.

3.1.8 Furfural estimation

(a) Furfural stock (0.10 mg/ml); (b) aniline (90%); (c) conc. HCl; (d) ethanol (50%).

3.1.9 Hydroxymethyl furfural

(a) Carrez I (15 g potassium ferrocyanide, $K_4FeCN_6.3H_2O$, was dissolved in d.w. and diluted to 100 ml); (b) Carrez II (30 g zinc acetate, $Zn(CH_3COO)_2.2H_2O$, was dissolved in d.w. and diluted to 100 ml); (c) sodium bisulfite: freshly prepared (0.2 g solid $NaHSO_3$ was dissolved in 100 ml d.w.).

3.1.10 Total phenolics estimation

(a) Gallic acid (0.5 g/L): stock solution was prepared by dissolving 0.05 g gallic acid in 10 ml ethanol and diluted to 100 ml with d.w.; (b) Folin-Ciocalteau reagent; (c) sodium carbonate solution (200 g anhydrous Na_2CO_3 was dissolved in 800 ml d.w. and boiled and after cooling few crystals of Na_2CO_3 was added, the solution was kept at room temperature for 24 h then filtered and d.w. was added to bring the volume to 1000 ml).

3.1.11 Media for viable cell estimation

(a) glucose-yeast extract-peptone (GYP) media contained (g/L): glucose 10; yeast extract 5; peptone 5; agar 15; (b) xylose-yeast extract-peptone (XYP) media contained (g/L): xylose 10; yeast extract 5; peptone 5; agar 15.

3.2 Analytical methods

3.2.1 Cellulose estimation

A simple and rapid colorimetric method was used for quantitative estimation of cellulose in the KGB samples [Updegraff, 1969]. In this method lignin and hemicellulose fractions were extracted with acetic/nitric acid reagent and remaining cellulose was dissolved in 67% (v/v) H_2SO_4 and determined by anthrone reagent. The method was based on the principle that cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cellodextrins which get dissolved and hydrolyzed to form glucose molecules on treatment with 67% H_2SO_4. The glucose molecules are dehydrated to form hydroxymethyl furfural which forms green colored product with anthrone and the color intensity is measured at 620 nm.

Preparation of standard curve

To prepare stock standard, 50 mg pure cellulose was dried for 6 h at 105°C and cooled in desiccators and dissolved in 10 ml 67% H_2SO_4. Then the solution was diluted to 500 ml with distilled water to obtain 100 µg cellulose/ml concentration. This stock solution was further diluted to obtain 50, 100, 150, 200, 250 µg/ml concentration and final volume was adjusted to 5 ml. Standard curve was prepared between cellulose concentration (µg/ml) and OD at 620 nm and given in figure 3.2.

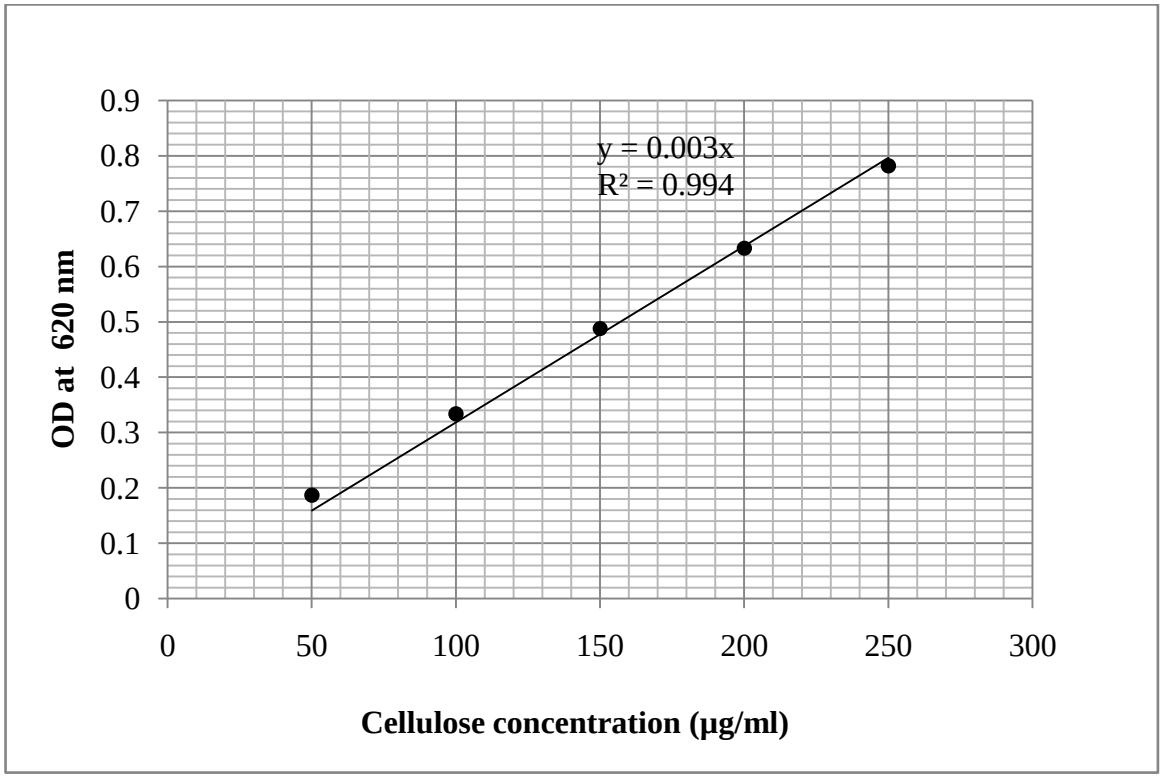


Figure 3.2 Calibration curve for cellulose estimation.

3.2.2 Hemicellulose estimation

The hemicellulose content of KGB was determined quantitatively by subtracting NDF to ADF [Goering and Van Soest, 1970]. Refluxing the lignocellulosic material with neutral detergent solution removes the water soluble materials other than the fibrous component. The left out material was weighed after filtration and expressed as Neutral Detergent Fiber (NDF). An acidified quaternary detergent solution was used to dissolve cell soluble and hemicellulose, leaving the residue of cellulose, lignin and minerals (ash). Acid detergent fiber (ADF) was determined gravimetrically as the residue remaining after extraction. A flow diagram of the process is given in Appendix A2.

Calculations: Following formulae were used:

$$\% \text{ NDF or } \% \text{ ADF} = \frac{W3 - w1}{w2 \times DM} \times 100$$

where, w_1 = weight of crucible (g); w_2 = initial sample weight (g); w_3 = weight of crucible and dried fiber (g) after treatment

DM = total dried matter in sample (estimated separately and given as

$$\% \text{ DM} = \frac{(\text{weight of dried sample and dish}) - \text{weight of dish}}{\text{weight of sample as received}} \text{ X } 100$$

$$\% \text{ Hemicellulose} = (\text{NDF} - \text{ADF})$$

3.2.3 Lignin estimation

The quantitative estimation of total lignin in a given lignocellulosic biomass was done according to Laboratory Analytical Procedures [Templeton and Ehrman, 1995 and Ehrman, 1996]. The Klason lignin determination was based on the principle that the residue remaining after extensive acid hydrolysis of the biomass sample, corrected for its ash content is referred to as acid insoluble lignin (AIL). A small portion of lignin was solubilized during the hydrolysis process and referred as acid soluble lignin (ASL). Addition of these two (AIL and ASL) gives the total lignin content of the given biomass sample. A process flow diagram is given in Appendix A3.

Calculations

Following formulae were used:

$$\% \text{ AIL} = \frac{W2 - W3}{W1} \times 100$$

where, w_1 = initial sample weight (dried at 105°C) in g; w_2 = weight of crucible, AIL and acid insoluble ash in g

w_3 = weight of crucible and acid insoluble ash in g

$$\% \text{ ASL} = \frac{A}{b \times a} \times df \times V \times 100$$

where, A = absorbance at 205 nm

b = cell path length (1 cm)

a = absorptivity (110 L/g cm)

df = dilution factor

V = volume of filtrate in L

% Total lignin = (AIL + ASL)

3.2.4 Ash estimation

The ash content is an approximate measure of the mineral content and other inorganic matter present in biomass. The quantitative estimation of ash content in the KGB was done according to the method described in Laboratory Analytical Procedure [Ehrman, 1994]. The residue remaining after the dry oxidation of (oxidation at 550 to 600°C) of biomass gives the ash content present in that biomass. The process flow diagram is given in Appendix A4.

Calculations

Following formula was used:

$$\% \text{ Ash} = \frac{W1}{W2} \times 100$$

where, W_1 = weight of (crucible + ash) – weight of crucible in g

W_2 = weight of (crucible + sample) – weight of crucible in g

3.2.5 Total reducing sugar estimation

Total reducing sugar (TRS) was estimated by using dinitrosalicylic acid (DNS) reagent [Miller, 1959]. It is rapid, sensitive and adoptable during handling of a large number of samples at a time. Stock solution of glucose was used for preparation of calibration curve.

Procedure

1 ml properly diluted sugar solution was taken and 2 ml freshly prepared DNS reagent was added. The content was kept in a boiling water bath for 5 min. When the content was still at warm condition, 1ml 40% Rochelle salt solution was added. Cooled and 17 ml distilled water was added.

The intensity of the color developed was recorded at 575 nm against blank. The blank was prepared by replacing sugar solution with equal amount of distilled water. A series of standards using glucose stock solution was run to prepare the calibration curve (glucose, mg/ml vs. OD). The calibration curve of glucose for TRS estimation is given in figure 3.3. For estimation of reducing sugar concentration in the sample, 1 ml properly diluted sample was used in place of glucose stock solution and rest procedure was same as mentioned above. The calibration curve was used to calculate final concentration of reducing sugar in the sample.

3.2.6 Xylose estimation

The method was based on the formation of furfural from xylose (pentose sugar) in acetic acid containing thiourea at 70°C and the reaction of furfural with p-bromoaniline acetate to form a pink colored compound [Roe and Rice, 1947]. Stock solution of xylose was used for preparation of calibration curve.

Procedure

In a routine assay, 1 ml of p-bromoaniline reagent was added to 0.2 ml sample and standard solutions for both the tests and the corresponding blank. Blank was used to correct the furfural already present in the sample. The tests and blanks were prepared in an identical manner, except that all tests were placed in 70°C water bath for 10 min while all blanks were kept at room temperature only. All the tubes were then incubated in dark at room temperature for 70 min. the absorbance of all tests and corresponding blanks were read at 520 nm using spectrophotometer. A calibration curve was prepared using different concentrations of xylose (prepared from stock solution) vs. OD and given in figure 3.4.

3.2.7 Ethanol estimation

(A) Colorimetric method: This method is based on the principle that under acidic conditions ethanol is oxidized to acetaldehyde by potassium dichromate and acetaldehyde is self oxidized to acetic acid:

$$3CH_3CH_2OH + K_2Cr_2O_7 + 4H_2SO_4 \longrightarrow 3CH_3CHO + Cr_2(SO_4)_3 + K_2SO_4 + 7H_2O$$

$$3CH_3CHO + K_2Cr_2O_7 + 4H_2SO_4 \longrightarrow 3CH_3COOH + Cr_2(SO_4)_3 + K_2SO_4 + 4H_2O$$

The intensity of green color thus produced can be measured by spectrophotometer and compared to known ethanol concentration using calibration curve to estimate the ethanol concentration in the original sample. The stock solution of ethanol was used to prepare calibration curve of ethanol.

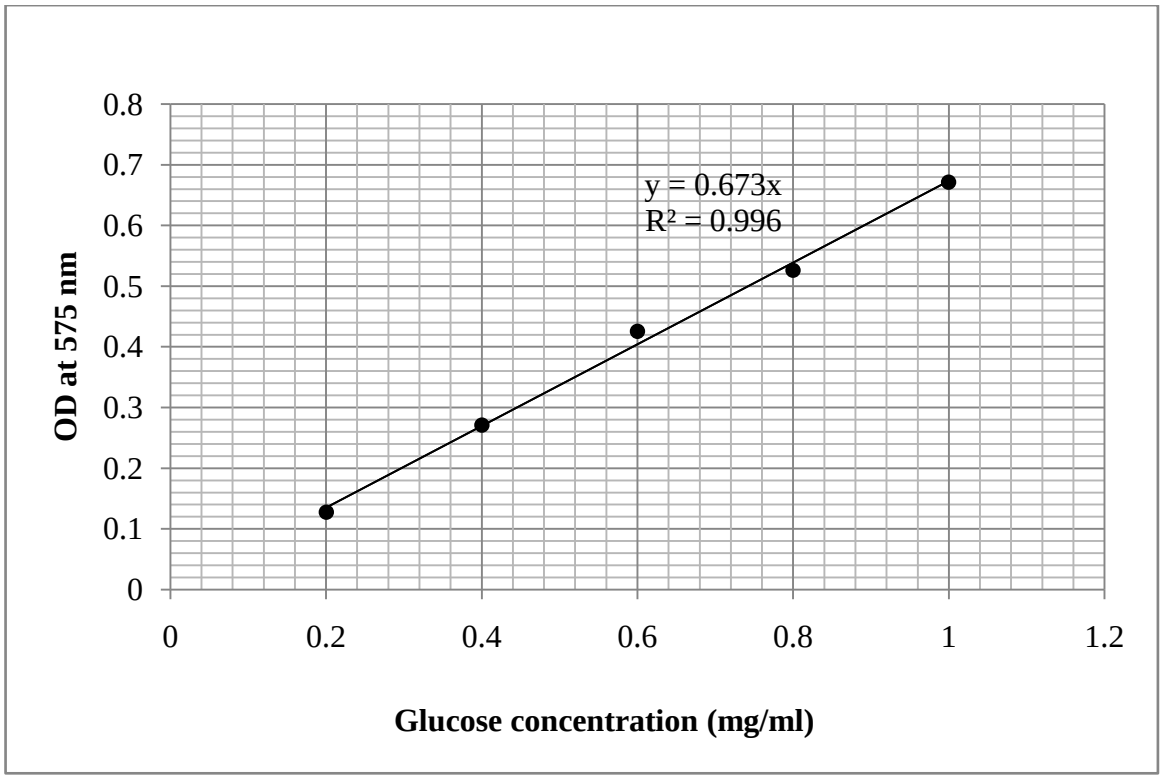


Figure 3.3 Calibration curve of glucose for reducing sugar estimation.

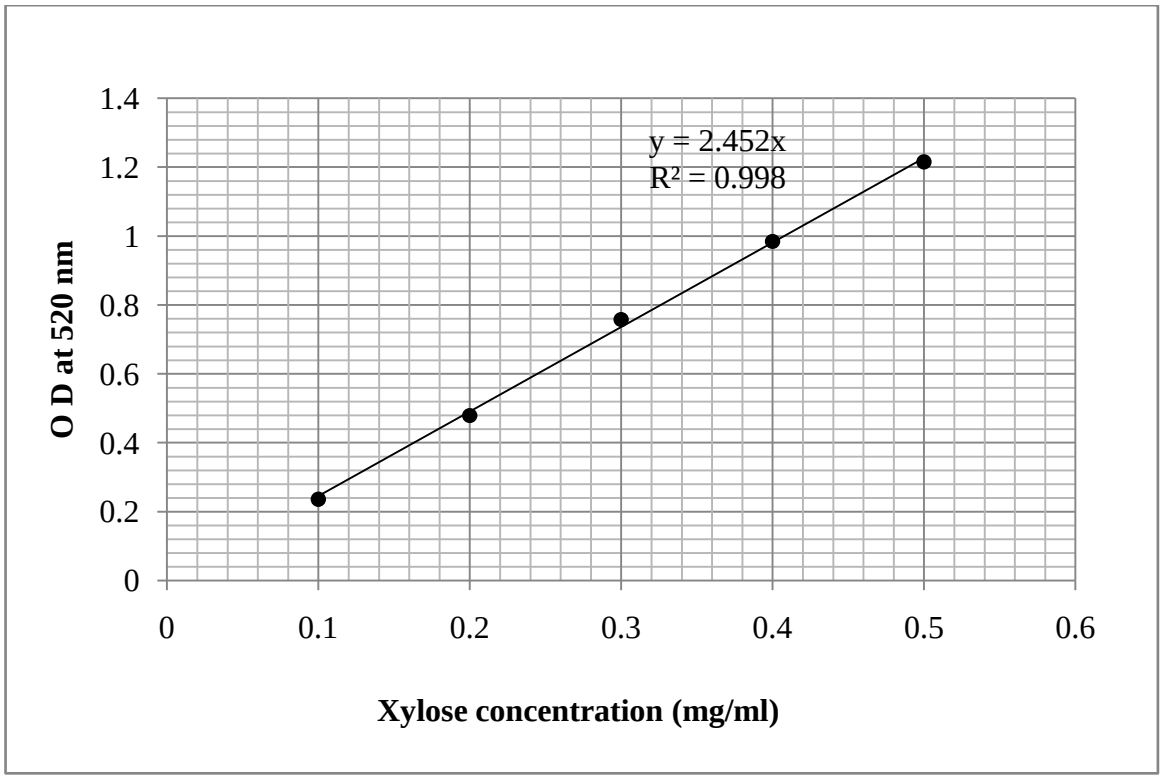
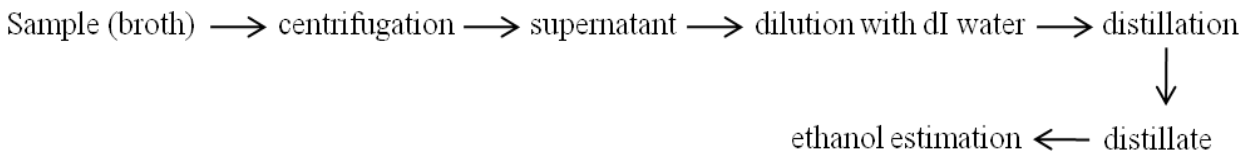


Figure 3.4 Calibration curve of xylose for pentose sugar estimation.

Procedure

1 ml sample of different concentrations of the stock solution were taken in test tubes and 3 ml $K_2Cr_2O_7$ reagent was added to each. Then kept at 60°C in a water bath for 30 min. ca. 2 ml 2M NaOH was added to remove excess dichromate. Absorbance was recorded at 600 nm against blank (where ethanol sample was replaced with deionized water and processed simultaneously with the samples). A calibration curve was prepared between OD vs. ethanol concentration figure 3.5. For estimation of ethanol concentration in the broth sample the following steps were followed:



(B) GC method: Ethanol was estimated by Gas Chromatography (DANI) equipped with a flame ionization detector and Solgel wax column. Nitrogen gas was used as mobile phase at a flow rate of 30 ml/min. The injector, detector and oven temperatures were maintained at 200°C, 200°C and 150°C respectively. Prior to GC analysis, the collected samples were diluted appropriately with deionized water and filtered using 0.2 µm membrane filter and kept at 4°C to prevent microbial growth.

3.2.8 Biomass estimation

(a) *P. stipitis*: Microbial biomass growth of *P. stipitis* was measured turbidometrically at 600 nm by diluting samples in the ratio 1:5 with 1 N HCl (to dissolve calcium salts), using a cuvette with 1 cm light path in Double Beam UV-VIS spectrophotometer (Cary 100, Varian) and culture dry weight was measured by centrifugation and drying cells at 105±2°C for 4 h, cooled in desiccators at room temperature and reweighed until no weight change between consecutive measurements was observed. A calibration curve of OD vs. cell concentration was prepared figure 3.6.

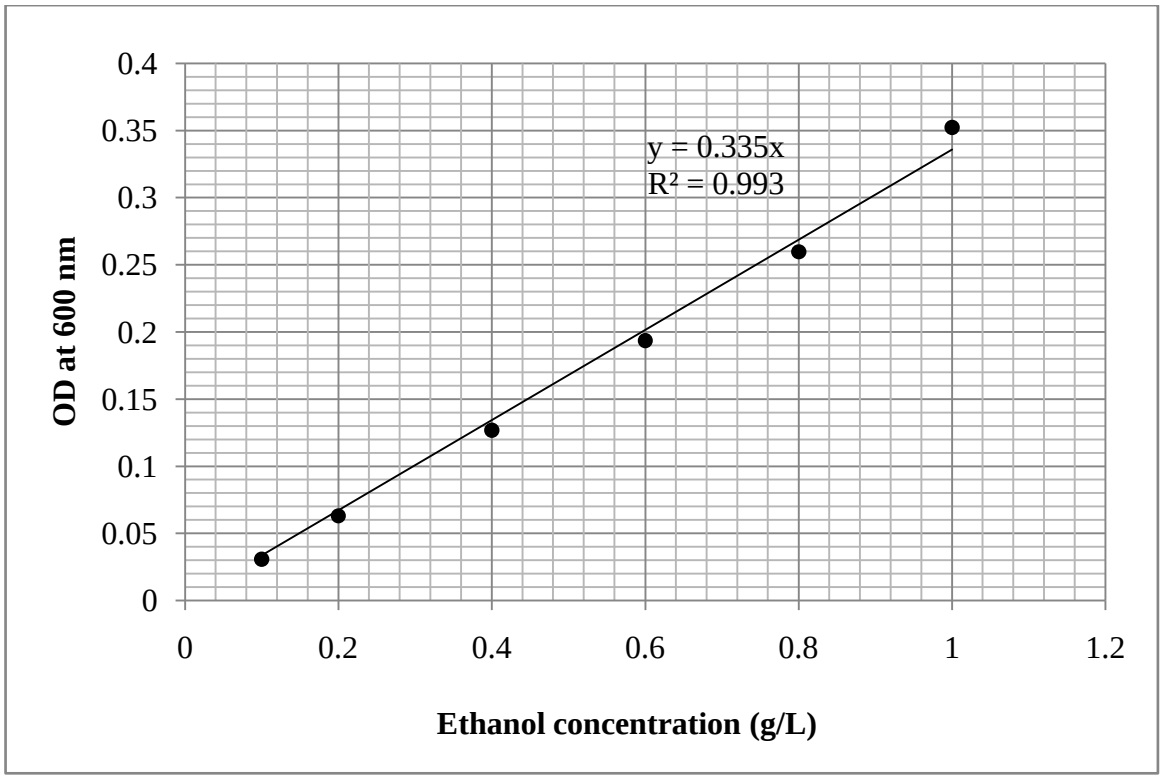


Figure 3.5 Calibration curve for ethanol estimation.

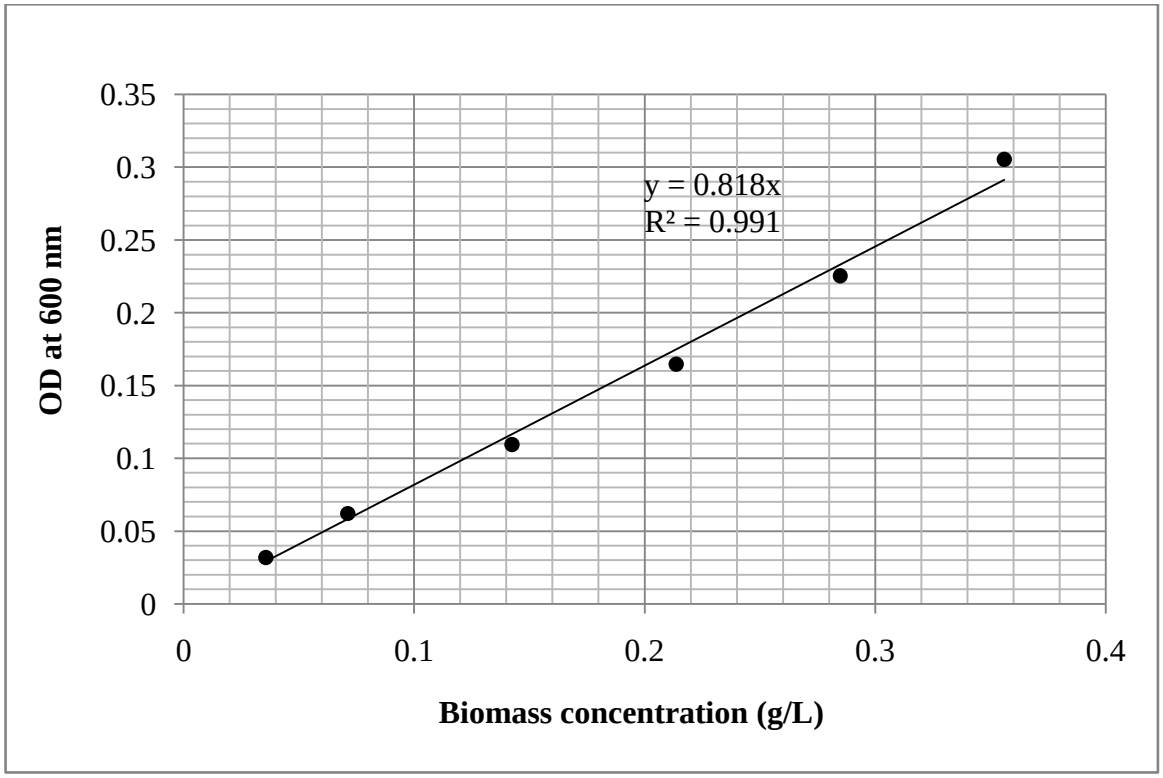


Figure 3.6 Calibration curve for biomass estimation of *P. stipitis*.

(b) *Z. mobilis*:

The broth samples collected at different time intervals were centrifuged at 5000 rpm for 10 min and pellets were re-suspended in minimum amount of distilled water. After appropriate dilution absorbance was recorded at 600 nm and cell concentration was obtained by using calibration curve of *Z. mobilis*. The calibration curve for *Z. mobilis* was prepared in the similar manner as mentioned for *P. stipitis* and given in figure 3.7.

(c) Viable cell concentration

The samples withdrawn from the bioreactor were immediately centrifuged at 5000 rpm at 8°C for 10 min. The supernatant and pellets were separated aseptically. The pellets were re-suspended appropriately diluted appropriately (ca. 10^5 times) with sterilized distilled water and used for viable cell count. The diluted cell suspension was plated out onto appropriate agar plate and incubated for 48 h. Plates containing maximum up to 200 colonies were used to calculate the viable cell concentration (cfu/ml). Two types of agar plates were used (GYP and XYP media). The plates were incubated at 30°C for 48 h. The colonies of both type of microorganisms, yeast and bacterium were appeared on the first type of plate (GYP) whereas, only yeast colonies were present on the second type of plate (XYP) as xylose was assimilated by *P. stipitis* cells and not by the *Z. mobilis*. The concentration of cells was calculated through the enumeration of cells from GYP and XYP plates. The difference between numbers of colonies on GYP to XYP was referred as the *Z. mobilis* cell concentration (cfu/ml), whereas numbers of cells on XYP was referred as *P. stipitis* cell concentration (cfu/ml). Furthermore the *P. stipitis* colonies were larger and opaque, whereas *Z. mobilis* colonies were smaller with a clear edge. For a particular co-culture sample the number of colonies of *P. stipitis* on GYP and XYP were more or less (maximum 5% difference was allowed).

3.2.9 Furfural estimation

Furfural derived from xylose degradation during the hydrolysis of lignocellulosic biomass need to be estimated due to its toxicity in further fermentation process. The estimation of furfural in the lignocellulosic hydrolysate was based on the method given by Al-Showiman (1998). A calibration curve was prepared with different concentrations of furfural (ranging from 0.01 to 0.10 mg/ml). For this a stock of furfural (0.10 mg/ml) was prepared and serially diluted in 50% ethanol. 1 ml of stock solution of various concentrations of furfural was taken in test tubes and 1 ml aniline (90%) and 0.25 ml conc. HCl were added and volume was made up to 5 ml. All the tubes along with blank were kept in dark for 15 min at room temperature. The absorbance was recorded at 510 nm (obtained by scanning the standard sample). The blank was prepared

by replacing furfural to ethanol (50%). All the samples were prepared in triplicate and average OD values were taken for plotting calibration graph. The calibration curve was a plot of absorbance vs concentration of standard furfural samples and shown in figure 3.8. The calibration graph gave a linear relationship (R^2=0.990) over the range of 10 ppm to 100 ppm.

3.2.10 Hydroxymethyl furfural estimation

Hydroxymethyl furfural (HmF) generated during acid hydrolysis of lignocellulosic biomass as a result of glucose (C6) degradation. The HmF concentration was measured in the hydrolysate by using the method proposed by White (1979) and Beer-Lambert law was used for calculations. The estimation of HmF was based on the measurement of UV absorbance of HmF at 284 nm. In order to avoid the interference of other components at this wave length, the difference between the absorbance of a clear aqueous solution and the same solution after addition of bisulfate was determined. The HmF content was calculated after subtraction of the background absorbance at 336 nm. Appropriately diluted 5 ml hydrolysate solution was taken in a test tube and 0.5 ml Carrez I (15 g potassium ferrocyanide, $K_4FeCN_6.3H_2O$, was dissolved in d.w. and diluted to 100 ml) solution was added to it. After mixing the content freshly prepared 0.5 ml Carrez II (30 g zinc acetate, $Zn(CH_3COO)_2.2H_2O$, was dissolved in d.w. and diluted to 100 ml) solution was added and mixed well using vortex mixer. The solution was then filtered and first 2 ml was discarded and 1 ml sample filtrate was taken in a separate test tube. 5 ml d.w. was then added to the sample filtrate tube and 5 ml freshly prepared sodium bisulfite (0.2 g solid $NaHSO_3$ was dissolved in 100 ml d.w.) solution was added to the reference tube. The content of all the tubes were well mixed and dilution (if required) were made with d.w. and sodium bisulfite respectively for sample and reference. Absorbance of sample and reference was measured at 284 nm and 336 nm respectively. All the sample and reference were prepared in duplicate and average value of OD was taken for calculation. Concentration of HmF was calculated by using Beer-Lambert law as follows:

$$c = \frac{A}{\epsilon \, . \, l} D$$

Where, A = (absorbance of sample at 284 nm, A_{284} – absorbance of reference at 336 nm, A_{336})

ϵ = molar absorptivity of HmF at 284 nm = 16830 L/ mol . cm

l = path length = 1 cm

D = dilution factor

$$\text{i.e.c } (\text{g/L}) = \frac{(\text{A284}-\text{A336})}{16830\ (\text{L}/(\text{mol}\,.\ \text{cm})\,.\ 1\ \text{cm}} \text{ D x } 126\ (\text{g/mol})$$

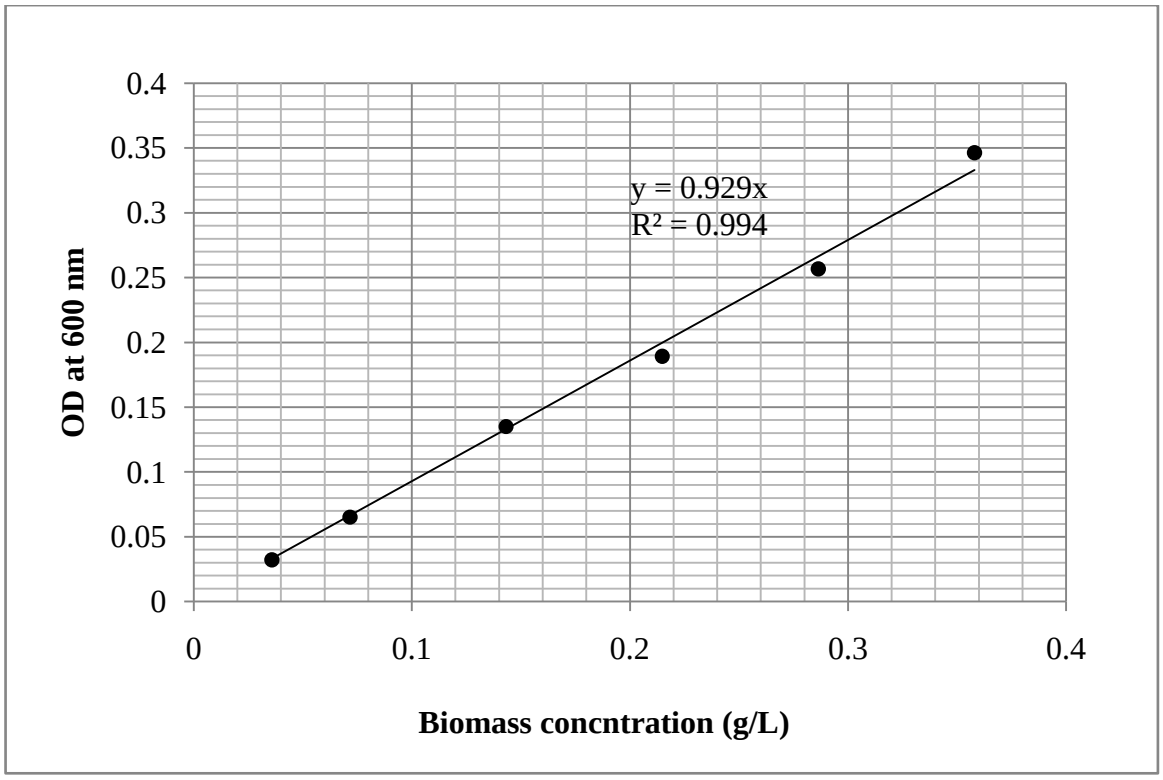


Figure 3.7 Calibration curve for biomass estimation of *Z. mobilis*.

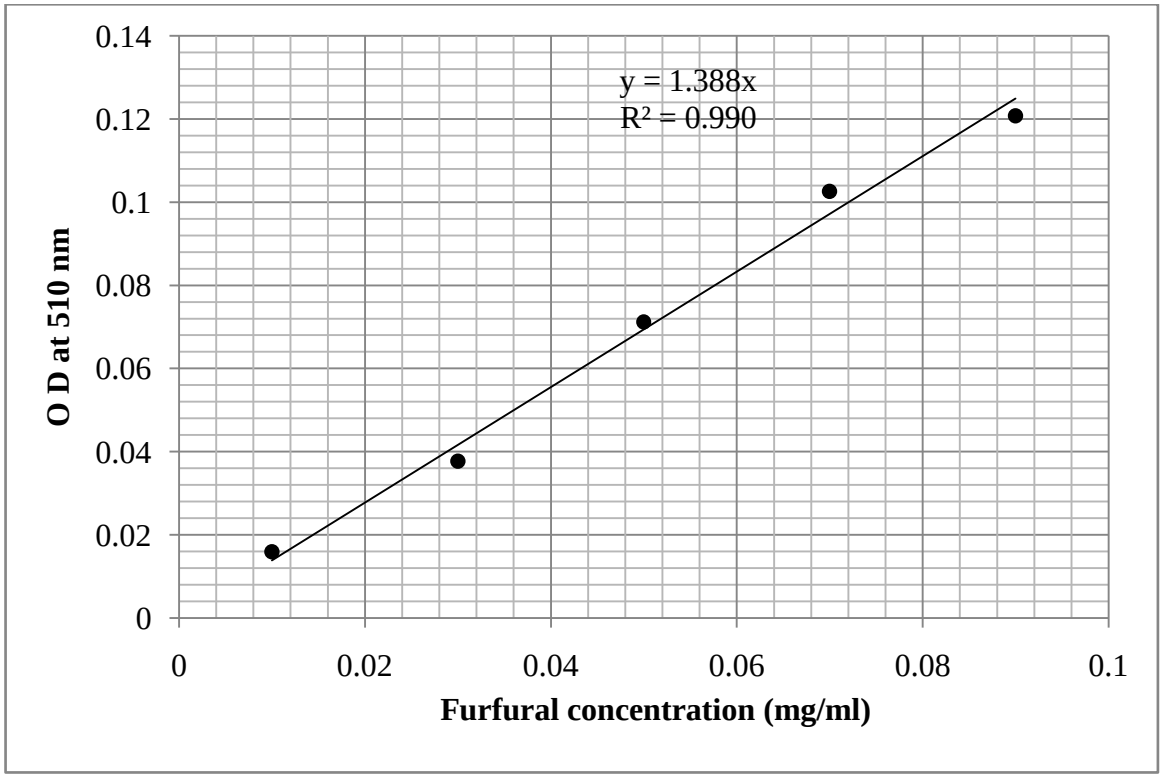


Figure 3.8 Calibration curve of furfural.

3.2.11 Total phenolics estimation

A variety of phenolics or aromatic compounds generated from lignin fraction of lignocellulosic biomass during its hydrolysis. These compounds are known to be inhibitory to the microorganisms. Estimation of these phenolic compounds and reporting the values in a single number is essentially required to monitor the concentration of these toxics in hydrolysate. The widely used method, Folin-Ciocalteau (FC) colorimetry[Waterhouse, 2002] was used and gallic acid was taken as model compound. Folin-Ciocalteaucolorimetry is based on the chemical reduction of the reagent, a mixture of tungsten and molybdenum oxides. The products of metal oxide reduction have a blue color that exhibit a broad light absorbance with a maximum at 765 nm wave length. The intensity of light absorbance is proportional to the concentration of phenols.

A calibration curve was prepared using different concentrations of gallic acid. The stock solution (0.5 g/L) of gallic acid was prepared by dissolving 0.05 g gallic acid in 10 ml ethanol and diluted to 100 ml with d.w. Different dilutions ranging from 50 to 500 mg/ml were made using d.w. From these dilutions, 20 µL standard solution was taken in glass cuvette. 1.58 ml d.w. and 100 µL FC reagent was added. The content was thoroughly mixed by pipetting and kept for 5 min. 300 µL sodium carbonate solution (200 g anhydrous Na_2CO_3 was dissolved in 800 ml d.w. and boiled and after cooling few crystals of Na_2CO_3 was added, the solution was kept at room temperature for 24 h then filtered and d.w. was added to bring the volume to 1000 ml) mixed and incubated at room temperature for 24 h. The absorbance was recorded at 765 nm against the blank which was prepared by replacing gallic acid solution with d.w. in the same amount. All the samples were prepared in triplicate and average value of absorbance was used to prepare calibration curve. The calibration graph (figure 3.9) between gallic acid concentration (mg/L) and OD values was found linear with $R^2 = 0.994$. The calibration curve was used to determine the total phenolics in the hydrolysate sample in terms of gallic acid equivalents (GAE) with unit mg/L.

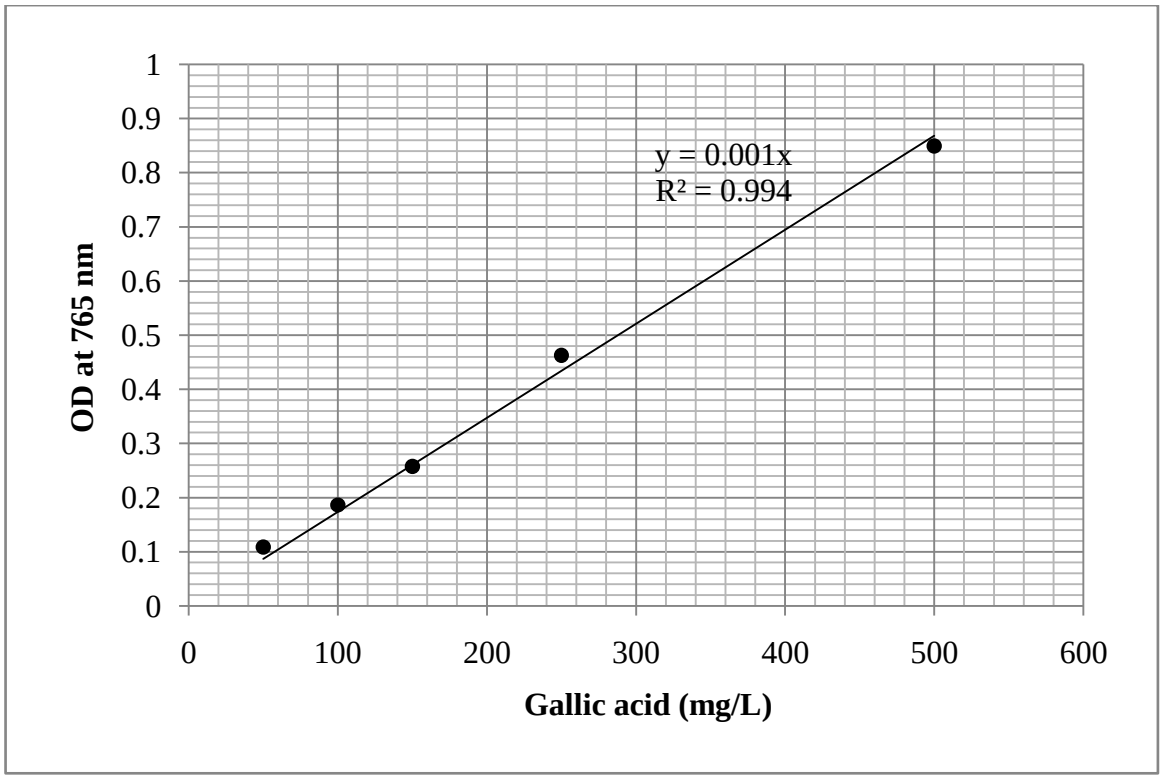


Figure 3.9 Calibration curve for total phenolics estimation as gallic acid equivalant (GAE).

3.2.12 Scanning Electron Microscopy

The structural changes in the KGB during treatment were qualitatively studied using Scanning electron microscopy (SEM). The native and treated samples of KGB were washed twice with deionized water and dried. The dry samples were mounted on aluminum stubs. A thin layer of gold was sputtered on to the mounted samples to reduce electron altering effects. Finally, the gold coated samples were observed with a scanning electron microscope (LEO Electron Microscopy Ltd, Cambridge, England; model: LEO 435 VF) equipped with Robinson detector and at the accelerating voltage of 15kV.

3.3 Processes

3.3.1 Characterization of KGB

The aforementioned (section 3.1.1) KGB sample was analyzed for its major components viz. cellulose, hemicellulose, lignin (soluble and insoluble) and ash content. The complete process flow sheets for cellulose, hemicellulose (NDF and ADF estimation), lignin (ASL and AIL) and ash content are given in Appendix A1, A2, A3 and A4 respectively.

3.3.2 Optimization of TRS production from KGB by single step hydrolysis

The effect of variables, acid concentration (A), biomass loading (B) and reaction time (C) were studied for total reducing sugar production from KGB. The acid used in the present investigation was H_2SO_4.

Scheme

Acid hydrolysis experiments of KGB were carried out in Erlenmeyer flasks as per the experimental plan. The experimental range and levels of independent variables i.e. acid concentration (A), biomass loading (B) and reaction time (C) are laid down in table 3.1. Operating temperature was kept constant for all experiments at 100°C i.e. normal boiling temperature of water at standard atmospheric condition. All the experiments were carried out in triplicate and mean of three readings were taken as result for a particular experiment.

Table 3.1 Experimental range and levels of independent variables

Independent variables	Symbol	Range and levels				
		-α	**-1**	**0**	**+1**	**+ α**
Acid Concentration, % (w/w)	A	33.18	40	50	60	66.82
Biomass loading, % (w/v)	B	1.59	5	10	15	18.41
Reaction time (min)	C	9.55	30	60	90	110.45

Experimental design and RSM

In the experimental plan, response surface methodology (RSM) was utilized to optimize the hydrolysis process and a 2^3 rotatable central composite design (CCD) was adopted in order to fit a second order model and the design consisted of 20 set of experiments. It included eight experiments for factorial portion (2^k=8, where k is the number of independent variables, 3 in this case), six experiments for axial points (2k=6) and six replications of the center point used to check the reliability of the data for lack of fit test (Montgomery, 2001). The value of α was calculated as 1.682 where $\alpha = 2^{k/4}$, (k=3, the number of variables).The details of experimental design with coded and actual levels of each factor are summarized in table 3.2.

The second order empirical model was selected that relates the response to the independent factors for predicting the optima point. The complete second order polynomial model equation (3.1) to be fitted to the yield values was expressed as:

$$Y=\beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (3.1)$$

where, Y represents response variable i.e. total reducing sugar in % (g TRS/g total carbohydrate content, TCC). $\beta_0$is offset value, β_1, β_2 and β_3 are coefficients of linear terms, β_{11}, β_{22} and β_{33} are coefficients of quadratic terms and β_{12}, β_{13} and β_{23} are coefficients of interactive terms.

Statistical analysis

The statistical software package Design–Expert®8.0, Stat–Ease Inc., Minneapolis, MN, USA was used for experimental design and subsequent regression and graphical analysis of the experimental data. All experiments were done in triplicate, and the average TRS produced was taken as the response.

Table 3.2 Central composite design consisting of 20 experiments (coded and actual units).

Run no.	Variables					
	Coded			Actual		
	A	B	C	A	B	C
1	-1	-1	-1	40	5	30
2	1	-1	-1	60	5	30
3	-1	1	-1	40	15	30
4	1	1	-1	60	15	30
5	-1	-1	1	40	5	90
6	1	-1	1	60	5	90
7	-1	1	1	40	15	90
8	1	1	1	60	15	90
9	-1.682	0	0	33.18	10	60
10	1.682	0	0	66.82	10	60
11	0	-1.682	0	50	1.59	60
12	0	1.682	0	50	18.41	60
13	0	0	-1.682	50	10	9.55
14	0	0	1.682	50	10	110.45
15	0	0	0	50	10	60
16	0	0	0	50	10	60
17	0	0	0	50	10	60
18	0	0	0	50	10	60
19	0	0	0	50	10	60
20	0	0	0	50	10	60

3.3.3 Dilute acid hydrolysis (single step) and pentose fermentation

Dilute acid hydrolysis of KGB

The oven dried sample of KGB was refluxed with 2% sulfuric acid (1:10 biomass to acid ratio) for a period of 6 h at 100°C, in conical flasks with occasional stirring. Hydrolysate (liquid portion) was filtered to separate the unhydrolysed solid residue. Solid residue was then rinsed with warm water to recover the rest of soluble sugar and added to the filtrate.

Conditioning of KGB acid hydrolysate medium

The two steps process was used to remove or reduce the concentration of toxic compounds, generated during the acid hydrolysis of KGB. In first step the concentration of volatile components were reduced by heating the acid hydrolysate (1L) at 100°C for 15 min and made up the loss with hot distilled water. In the second step pH of the acid hydrolysate was raised to 2.0 with 1N NaOH and then overlimed with solid $Ca(OH)_2$ up to pH 10.0 in combination with 0.1% sodium sulfite. Liquor was then filtered to remove insolubles and re-acidified to pH 6.0 ± 0.2 with 1 N sulfuric acid. The filtrate was concentrated under vacuum at 60°C to achieve 60 to 65 g/L of xylose concentration. The total reducing sugar and pentose sugar composition in the acid hydrolysate were determined. The main fermentable sugar found was xylose, derived from hemicellulosic fraction of the KGB; therefore, the acid hydrolysate should be referred as hemicellulose acid hydrolysate hence forth in this experiment. The resulting solution was stored at -10°C for further use as substrate. The fermentation media was prepared as mentioned in section 3.1.3 (a). The synthetic medium was used to compare the fermentation parameters.

Recovery of ethanol

After separation of cells by centrifugation (REMI cooling centrifuge C-24 BL, REMI Instruments, India) at 8°C, the fermented broth was diluted with demineralized water and distilled in a distillation assembly at 79 ± 1°C. Temperature was controlled accurately to prevent mixing of higher boiling distillate like water in the broth.

Software

The estimation of the parameters of the mathematical model and the statistical analysis were carried out using the software "Polymath" version 6.10 (CACHE Corporation, USA).

Model development

To develop basic understanding for the bioconversion of xylose to ethanol, kinetic models were proposed with the assumptions that the yeast cells were active and the agitation speed of 150

rev min^{-1} was in excess to maintain uniform conditions inside the fermentation flasks to provide adequate mass transfer. The energy system as controller of cells and product formation as proposed is given in figure 3.10.

Fermentation

Batch fermentation was conducted in a 500 ml Erlenmeyer flask with a working volume of 125 ml. The fermentation medium was inoculated with 5% v/v inoculum (18 h culture, $1x10^7$cells/ml). The fermentation temperature was kept constant at 30 ± 0.2°C in an incubator shaker (Lab Therm LT-X Kuhner, Switzerland). The broth was kept under agitation at 150 rev/min. Samples were taken at regular time intervals during fermentations to determine the concentrations of cell mass, ethanol and residual sugars in the broth. All experiments were carried out in duplicate.

Calculation of Kinetic Parameters

The non-linear regression technique was used to estimate model parameters. The parameters were estimated using the experimental data of biomass concentration (x), reducing sugar concentration (s) and ethanol concentration (p). The rate of growth of cells r_x (dx/dt) and rate of ethanol formation r_p (dp/dt) were calculated using these data. The coefficients of the models were estimated using software package "Polymath" version 6.10.

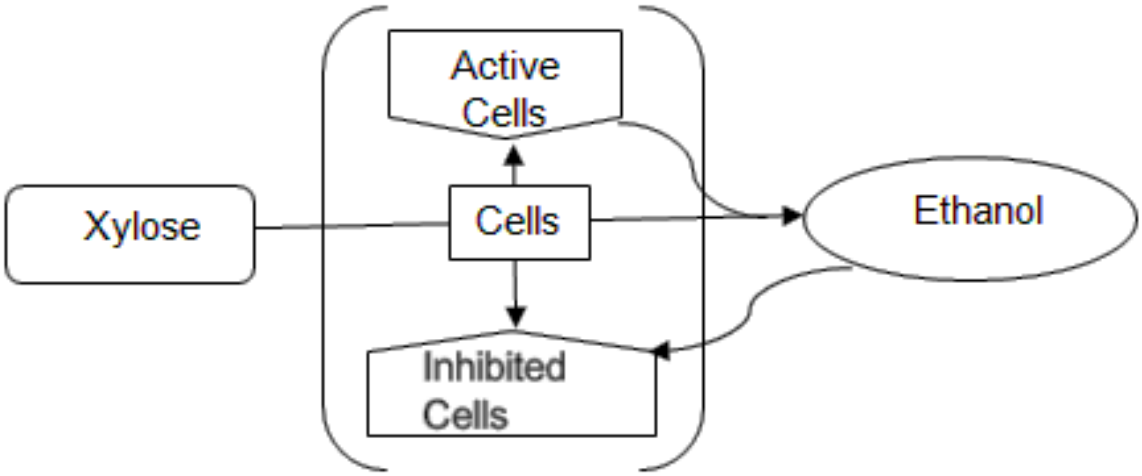


Figure 3.10 The energy system as controller of cells and product formation.

3.3.4 Effect of toxics on microbial growth

(a) Effect of furfural on specific growth rate of *Pichia stipitis*

The effect of furfural on the growth of *P. stipitis* NCIM 3498 was evaluated by adding different concentrations of furfural to the sterilized growth media given in section 3.1.2 (glucose was replaced with 20 g/L xylose) by using membrane syringe filter of pore size 0.2 µm in a laminar flow inoculation chamber. Various concentrations of furfural added were 0.1, 0.2, 0.3, 0.4 and 0.5 g/L whereas; no furfural was added in the control flask.

Fermentation conditions

Batch fermentation was conducted in 250 ml Erlenmeyer flask with a working volume of 50 ml. The fermentation medium was inoculated with 5% v/v inoculum (18 h culture, $1x10^7$cells/ml). The fermentation temperature was kept constant at 30 ± 0.2°C in an incubator shaker (Lab Therm LT-X Kuhner, Switzerland). The broth was kept under agitation at 150 rev/min. Samples were withdrawn at regular time intervals (from 4 h to 16 h) during fermentations to observe the exponential phase data and determination of the concentrations of cell mass in the broth. All the flasks (total six set of experimental flasks: 5 with furfural + 1 control) were incubated in duplicate. The broth samples were collected at regular intervals.

Calculations

Specific growth rate, µ (h^{-1}) of *P. stipitis* in the above mentioned experimental setups were determined by using their corresponding curve of ln (x/x_0) vs. t.

Where, t is fermentation time in h; x_0 is the cell concentration (g/L) at the onset of exponential phase and x is the cell concentration (g/L) at time t (h).

The % relative specific growth rate, μ_{rel} (%) was calculated by taking specific growth rate µ (h^{-1}) of *P. stipitis* from control flask as 100%.

The % reduction in specific growth rate μ_{red} (%) was calculated by subtracting μ_{rel} (%) from 100.

(b) Effect of vanillin on specific growth rate of *P. stipitis*

Vanillin was selected as the model phenolic compound to investigate its effect on the growth of *P. stipitis*. The growth media of *Pichiastipitis*NCIM 3498 was same as mentioned in the previous section except the inhibitor. In place of furfural different concentrations of vanillin (0.2, 0.5, 1.0, 1.5 and 2.0 g/L) was added to the growth media aseptically using membrane syringe filter of pore size 0.2 µm. No vanillin was added in the control flask. All the flasks (total six set of experimental flasks: 5 with vanillin + 1 control) were incubated in duplicate.

Fermentation conditions and calculations for μ (h^{-1}), μ_{rel} (%), μ_{red} (%) were same as mentioned in the previous section 3.3.4 (a).

(c) Effect of hydroxymethyl furfural on specific growth rate of *Z. mobilis*

Hydroxymethyl furfural (HmF) is primarily derived from glucose (hexose sugar) degradation during the acid hydrolysis of lignocellulosic biomass. It is also known to be inhibitory for microbial growth. Varying amount of HmF were added to the growth media of *Z. mobilis* and its effect were estimated on specific growth of *Z. mobilis*. Different concentration of HmF was added to the sterilized growth media (given in section 3.1.2)by using membrane syringe filter of pore size 0.2 μm in a laminar flow inoculation chamber. Various concentrations (0.2, 0.5, 1.0, 1.5 and 2.0 g/L) of HmF were added in growth media to estimate the effect of HmF to *Z. mobilbis*.

Fermentation conditions

Batch fermentation was conducted in 250 ml Erlenmeyer flask with a working volume of 50 ml. The fermentation medium was inoculated with 5% v/v inoculum (10 h culture, $1x10^8$cells/ml). The fermentation temperature was kept constant at 30 ± 0.2°C in an incubator (Lab Therm LT-X Kuhner, Switzerland) under static condition. The mouth of flask was sealed using parafilm. Samples were withdrawn at regular time intervals (from 2 h to 8 h) during fermentations to observe the exponential phase data and determination of the concentrations of cell mass in the broth. All the flasks (total six set of experimental flasks: 5 with HmF + 1 control) were incubated in duplicate.

Calculations

Calculations for specific growth rate of *Z. mobilis* μ (h^{-1}), μ_{rel} (%), μ_{red} (%) were done in similar manner as used for *P. stipitis* and mentioned in the section 3.3.4 (a).

(d) Effect of vanillin on specific growth rate of *Z. mobilis*

Vanillin (4-hydroxy-3- methoxybenzaldehyde) was used as the model phenolic compound to investigate its inhibitory effect on the growth of *Z. mobilis*. The growth media of *Z. mobilis* was same as mentioned in the previous section except the inhibitor. In place of HmF different concentrations of vanillin (0.2, 0.5, 1.0, 1.5 and 2.0 g/L) was added to the growth media aseptically using membrane syringe filter of pore size 0.2 μm. No vanillin was added in the control flask. All the flasks (total six set of experimental flasks: 5 with vanillin + 1 control) were incubated in duplicate. Fermentation conditions and calculations for μ (h^{-1}), μ_{rel} (%), μ_{red} (%) were same as mentioned in the previous section 3.3.4 (c).

3.3.5 Effects of ethanol concentration on growth of microbes

(a) Effect of ethanol concentration on specific growth rate of *P. stipitis*

For quantification of ethanol tolerance of *P. stipitis* the present experiment was designed. Batch fermentation was conducted in 250 ml Erlenmeyer flask with a working volume of 50 ml. Different concentration of ethanol ranging from 15-40 g/L (15, 20, 25, 30, 35 and 40 g/L) were exogenously added to the growth media of *P. stipitis* (section 3.2.2.4(a). All the flasks were inoculated with same inoculum concentration (5% v/v inoculum; 18 h culture; $1x10^7$cells/ml) and incubated at the same conditions (30 ± 0.2°C; 150 rev/min). The samples were withdrawn at regular interval during the growth phase of the microorganism to calculate specific growth rate. For calculation of the concentration of ethanol over which the growth of *P. stipitis* was inhibited, model proposed by Luong (1985) was used. All the flasks were incubated in duplicate and average value taken for the calculations.

(b) Effect of ethanol concentration on specific growth rate of *Z. mobilis*

For quantification of ethanol tolerance of *Z. mobilis* the present experiment was designed. Batch fermentation was conducted in 250 ml Erlenmeyer flask with a working volume of 50 ml. Different concentration of ethanol ranging from 60-110 g/L (60, 70, 80, 90, 100 and 110 g/L) were exogenously added to the growth media of *Z. mobilis* (section 3.1.2). The fermentation medium was inoculated with 5% v/v inoculum (10 h culture, $1x10^8$cells/ml). The fermentation temperature was kept constant at 30 ± 0.2°C in the incubator under static condition. The mouth of flask was sealed using parafilm. Samples were withdrawn at regular time intervals (from 2 h to 8 h) during fermentations to observe the exponential phase data for calculating specific growth rates. All the flasks were inoculated with same inoculum concentration and incubated at the same conditions. For calculation of the concentration of ethanol over which the growth of *Z. mobilis* was inhibited, model proposed by Luong (1985) was used. All the flasks were incubated in duplicate and average value taken for the calculations.

3.3.6 Novel process for fractionating hydrolysis of KGB (Multi-step)

Description of experimental setup

The heart of the experimental setup was an especially design glass (Borosil) column of 300 mm height with 60 mm internal diameter. The column was closed at the bottom and at the top a narrow mouth was provided with 20 mm diameter. A 10 mm diameter glass rod was centrally inserted from the bottom of the column. The total height of the rod was 300 mm (200 mm

inside the column and 100 mm outside the column). Small holes of 2 mm diameter were provided all around the rod inside the column i.e. 200 mm. The distance between two successive holes was 20 mm. The rod was closed at the top and opened at the bottom. Two openings have been provided to the column, one at the side of top section for collection of exhaust steam and other at the side of the bottom section to collect the liquid fractions from the column. The top side opening was attached to a condenser with rubber tubing and a stopper/valve. The bottom side opening was attached with high quality rubber tubing and a stopper/valve. The bottom end of the glass rod was also attached with the high quality rubber tubing with a stopper/valve and the other end of the tubing was connected with steam generator. A 50 ml syringe was attached to mouth of the column. To hold the column and condenser cast iron stands were used. A schematic diagram is shown in figure 3.11.

Operation

Different concentrations of sulfuric acid were prepared (1, 2, 5, 10, 15, 20, 25, 30 and 35%). All the parts were assembled according to the experimental setup. 10 g of oven dried KGB was charged in to the column. The bottom side stopper was closed and the other two were opened. After 45 min, steam insertion was stopped by closing the valve and the desired concentration of acid was introduced slowly from the mouth of the column with the help of syringe. Then the syringe was removed and mouth was closed. Again the steam insertion valve was opened. After 30 min of reaction the steam insertion was stopped and bottom side valve was opened to collect the liquid fraction. Then this valve was closed and next level of acid was introduced and steam was inserted similarly. The reaction time was kept constant for each level of acid concentration to 30 min. Total nine liquid fractions were collected and analyzed for the TRS, xylose, HmF, furfural and total phenolics. The care was taken to connect the various parts and the timings of opening and closing of the valves otherwise excess or low pressure may cause the damage to the system.

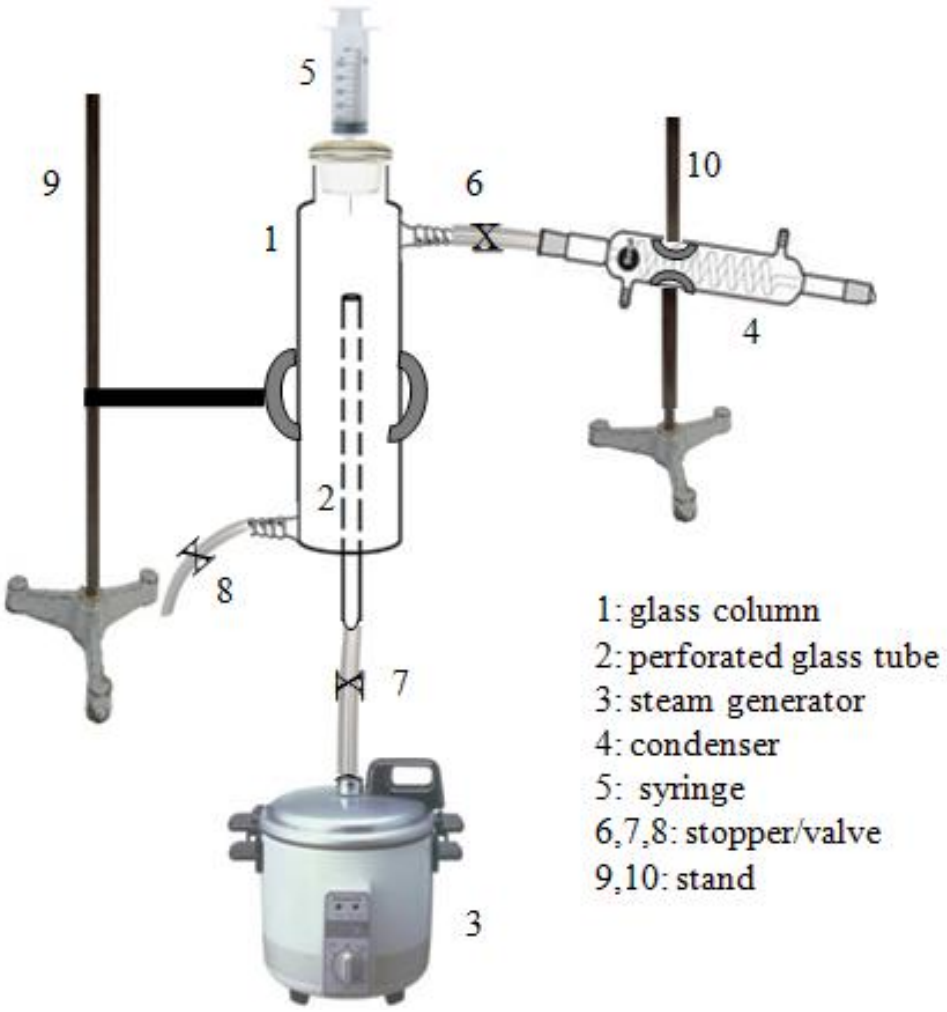


Figure 3.11 Schematic diagram of experimental setup for fractionating hydrolysis of KGB.

Conditioning of KGB acid hydrolysate production medium

After analysis of the desired compounds the fractions were mixed in such a way that two main streams of sugars were generated, first xylose rich fraction (XRF) and second glucose rich fraction (GRF). The pH of both the fractions were raised to 2.0 with 1N NaOH then 6.0 ± 0.2 by using $Ca(OH)_2$. The fractions were filtered to remove any precipitate. The filtrate were concentrated under vacuum at 60°C to achieve ~60 g/L of xylose concentration in first fraction and ~100 g/L glucose concentration in the second fraction. The resulting solution was stored at -10°C for further use as substrate.

The above mentioned sugar solutions were supplemented with the nutrients for further fermentation by the respective microorganism. The XRF to be fermented by *P. stipitis* was supplemented with media constituents as given in section 3.1.3 (a) and GRF to be fermented by *Z. mobilis* was supplemented with the media constituents as given in section 3.1.3 (b). The synthetic media were also prepared with the same composition as present in the respective hydrolysate medium without providing any toxic compounds. The synthetic medium was used to compare the fermentation parameters.

Fermentation of XRF by *P. stipitis*

Batch fermentations were conducted in a 250 ml Erlenmeyer flask with a working volume of 100 ml. The XRF was fermented with 5% v/v inoculum (18 h culture, $1x10^7$ cells/ml). The fermentation temperature was kept constant at 30 ± 0.2°C in an incubation shaker (Lab Therm LT-X Kuhner, Switzerland). The broth was kept under agitation at 150 rev/ min. All experiments were carried out in duplicate along with the control (inoculated with same inoculum density and kept under same conditions). Samples were taken at regular time intervals during fermentation to determine the concentrations of biomass, ethanol and residual sugars in the broth.

Fermentation of GRF by *Z. mobilis*

Batch fermentations were conducted in a 500 ml Erlenmeyer flask with a working volume of 150 ml. The GRF was fermented with 5% v/v inoculum (8 h, $1x10^8$ cells/ml). The fermentation temperature was kept constant at 30 ± 0.2°C in the same incubator under static condition. The mouth of the flasks was sealed with paraffin wax. Samples were taken at regular time intervals during fermentation to determine the concentrations of biomass, ethanol and residual sugars in the broth. All experiments were carried out in duplicate along with the control (inoculated with same inoculum density and kept under same conditions).

3.3.7 Development of sequential-co-culture system

The development of a suitable co-culture system (*P. stipitis* and *Z. mobilis*) for utilization of both types of sugars, xylose and glucose to produce ethanol in a single reactor system was done in two stages: (i) flask level (total working volume, 300 ml) using culture bottle and (ii) bioreactor level (total working volume, 4 L) using New Brunswick Bio Flow 110 bioreactor system. A schematic diagram for the development of sequential-co-culture system is presented in figure 3.12.

(a) Sequential-co-culture system in flask using synthetic fermentation media

Two strategies have been investigated at flask level. In first strategy, xylose was initially fermented by *P. stipitis*, followed by second and third stages where 100 g/L and 200 g/L glucose media was introduced respectively. The *Z. mobilis* cells (as inoculum) were introduced only in the beginning of second stage to the system. During first stage culture bottles were incubated in orbital incubator shaker (150 rpm). At the onset of second stage the shaking was stopped and vent of air filter was closed to provide better fermentation environment to the *Z. mobilis* cells for bioconversion of glucose to ethanol. The fermentation conditions as adopted in Strategy I are given in table 3.3.

In the second strategy, same process steps were adopted as in the first strategy with the difference that Nitrogen gas was sparged for 30 min into the fermentation medium in the beginning of second and third stages to create strictly anaerobic environment suitable for *Z. mobilis* fermentation and then culture bottles were kept under static condition. All experiments were performed in triplicate and the data were presented as the mean ± SD.

(b) Sequential-co-culture system in bioreactor using synthetic fermentation media

Sequential-co-culture system of *P. stipitis* and *Z. mobilis* was also developed in a bioreactor (working volume 4 L, New Brunswick Bio Flow 110 bioreactor, capacity 7 L) with fermentation media containing xylose and glucose sugars. The two stage process was used and the fermentation conditions are summarized in table 3.4. In this case a two stage process was adopted: in stage I, the fermentation media (except xylose) was sterilized in bioreactor in an autoclave at 121°C for 20 min. Xylose solution was sterilized separately and added to the bioreactor before addition of inoculum. This first step fermentation (150 rpm, 0.05 vvm) process was carried out for 24 h, in the second stage (after exhaustion of xylose), separately prepared and sterilized *Z. mobilis* fermentation medium (as given in section 3.6.4 with 200 g/L glucose) was added to the bioreactor aseptically. The agitator was stopped and N_2 gas was spargedthrough for 2 h as soon as the medium and inoculum (*Z. mobilis*; 5% v/v, $1x10^8$ cells/ml

based on the media added) were introduced to the bioreactor. The samples (5 ml) were drawn at regular interval (8 h and 4 h for stage I and stage II respectively) for analysis of cell concentration (cfu/ml), residual TRS (g/L) and ethanol concentration (g/L). Before taking samples during step II agitator was set on at 250 rpm for 5 min to homogenize the contents in the broth. The experiments were performed in duplicate and the data were presented as the mean ± SD.

(c) Sequential-co-culture system in bioreactor using KGB hydrolysate media

The two stage process strategy was adopted in a bioreactor (working volume 4 L, New Brunswick Bio Flow 110 bioreactor, capacity 7 L) with fermentation media containing KGB hydrolysate and the fermentation conditions were same as used in the case of synthetic media fermentation with the difference that xylose and glucose were replaced with XRF (60 g/L) in stage-I and GRF (200 g/L) in stage-II respectively in the fermentation media. The fermentation duration for stage I was 56 h (0-56 h) and that for stage II was 24 h (56-80 h). The sampling was done in similar manner as described for synthetic sugar fermentation in bioreactor. The experiments were performed in duplicate and the data were presented as the mean ± SD.

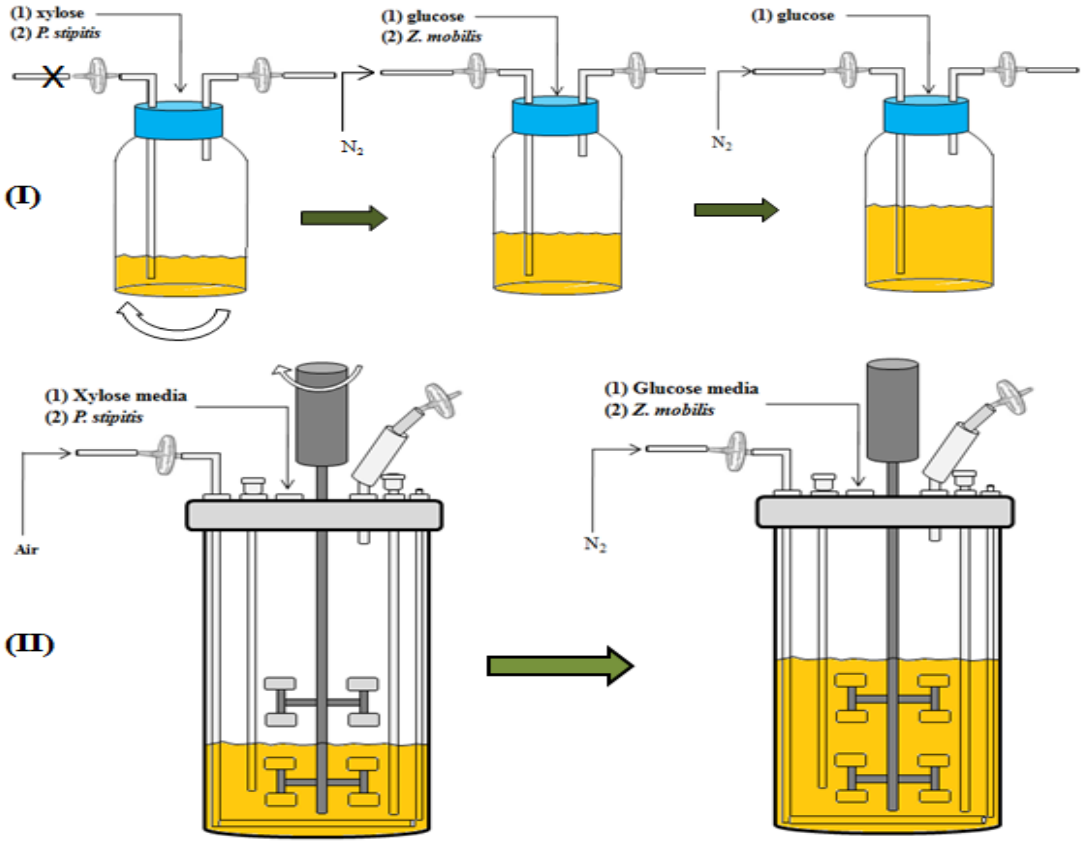


Figure 3.12 The schematic diagram of the development of sequential-co-culture system (I) flask level and (II) bioreactor level.

Table 3.3 The fermentation conditions adopted in strategy I for studies in shake flask.

Fermentation conditions	**I Stage**	**II Stage**	**III Stage**
Total volume of media (ml)	100	200	300
Temperature (°C)	30	30	30
Agitation/aeration	150 rpm	Static	Static
Type of cells present	*P. stipitis*	*P. stipitis* and *Z. mobilis*	*P. stipitis* and *Z. mobilis*
Strength of inoculum added (cfu/ml) (a) *P. stipitis*	1.0×10^7 (5%v/v)	0	0
(b) *Z. mobilis*	0	*1.0×10^8 (5%v/v)	0
Strength of sugar added (g/L)	60 (xylose)	100 (glucose)	200 (glucose)
Fermentation duration	0 – 28 h	28 – 42 h	42 – 56 h

*based on media added.

Table 3.4 The fermentation conditions adopted for two step studies in bioreactor.

Fermentation conditions	**I Stage**	**II Stage**
Total volume of media (ml)	2000	4000
Temperature (°C)	30	30
Agitation/aeration	150 rpm/0.05vvm	Static and N_2sparging
Type of cells present	P. stipitis	P. stipitis and Z. mobilis
Strength of inoculum added (cfu/ml)		
(a) *P. stipitis*		
(b) *Z. mobilis*	1.0×10^7 (5%v/v)	0
	0	*1.0×10^8 (5%v/v)
Strength of sugar added (g/L)	60 (xylose)	200 (glucose)
Fermentation duration	0 – 24 h	24 – 44 h

*based on media added.

Calculation

Product Yield ($Y_{p/s}$): The amount of product formed per unit of substrate consumed by the organism is an important way to report fermentation performance. The yields of fermentation are expressed on either molar or weight basis. The primary stoichiometric equations for the bioethanol production are as follows:

Pentosan to pentose:

$$\underset{1\ g}{n\ C_5H_8O_4} + \underset{0.136\ g}{n\ H_2O} \longrightarrow \underset{1.136\ g}{n\ C_5H_{10}O_5} \quad (1)$$

Hexosan to hexose:

$$\underset{1\ g}{n\ C_6H_{10}O_5} + \underset{0.111\ g}{n\ H_2O} \longrightarrow \underset{1.111\ g}{n\ C_6H_{12}O_6} \quad (2)$$

Pentose to bioethanol:

$$\underset{1\ g}{3\ C_5H_{10}O_5} \longrightarrow \underset{0.511\ g}{5\ C_2H_5OH} + \underset{0.489\ g}{5\ CO_2} \quad (3)$$

Hexose to bioethanol:

$$\underset{1\ g}{C_6H_{12}O_6} \longrightarrow \underset{0.511\ g}{2\ C_2H_5OH} + \underset{0.489\ g}{2\ CO_2} \quad (4)$$

A reduction in yield below theoretical always occurs since the microorganism requires a portion of the substrate for cell growth and maintenance.

CHAPTER 4 RESULTS AND DISCUSSION

(SECTION A)

4.1 Compositional analysis of KGB

Kans grass (*Saccharum spontaneum*) explored for the present investigation is a C_4 plant which have the potential to achieve higher rates of photosynthesis and greater water use efficiency than their C_3 counterpart under the same environmental conditions [Osborne and Freckleton, 2009]. The average composition of Kans grass and other extensively studied grasses used for ethanol production have been given in table 4.1. KGB contained 68% total carbohydrate content (TCC) on dry weight basis, where cellulose and hemicellulose content were obtained as 43.78% and 24.22% by weight respectively. The total carbohydrate content in the KGB and thus total reducing sugars available for bioconversion to ethanol was comparatively higher than those reported in various other grasses. This showed Kans grass is a potential candidate for bioethanol production.

Table 4.1 The composition of various grasses investigated for ethanol production.

Name of Grass	**Average composition (% dry weight basis)**				
	Cellulose	**Hemicellulose**	**Lignin**	**Ash**	**Reference**
Kans grass (*Saccharum spontaneum*)	43.78±0.4	24.22±0.5	23.45±0.3	4.62±0.2	Present work
Coastal Bermuda grass (*Cynodondactylon*)	30.4	29.3	23.2	4.8	[Lee et al. 2009; Sun and Cheng 2005]
Reed Canary grass (*Phalarisarundinacea*)	26.5	21.8	14.8	8.0	[Boateng et al., 2006]
Switch grass (*Panicumvirgatum*)	31.0	24.4	17.56	5.76	[Lee et al., 2009]
Eastern Gama grass (*Trypsacumdactyloides*)	37.0	22.1	20.4	4.3	[Ge et al., 2012]

4.2 Optimization of TRS production from KGB

[Singh, L. K., Chaudhary, G., Majumder, C.B., Ghosh, S. Explore the perennial Kans grass (*Saccharum spontaneum*) biomass for releasing reducing sugars and its optimization. Der ChemicaSinica. 2 (3): 154-163 (2011b)]

KGB is a low cost, renewable and prevalent source of reducing sugars which can further easily be utilized by the microorganisms to produce valuable chemicals. Concentrated sulfuric acid hydrolysis was preferred over other methods. It is advantageous as (1) it requires no enzyme, (2) it is applicable to broad range of biomass, and (3) operated at moderate temperature which limits the generation of toxic compounds. Objective of this experiment was to investigate the effect of H_2SO_4 concentration, biomass loading and reaction time for releasing reducing sugars from KGB.

4.2.1 Statistical modeling

A 2^3 rotatable CCD was adopted in designing the experiments and RSM was used to optimize the hydrolysis process parameters. The RSM was also used by other authors [Karunanithy and Muthukumarappan, 2011a, 2011b; Cheng et al., 2010]. According to the experimental plan, range and levels of independent variables, acid concentration (A), biomass loading (B) and reaction time (C) studied for the hydrolysis of KGB are shown in table 3.1. The coded values of all independent variables and the experimental values of the response variable Y (% g/g) along with predicted values are presented in table 4.2 where Y was defined by: [(g of total reducing sugar obtained/ g of total carbohydrate present initially) x 100]. The coefficients were calculated by using Design Expert v.8.0.

The quadratic model in terms of coded variables was found as

$$Y=+73.98+15.38A+1.22B-6.19C-7.29A^2-7.64B^2-7.55C^2+1.25AB-1.25AC+0.25BC \qquad (4.1)$$

To fit the response function and experimental data, regression analysis was performed and second order model for the response (Y) was evaluated by ANOVA which is presented in table 4.3. The regression for the response was statistically significant at 95% of confidence level. The Model F-value of 264.24 and low % CV value of 2.67 implies the model is highly significant. A poor F-value, 0.53 of the lack of fit test (table 4.3) confirming the reliability of the experimental data. The "Pred R-Squared" of 0.9838 is in reasonable agreement with the "Adj R-Squared" of 0.9920. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The very high signal to noise ratio of 46.745 indicates that the chance of the values could be due to noise is very less. The goodness of fit of the model was

checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2=0.9958) indicates that only 0.42% of the total variations are not explained by the model.

Table 4.2 Central composite design consisting of 20 experiments with the experimental and predicted response.

Run no.	**Coded values of the variables**			**Response (Y) total reducing sugar % (w/w of TCC)**	
	A	B	C	Experimental value	Predicted value
1	-1	-1	-1	42.00	41.32
2	1	-1	-1	72.00	72.09
3	-1	1	-1	40.00	40.77
4	1	1	-1	78.00	76.54
5	-1	-1	1	30.00	30.95
6	1	-1	1	58.00	56.72
7	-1	1	1	32.00	31.40
8	1	1	1	62.00	62.17
9	-1.682	0	0	28.00	27.49
10	1.682	0	0	78.00	79.23
11	0	-1.682	0	50.00	50.30
12	0	1.682	0	54.00	54.42
13	0	0	-1.682	62.50	63.02
14	0	0	1.682	42.00	42.21
15	0	0	0	76.00	73.98
16	0	0	0	72.00	73.98
17	0	0	0	76.00	73.98
18	0	0	0	74.00	73.98
19	0	0	0	72.00	73.98
20	0	0	0	74.00	73.98

Table 4.3 Analysis of variance (ANOVA) for total reducing sugar yield

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value Prob> F
Model	5827.93	9	647.55	264.24	< 0.0001
A-Acid conc.	*3231.91*	*1*	*3231.91*	*1318.83*	*< 0.0001*
B-Biomass loading	*20.49*	*1*	*20.49*	*8.36*	*0.0161*
C-Time	*522.55*	*1*	*522.55*	*213.23*	*< 0.0001*
AB	*12.50*	*1*	*12.50*	*5.10*	*0.0475*
AC	*12.50*	*1*	*12.50*	*5.10*	*0.0475*
BC	*0.50*	*1*	*0.50*	*0.20*	*0.6611*
A^2	*765.67*	*1*	*765.67*	*312.44*	*< 0.0001*
B^2	*841.75*	*1*	*841.75*	*343.49*	*< 0.0001*
C^2	*822.39*	*1*	*822.39*	*335.59*	*< 0.0001*
Residual	24.51	10	2.45		
Lack of Fit	*8.51*	*5*	*1.70*	*0.53*	*0.7476*
Pure Error	*16.00*	*5*	*3.20*		
Cor Total	5852.44	19			

The interactive behavior of the variables involved on the total reducing sugar release from KGB is given in figure 4.1(a), (b) and (c). The highly parabolic nature of the contours in figure 4.1(a) (interaction between acid concentration and biomass loading) and figure 4.1(b) (interaction between acid concentration and reaction time) states that total reducing sugar production is highly dependent on the interactive behavior on the respective parameters.

The interaction between H_2SO_4 concentration and biomass loading on reducing sugar release from KGB while reaction time was selected as 60 min is shown in figure 4.1(a). From the figure and experimental data it can be interpreted that maximum and minimum reducing sugar yield of 78% and 28% can be obtained by conducting hydrolysis experiment for 10% biomass loading and 70.23% and 19.77% acid concentration respectively. The effect of acid concentration and reaction time on reducing sugar release is shown in figure 4.1(b), when biomass loading was selected at 10% as the center point. From the figure and experimental data it is evident that as the acid concentration increases, the response also increases significantly but when the time increases, response decreases because some amount of sugar degraded into furfural and hydroxymethyl furfural. Interaction between biomass loading and reaction time is shown in figure 4.1(c), while acid concentration was kept constant at 45%. More circular nature of contours shown in the figure 4.1 (c) signifies that the production of reducing sugars is least dependent on interactive behavior of these two parameters.

On the basis of model, the optimum values of the parameters were calculated by setting the first order derivatives of the equation (2) (dY/dA,dY/dB and dY/dC) as zero. The optimum values of the variables A, B and C in coded and actual form thus obtained are given in table 4.4.

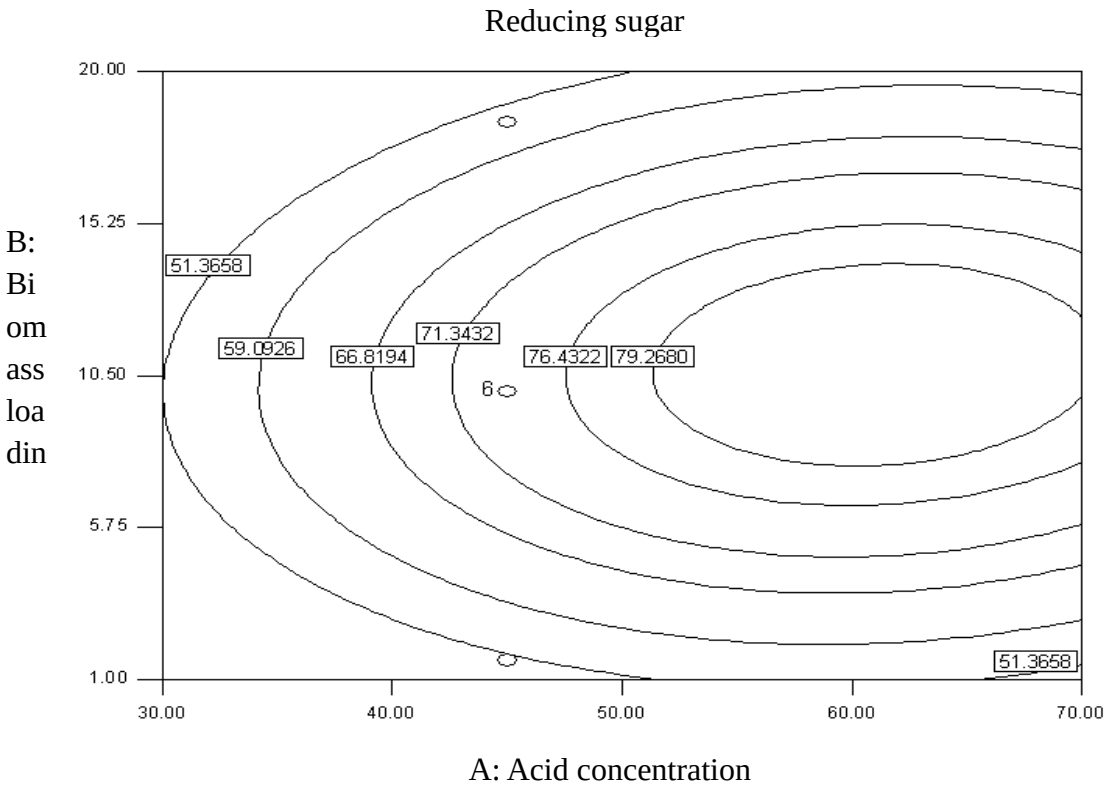


Figure 4.1(a) Effect of acid concentration and biomass loading on the release of total reducing sugar from KGB, reaction time 60 min.

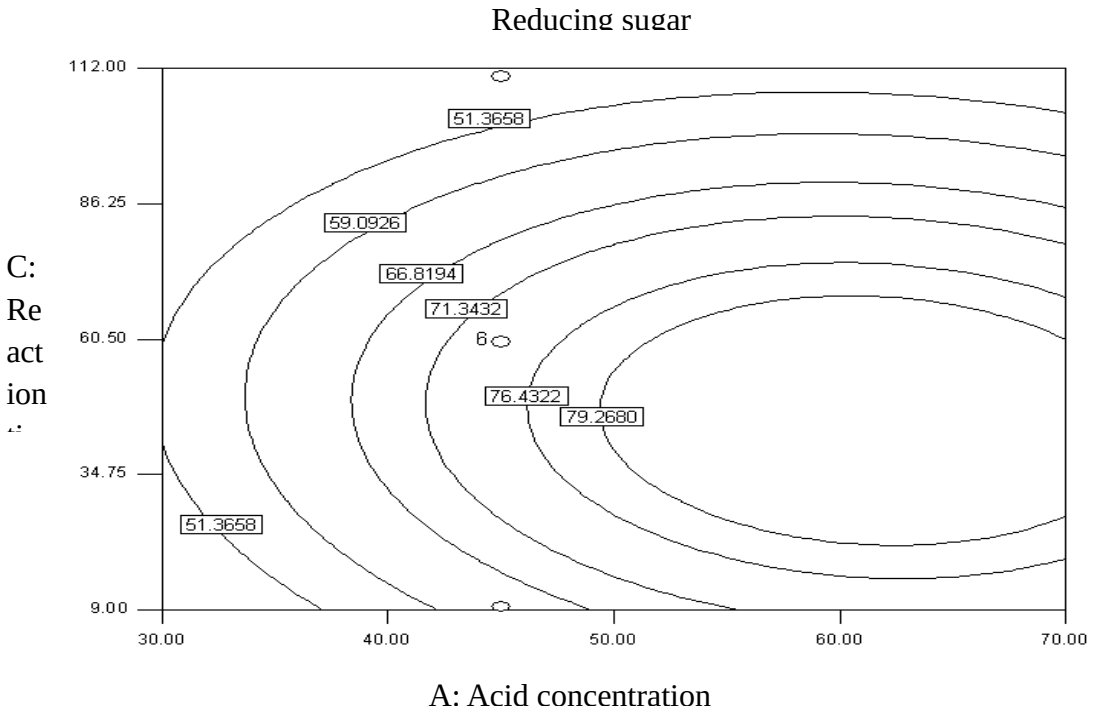


Figure 4.1(b) Effect of acid concentration and reaction time on the release of total reducing sugar from KGB, biomass loading 10%.

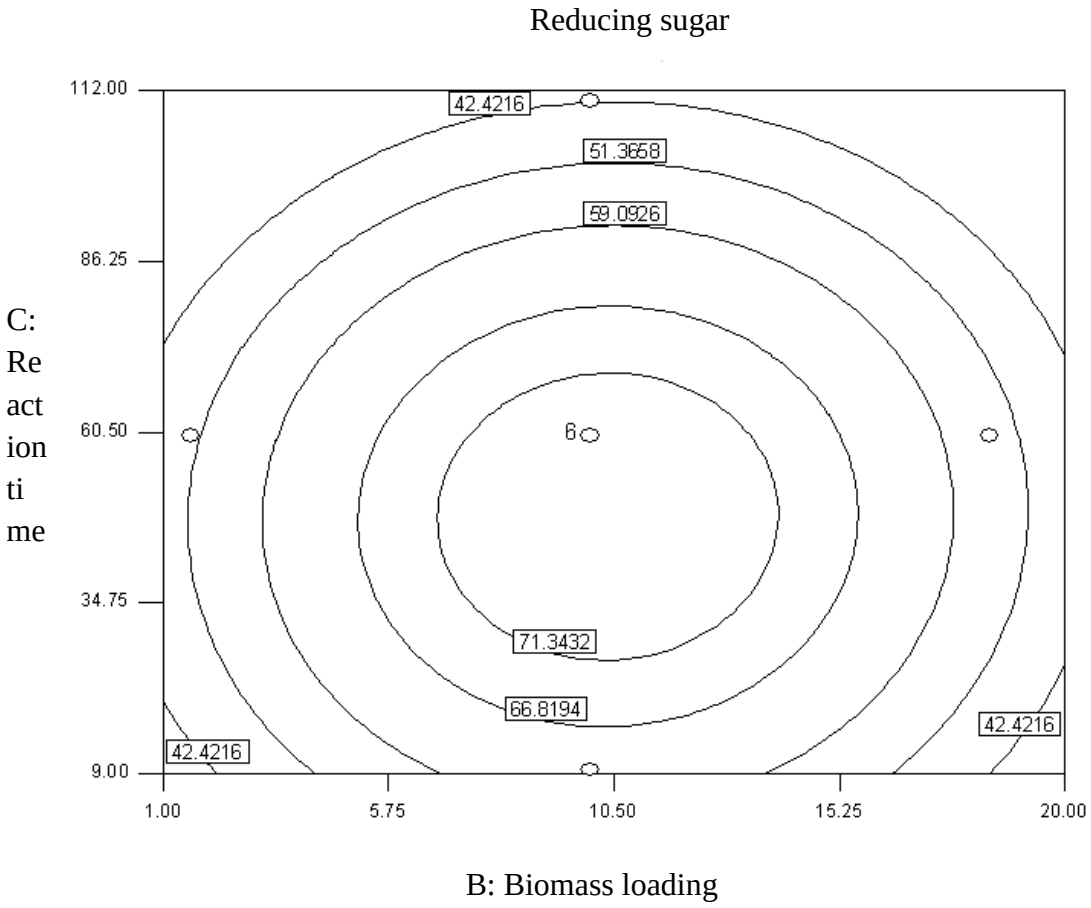


Figure 4.1(c) Effect of biomass loading and reaction time on the release of total reducing sugar from KGB, acid concentration 45%.

Table 4.4 Solution for optimum condition.

Parameters	Coded value	Actual Value
Acid concentration % (g/g)	+1.11	61.10
Biomass loading % (g/ml)	+0.16	10.80
Reaction time (min)	-0.50	45.00

4.2.2 Validation of model and significance of study

Three sets of experiments were conducted at the optimum conditions and the mean value of the reducing sugar produced was found to be 83.5% on total carbohydrate content basis. The same was theoretically evaluated from equation 2 for optimum value of A, B, C and was found to be 84.14%. The closeness of the theoretical value to that of experimental value validates the model.

In the present investigation we have used a single step hydrolysis process to convert the cellulosic and hemicellulosic fractions of KGB to reducing sugars. In many studies it was observed that two or more steps have been used to fractionate the various lignocellulosic materials to obtain the monomeric form of the polysaccharides. Saha et al., (2005) used dilute acid treatment (0.75% v/v, 121°C, 1 h) and cocktail of four commercial enzyme preparations for saccharifying wheat straw and obtained 74% saccharification yield. Coastal Bermuda grass was pretreated using autohydrolysis process (150°C, 60 min) followed by enzymatic hydrolysis and 70% of the theoretical sugar yield was obtained by Lee et al., (2009). Xu et al. (2010) used sodium hydroxide pretreatment (1% NaOH, 50°C, 12h) followed by enzymatic saccharification of switch grass and obtained 70.8% sugars on total available carbohydrate basis. In our study 83.5% yield of total reducing sugar was obtained which was significantly higher than the reported investigations. Also the proposed generalized model was successful in explaining the experimental facts and can be utilized for further large scale studies.

4.3 Hemicellulose hydrolysis and pentose fermentation

[Singh, L. K., Majumder, C. B., Ghosh, S. Bioconversion of Hemicellulosic Fraction of Perennial Kans Grass (*Saccharum spontaneum*) Biomass to Ethanol by *Pichia stipitis*: A Kinetic Study. International Journal of Green Energy.9(5): 409-420 (2012a)]

4.3.1 Kans grass hemicellulose acid hydrolysate preparation

The average composition of Kans grass biomass (KGB) and other extensively studied grasses used for ethanol production have been given in table 4.1 [Lee et al. 2009; Sun and Cheng 2005; Boateng et al 2006]. KGB contained 68% total carbohydrate content with 24.22 % hemicellulosic fraction was comparable with other grasses for ethanol production. Dilute sulfuric acid hydrolysis (2% v/v) of KGB under reflux was found very effective in solubilizing the hemicellulosic fraction. After 6 h of reflux at 100°C, total reducing sugar yield was 20.4 g per 100 g of dry biomass of which pentose sugar constituted 17.2 g, remaining was other reducing sugars. All these sugars were derived primarily from hemicellulosic fraction of KGB. A very low hexose sugar yield indicates that the cellulosic fraction of KGB remained practically unhydrolyzed. Dilute acid at moderate temperature effectively recovers most of the hemicellulose as soluble sugars. The complex nature of the structure of cellulose in the plant material requires much severe conditions for their degradation in contrast to the hemicellulose portion. The total reducing sugar and pentose sugar concentration in the acid hydrolysate were found to be 68.0 g/L and 61.5 g/L respectively. Besides sugars, the acid hydrolysate contained different and varying amounts of toxic components, such as acetic acid, furfural and soluble lignin derivatives, showed inhibitory effects on the growth of microorganisms during fermentation [Kadar et al., 2007].

4.3.2 Detoxification of the hemicellulose acid hydrolysate

The two steps process for detoxification of Kans grass hemicellulose acid hydrolysate by boiling and overliming were found very effective and resulted in better fermentability of the hydrolysate [Amartey and Jeffries, 1996]. During boiling the volatile compounds such as furfural and phenols were stripped followed by overliming with $Ca(OH)_2$ removed and/or reduced the concentration of other acid compounds e.g. acetic and tannic acid. Furthermore, the pH increase up to 10.0 due to overliming resulted in precipitation of heavy metals. The furfural is transformed into furfuryl acid, which condenses with other components of hydrolysate [Strickland and Beck, 1985]. Overliming resulted in the loss of glucose (8 %) and xylose (5 %). In order to reduce the sugar losses, the shift to higher pH during the overliming process must be kept short to minimize pentose decomposition [McMillan, 1994]. Very limited growth was

observed when the organism was tried to grow in the hemicellulose acid hydrolysate medium prior to the step of detoxification with lime. Similar observation was reported by Amartey and Jeffries (1996).

4.3.3 Fermentation of pretreated Kans grass hemicellulose acid hydrolysate medium

It was observed that the xylose utilization in pretreated hemicellulose acid hydrolysate medium and synthetic medium was 74% and 94% respectively whereas glucose was utilized completely in both the cases. This may be due to the left over toxic compounds in the hydrolysate. Utilization of different sugars has been reported by other authors also [Jeffries and Sreenath, 1988; Ferrari et al., 1992]. Time course for cell growth, utilization of sugars and ethanol production have been shown in figure 4.2 and figure 4.3 for simulated synthetic and Kans grass hemicelluloses acid hydrolysate medium respectively.

The time for completion of fermentation was more (96 h) for hemicellulose acid hydrolysate medium than that for synthetic medium (36 h) due to longer lag phase of the microorganism in the hemicellulose acid hydrolysate medium. The *P.stipitis*cells showed more time for adaptation in the hydrolysate medium than that in the synthetic medium. The ethanol yield ($Y_{p/s}$), productivity (q_p), biomass yield ($Y_{x/s}$) and maximum specific growth rate (μ_{max}) were found to be 0.429 g/g, 0.231 g_p/L/h, 0.065 g/g and 0.064 h^{-1} respectively. These fermentation parameters for the detoxified hemicellulose acid hydrolysate were lower than those obtained with synthetic medium. The reason might be some left over toxic components in the conditioned hemicellulose acid hydrolysate which were further concentrated during the later stage of evaporation under vacuum, used to enhance the concentration of sugars to the desired level and negatively affects the growth of cells and ethanol producing ability of *P. stipitis*. The similar observations were also reported by Nigam (2002).

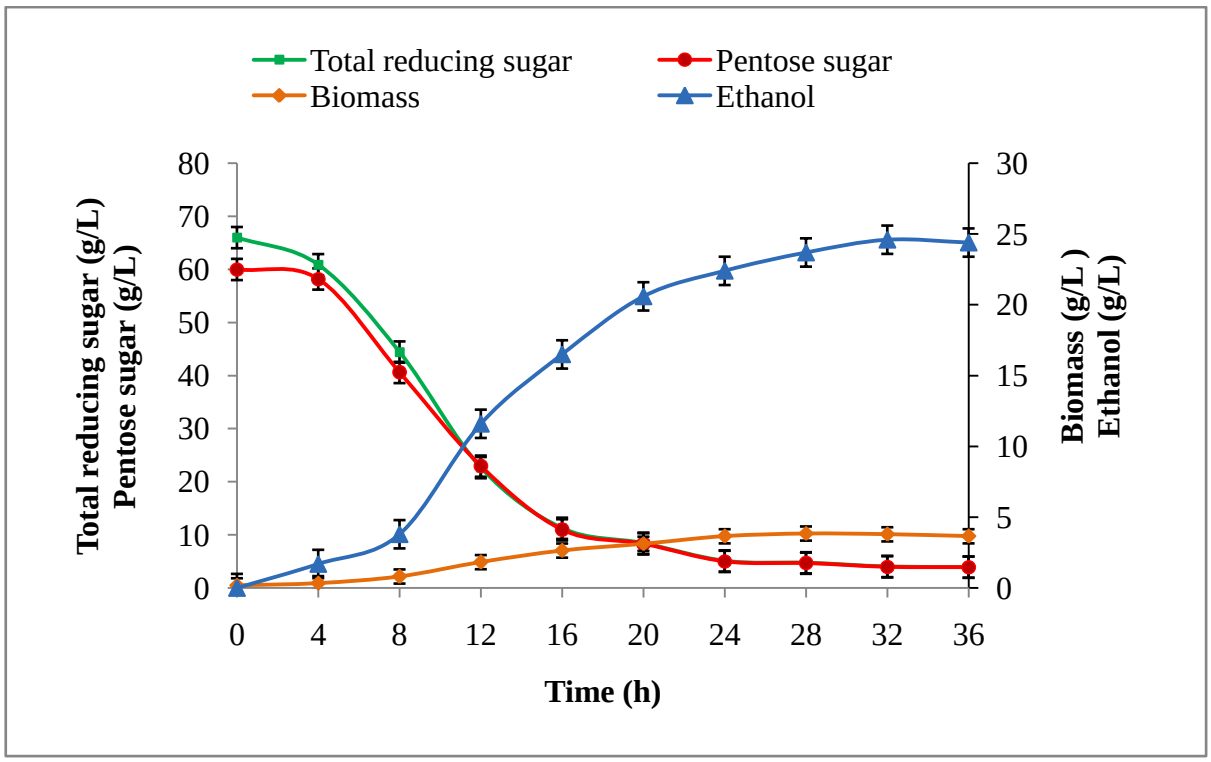


Figure 4.2 Concentration profiles of total reducing sugar, pentose sugar, biomass and ethanol during fermentation of synthetic media by *P. stipitis*.

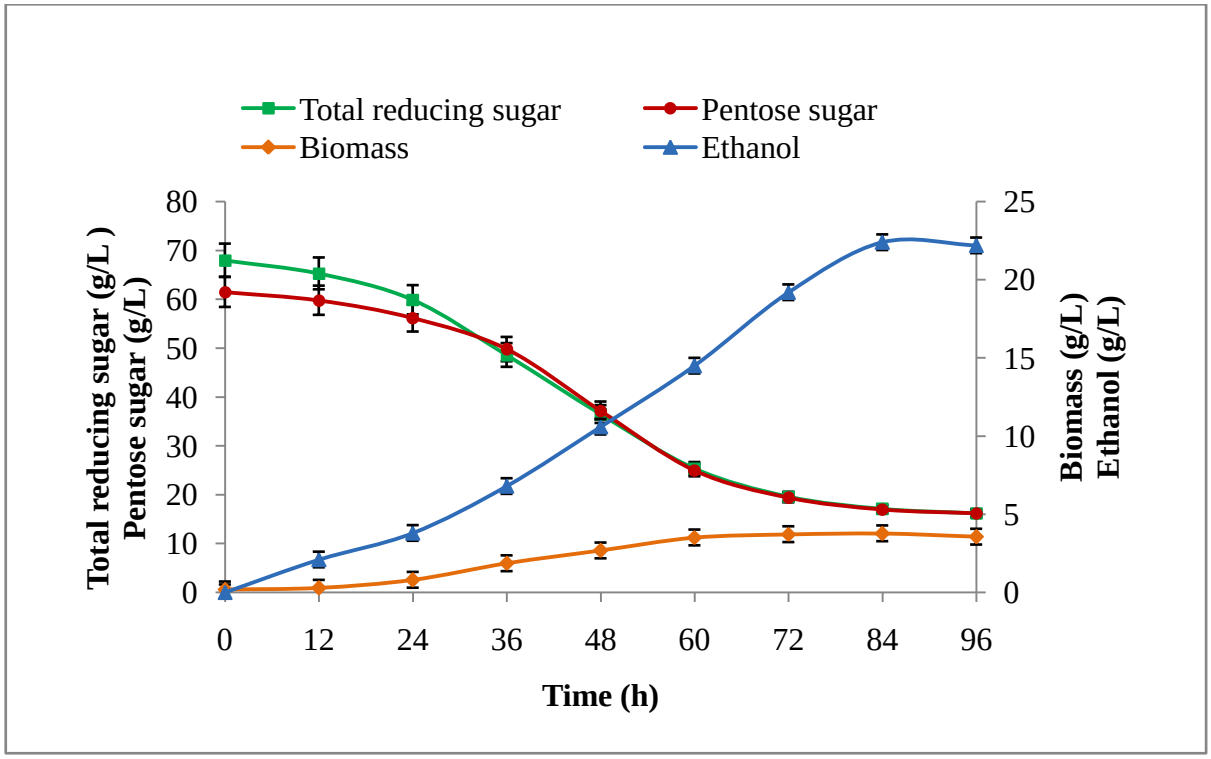


Figure 4.3 Concentration profiles of total reducing sugar, pentose sugar, biomass and ethanol during fermentation of Kans grass hemicellulose acid hydrolysate medium by *P. stipitis*.

4.3.4 Modeling of ethanol formation

The appropriate kinetic models were proposed for the interpretation of experimental results obtained by organized complex systems like microbial cells. The dynamics of the sugar degradation is assumed to be the result of an autocatalytic process that depends on the concentrations of substrate and the number of active cells. The proposed model takes account of the fact that the sugar substrate promotes an energy flow from the sugar to active cells for increasing its concentration. Ethanol concentration acts as a controller of the quantity of active cells, shown in figure 3.10.The systems can be described for rate of biomass growth (r_x) by the equation 4.2, in which sugar (s) degradation, ethanol production (p) and cell activation (x) are linked:

$$r_x = \frac{dx}{dt} = Cd \times s \times x - Ci \times x \times p \qquad (4.2)$$

where C_d and C_i represents coefficients of cell formation based on sugar degradation and inhibition of cells due to ethanol formation respectively.

A reasonably good correlation can be obtained if it is assumed that the rate of ethanol formation is related both to the rate of growth (dx/dt) and to the quantity (x) of microorganisms present. The equation for ethanol formation rate (r_p) can thus be written as follows (equation 4.3):

$$r_p = \frac{dp}{dt} = C_g \times (\frac{dx}{dt}) + C_{ng} \times x \qquad (4.3)$$

Where, C_g and C_{ng} represents growth associated constant and non-growth associated constant of the model respectively.

4.3.5 Calculation of kinetic parameters

The non-linear regression technique was used to estimate model parameters. The parameters were estimated using the experimental data of biomass concentration (x), reducing sugar concentration (s) and ethanol concentration (p).The rate of growth of cells r_x (dx/dt) and rate of ethanol formation r_p (dp/dt) were calculated using these data. The coefficients of the models were estimated using software package "Polymath" version 6.10 and found as C_d (L/g/h):0.0012994, C_i (L/g/h): - 0.0006741, C_g: 1.141467, and C_{ng}(h^{-1}): 0.1626586 with 95% confidence.

4.3.6 Interpretation and validation of regression models

The statistical indicators and the various plots can be used to assess the quality of the regression models. The plots between calculated and measured values of the dependent variables r_x and r_p are showing the same trend (figure 4.4 and figure 4.5 for r_x and r_p respectively), thus indicating the experimental data are accurately modeled. When the plots show different trends, this usually indicates an inappropriate model. If the difference between the measured and calculated points is large, but no clear trend exists, this may also indicate very noisy data (excessive experimental error) and cannot be accurately modeled.

The residual plot shows the difference between the calculated and measured values ($\Delta r_x = r_x$ experimental – r_x calculated and $\Delta r_p = r_p$ experimental – r_p calculated) of the dependent variable (r_x and r_p) as function of the measured values (r_x experimental and r_p experimental). It is immediately clear from the figure 4.6 that the residuals are randomly distributed around the line of zero error indicating that the regression model represents the data correctly. Thus this can be interpreted that the proposed model is appropriate for the present study. If the residuals show a clear trend, this indicates that an inappropriate model is being used. The coefficient of determination (R^2) and coefficient of determination adjusted (R^2_{adj}) are frequently used to judge whether the model represents the data correctly, implying that if they are close to unity then the regression model is correct. The R^2 and R^2_{adj} values for the model for rate of cell formation were 0.949 and 0.942 respectively and the same for rate of product formation were 0.985 and 0.983 respectively. Since the values are close to one, the proposed model is able to explain the experimental facts.

Just like coefficient of determination, the two statistical parameters, variance and root mean square deviation (RMSD) are recommended to be used for model testing. A model with smaller variance and RMSD represent the data more accurately than a model with larger values of these parameters. For RMSD calculation respective deviations were taken between experimental and calculated values at each data point (sampling time). The variance and RMSD were found to be 0.0002399 and 0.0045531 respectively for the rate of cell formation, and these were 0.0016283 and 0.0118624 respectively for rate of product formation. The R^2, R^2_{adj}, variance and RMSD values were calculated by the same software mentioned earlier by Shacham et al. (1996).

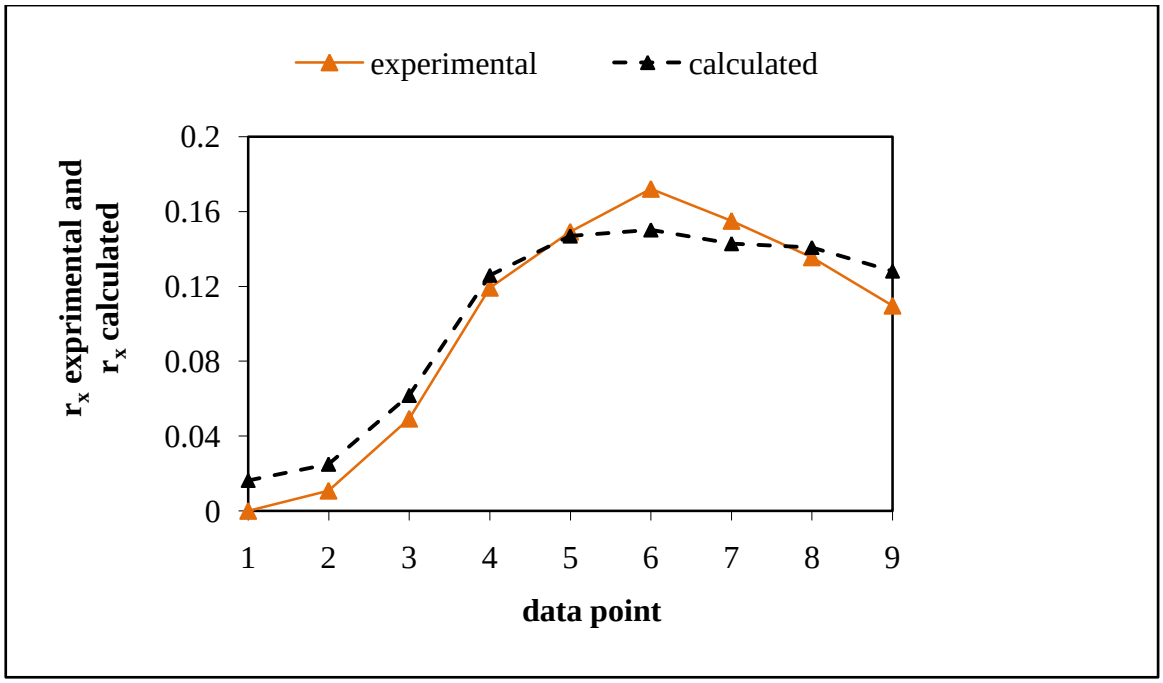


Figure 4.4 Nonlinear plot of calculated and experimental values of biomass formation rate (r_x=dx/dt) verses data point (sampling time) using hemicellulose acid hydrolysate medium.

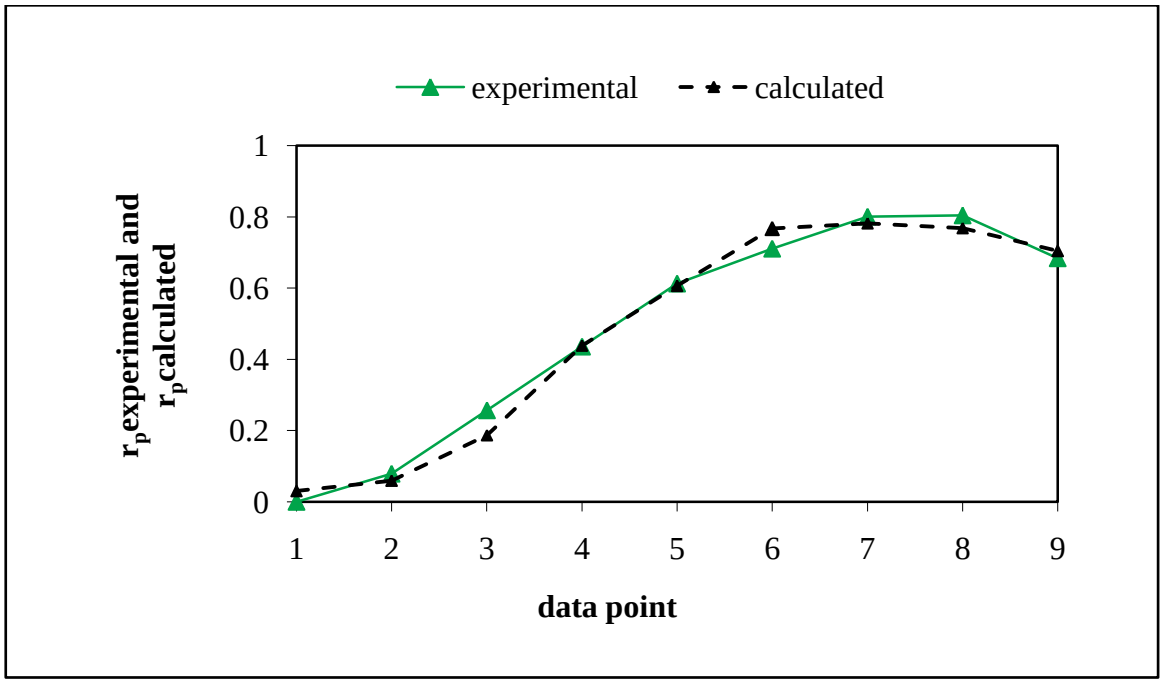


Figure 4.5 Nonlinear plot of calculated and experimental values of ethanol formation rate (r_p=dp/dt) verses data point (sampling time) using hemicellulose acid hydrolysate medium.

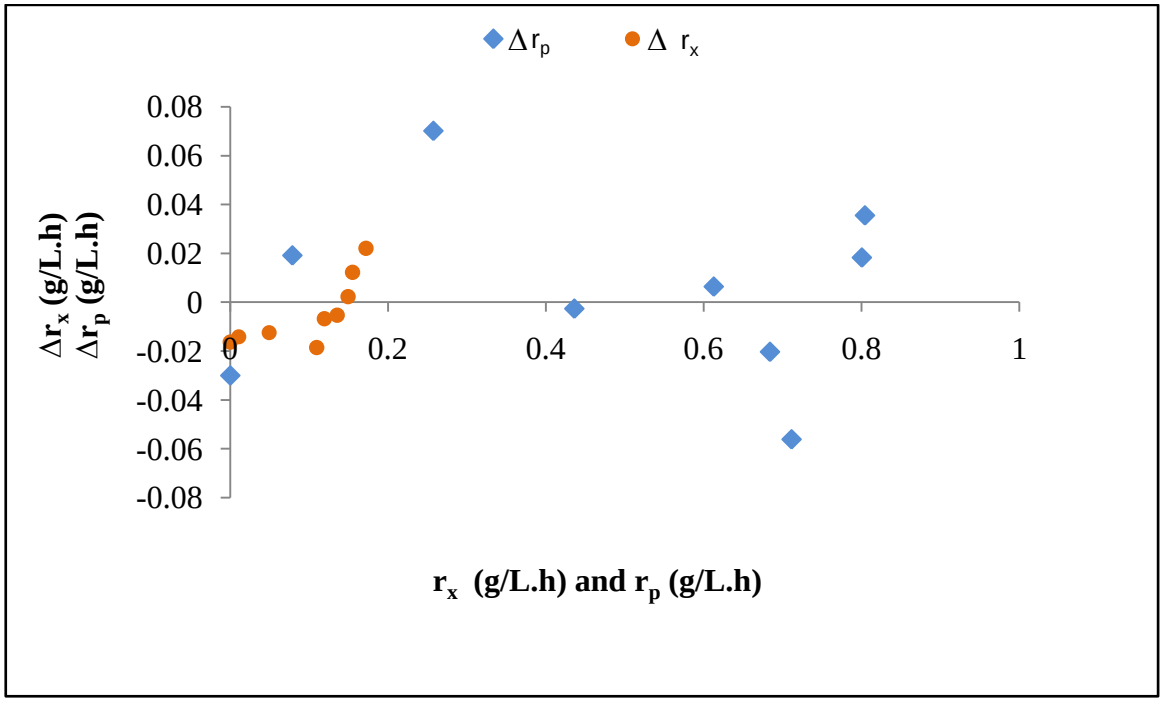


Figure 4.6 Nonlinear residual plot of difference between experimental and calculated values of biomass formation rate (Δr_x) and ethanol production rate (Δr_p) verses experimental values of r_x and r_p respectively using hemicellulose acid hydrolysate medium.

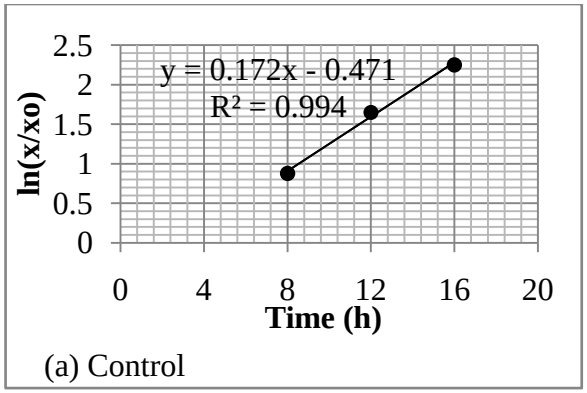
4.4 Effects of toxics on growth of microbes

4.4.1 Effect of furfural on specific growth rate of *Pichia stipitis*

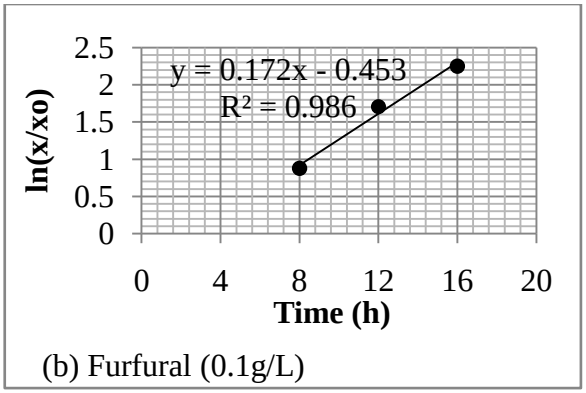
Xylose and other pentose sugars are liberated during the hydrolysis of hemicellulose fraction of lignocellulosic biomass. Further degradation of these sugars released furfural. Furfural is known to be inhibitory compound and affect the growth of microorganisms. Palmqvist et al. (1999) reported that the growth of microorganism is more susceptible to furfural concentration than for ethanol production. *P. stipitis*, a well known xylose bioconverter to ethanol is likely to encounter this compound during fermentation of lignocellulose hydrolysate to ethanol, thus the experiment was designed to examine the effect of furfural concentration on the growth of *P. stipitis*. Different concentration of furfural was introduced in the growth media of *P. stipitis* ranging from 0.1 to 0.5 g/L along with the control (no furfural). The specific growth rate was calculated during the exponential growth phase of the *P. stipitis* as the slope of the linear relationship from the curve $\ln(x/x_0)$ vs. time, shown in figure 4.7.

The effect of furfural on specific growth rate of *P. stipitis* is given in table 4.5. The % reduction in specific growth rate of *P. stipitis* was calculated. It was observed that the effect of furfural on the specific growth rate of *P. stipitis* was more prevalent on 0.4 g/L or more concentration than that with the lower values of furfural concentration. The relative specific growth rate (μ_{rel}) was also calculated by considering specific growth rate in control as 100% and shown in figure 4.8. From the analysis of results ca. 9 % reduction in specific growth rate was observed with 0.4 g/L furfural concentration whereas ca. 23 % reduction was found with 0.5 g/L furfural concentration. Thus 0.4 g/L furfural concentration may be considered as tolerable to *P. stipitis* growth.

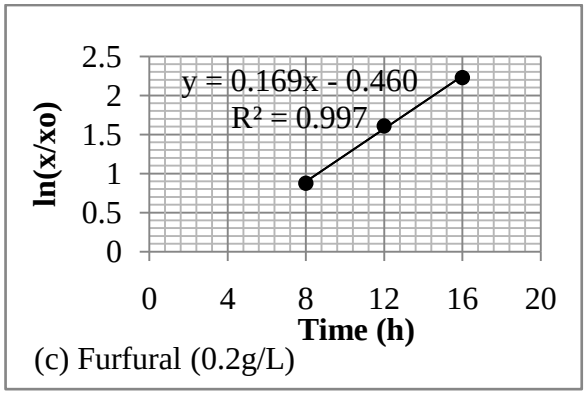
Delgenes et al. (1996) have reported that the growth of *P. stipitis* was reduced by 25% at furfural level 0.5 g/L whereas, 47% and 99% reduction was found with 1.0 g/L and 2.0 g/L furfural concentration respectively. Nigam (2001) reported that a furfural concentration of 1.5 g/L interfered in respiration and resulted ca. 90% reduction in ethanol yield, whereas furfural concentration of 0.25 g/L was not sufficient to reduce ethanol formation. Studies done by Boyer et al. (1992); Navarro (1994) also revealed that furfural reduce the specific growth rate of microorganisms and biomass yield per ATP [Palmqvist et al., 1999].



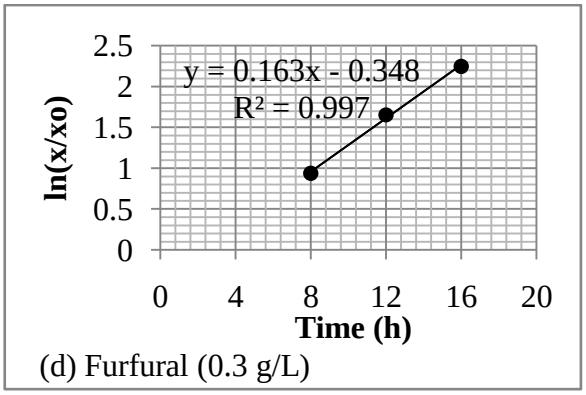
(a) Control



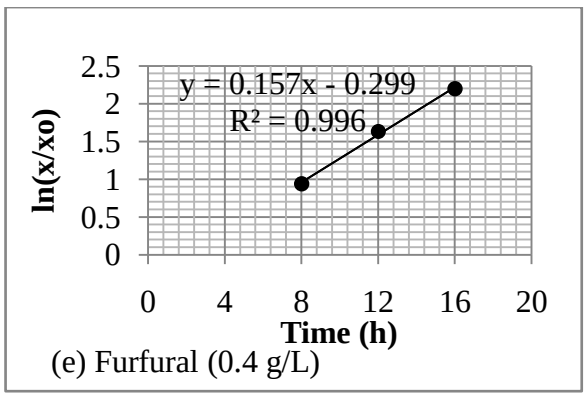
(b) Furfural (0.1g/L)



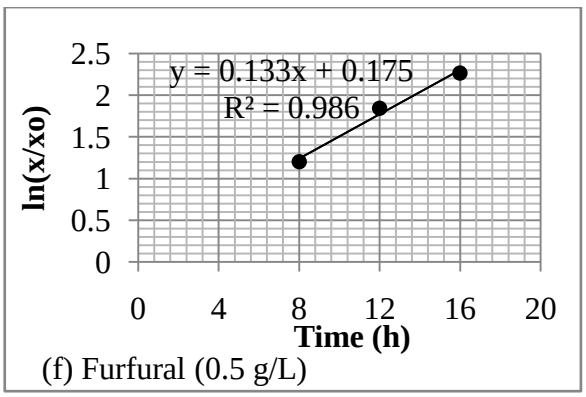
(c) Furfural (0.2g/L)



(d) Furfural (0.3 g/L)



(e) Furfural (0.4 g/L)



(f) Furfural (0.5 g/L)

Figure 4.7 Curve for specific growth rate (μ) calculation of *P. stipitis* with different concentration of furfural in the growth media.

Table 4.5 Effect of furfural concentration on specific growth rate of *P. stipitis*.

S. no.	Furfural concentration (g/L)	Specific growth rate μ (h^{-1})	Reduction in specific growth rate (%)
1	0.0	0.172	0.00
2	0.1	0.172	0.00
3	0.2	0.169	1.74
4	0.3	0.163	5.23
5	0.4	0.157	8.72
6	0.5	0.133	22.67

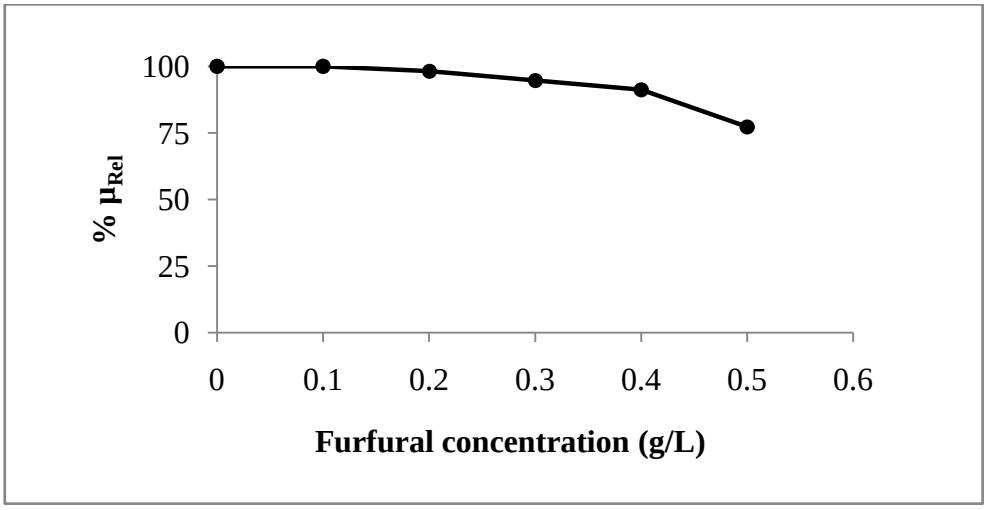


Figure 4.8 Effect of furfural concentration on the specific growth rate of *P. stipitis*.

4.4.2 Effect of phenolics on specific growth rate of *Pichia stipitis*

A large number of phenolic or aromatic compounds have been detected in lignocellulose hydrolysate. These are believed to be degradation products of lignin during acid hydrolysis. Palmqvist and Hahn-Hagerdal (2000) reported that the biomass growth and sugar assimilation are reduced due to the presence of these phenolic compounds because the biological membranes lost their integrity, thereby affecting ability to serve as selective barriers. Among the phenolic compounds, vanillin (4-hydroxy-3-methoxy benzaldehyde) is one of the important and strong inhibitor for the growth of microorganisms. The degradation of guaiacylpropane units of lignin produced vanillin and has been reported in hydrolysate of willow [Jonsson et al., 1998] and poplar [Ando et al., 1986]. Thus vanillin was selected as the model phenolic compound to investigate its effect on the growth of *P. stipitis*. Different concentration of vanillin was introduced in the growth media of *P. stipitis* ranging from 0.2 to 2.0 g/L along with the control (no vanillin). The specific growth rate (μ) was calculated during the exponential growth phase of the *P. stipitis* from the slope of the linear relationship of the curve $\ln(x/x_0)$ vs. time, shown in figure 4.9.

The effect of vanillin on specific growth rate of *P. stipitis* is given in table 4.6. The % reduction in specific growth rate of *P. stipitis* was calculated. It was found that the effect of vanillin on the specific growth rate of *P. stipitis* was nominal at 0.5 g/L whereas, at 1.0 g/L or more concentration of vanillin the specific growth rate of *P. stipitis* decreased rapidly. The relative specific growth rate (μ_{rel}) was also calculated by taking specific growth rate in control as 100% and is shown in figure 4.10. From the analysis of results it was found that ca. 7 % reduction in specific growth rate was observed with 0.5 g/L vanillin concentration whereas, ca. 15 % reduction was found with 1.0 g/L initial vanillin concentration. Thus 0.5 g/L vanillin concentration was noted as tolerable phenolics concentration for *P. stipitis* growth.

Larsson (2000) reported the effect of lignocellulose derived aromatic compounds on growth and ethanol production by *S. cerevisiae*. It was revealed that a position of substituent is very important for the inhibitory effect of hydroxymethoxybenzaldehydes and the oxidized form being more toxic than the reduced form of a diphenol/quinine. Less heavily substituted phenolics are the most toxic materials in the hydrolysate [Clark and Mackie, 1984;Nishikawa et al., 1988]. Delgenes et al., (1996) have reported that among the phenolic compounds, vanillin and syringaldehyde are the important inhibitors.

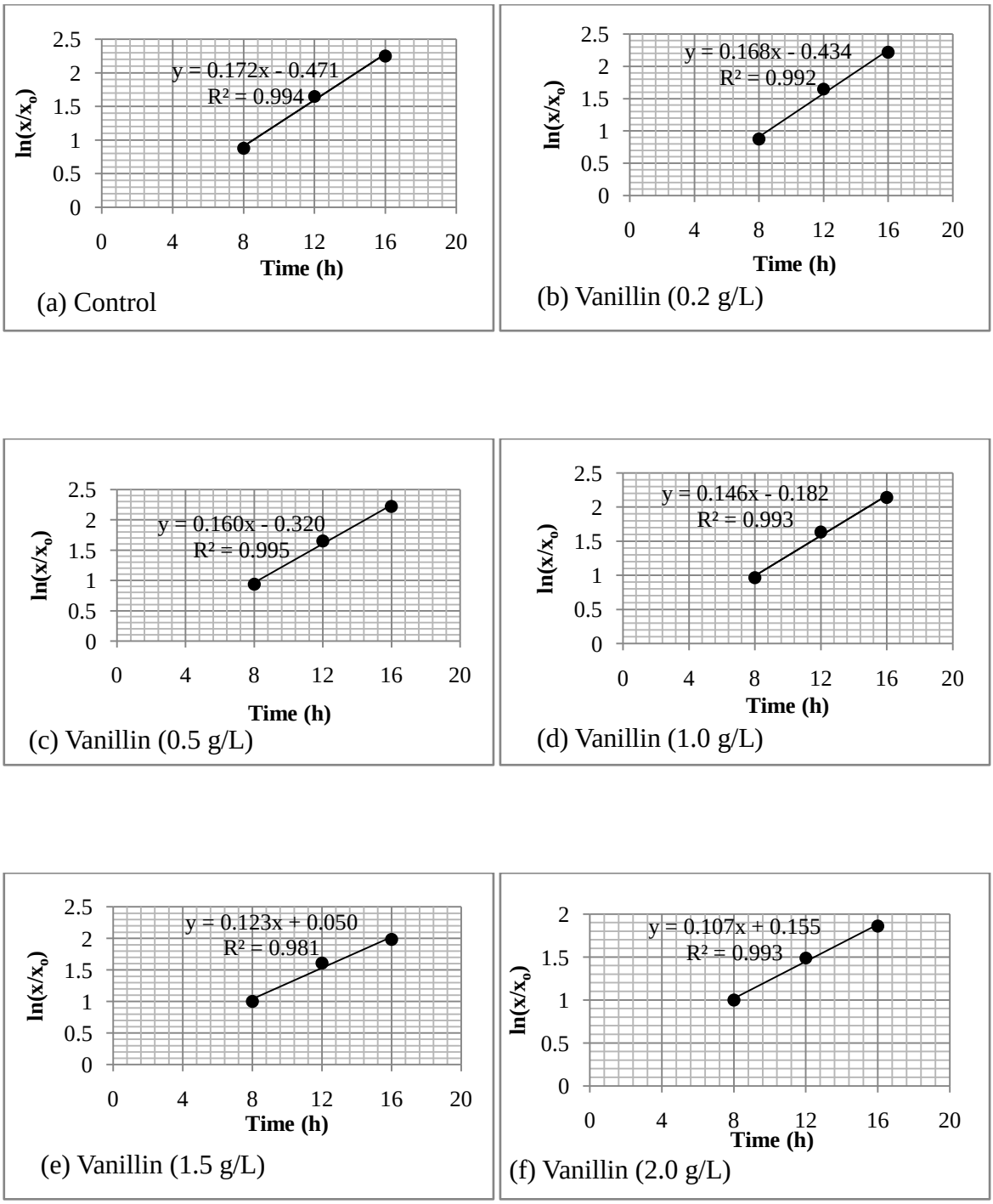


Figure 4.9 Curve for specific growth rate (μ) calculation of *P. stipitis* with different concentration of vanillin as model phenolic compound in the growth media.

Table 4.6 Effect of vanillin concentration on specific growth rate of *P. stipitis*.

S. no.	Furfural concentration (g/L)	Specific growth rate μ (h^{-1})	Reduction in specific growth rate (%)
1	0.0	0.172	0.00
2	0.2	0.168	2.33
3	0.5	0.160	6.98
4	1.0	0.146	15.12
5	1.5	0.123	28.49
6	2.0	0.107	37.79

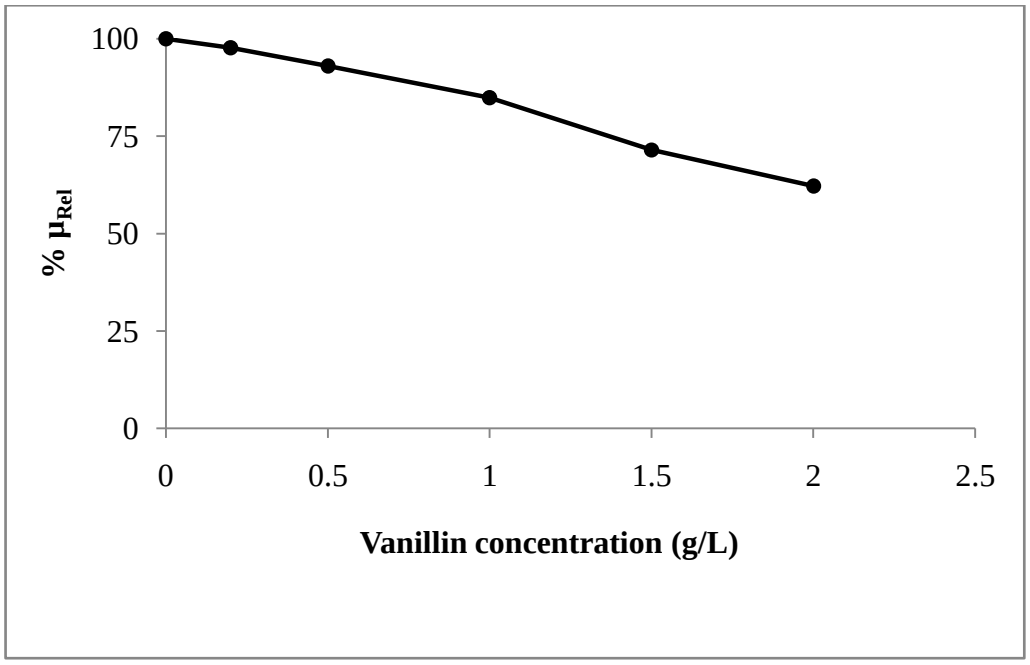


Figure 4.10 Effect of vanillin (used as model phenolic compound)concentration on the specific growth rate of *P. stipitis*.

4.4.3 Effect of hydroxymethyl furfural on specific growth rate of *Zymomonas mobilis*

Hydroxymethyl furfural (HmF) is a sugar degradation product generated during acid hydrolysis of lignocellulosic biomass. Larsson et al. (1998) noticed that presence of HmF caused longer lag phase and it affects growth of microorganism in similar way as that by furfural [Taherzadeh et al., 1999]. The concentration of HmF is normally low in hemicellulose hydrolysate because it is primarily derived from glucose (hexose) degradation [Ulbricht et al., 1984]. It is mainly present in cellulose hydrolysate thus a glucose bioconverter microorganism may likely to encounter HmF during fermentation. Therefore the present experiment was designed to evaluate the effect of HmF concentration on the growth of *Z. mobilis*. Different concentration of HmF was introduced in the growth media of *Z. mobilis* ranging from 0.2 to 2.0 g/L along with the control (no HmF). The specific growth rate was calculated during the exponential growth phase of the *Z. mobilis* as the slope of the linear relationship from the curve $\ln(x/x_0)$ vs. time, shown in figure 4.11.

The effect of HmF on specific growth rate of *Z. mobilis* is given in table 4.7. The % reduction in specific growth rate of *Z. mobilis* was calculated. It was observed that the effect of HmF on the specific growth rate of *Z. mobilis* was more intense above 1.0 g/L HmF concentration than that with the lower values. The relative specific growth rate (μ_{rel}) was calculated by considering specific growth rate in control (without HmF) as 100% and shown in figure 4.12. From the analysis of results ca. 8 % reduction in specific growth rate was observed with 1.0 g/L HmF concentration whereas, ca. 15 % reduction was found with 1.5 g/L HmF concentration. Thus 1.0 g/L HmF concentration may be considered as tolerable to *Z. mobilis* growth.

The growth rate and cell yield is reduced due to presence of HmF in the media because HmF inhibit glycolysis especially the activity of dehydrogenases [Banerjee et al., 1981]. Azhar et al. (1981) observed that HmF concentration of 1.0 g/L was sufficient to inhibit cell growth and ethanol production by *S. cerevisiae*. Wikandari et al. (2010) reported that the presence of 1.0 g/L HmF declined ethanol productivity by 71.42% by an isolated strain however, up to 0.5 g/L HmF, glucose was completely consumed and very little effect was observed on ethanol productivity and yield. Fein et al. (1984) studied the effect of potential wood hydrolysate compounds on growth and morphology of *Z. mobilis* and found that minimum inhibitory concentration for HmF was 1.47 % w/v with 0.49-0.98 % w/v causative concentration for morphological disturbance of *Z. mobilis*.

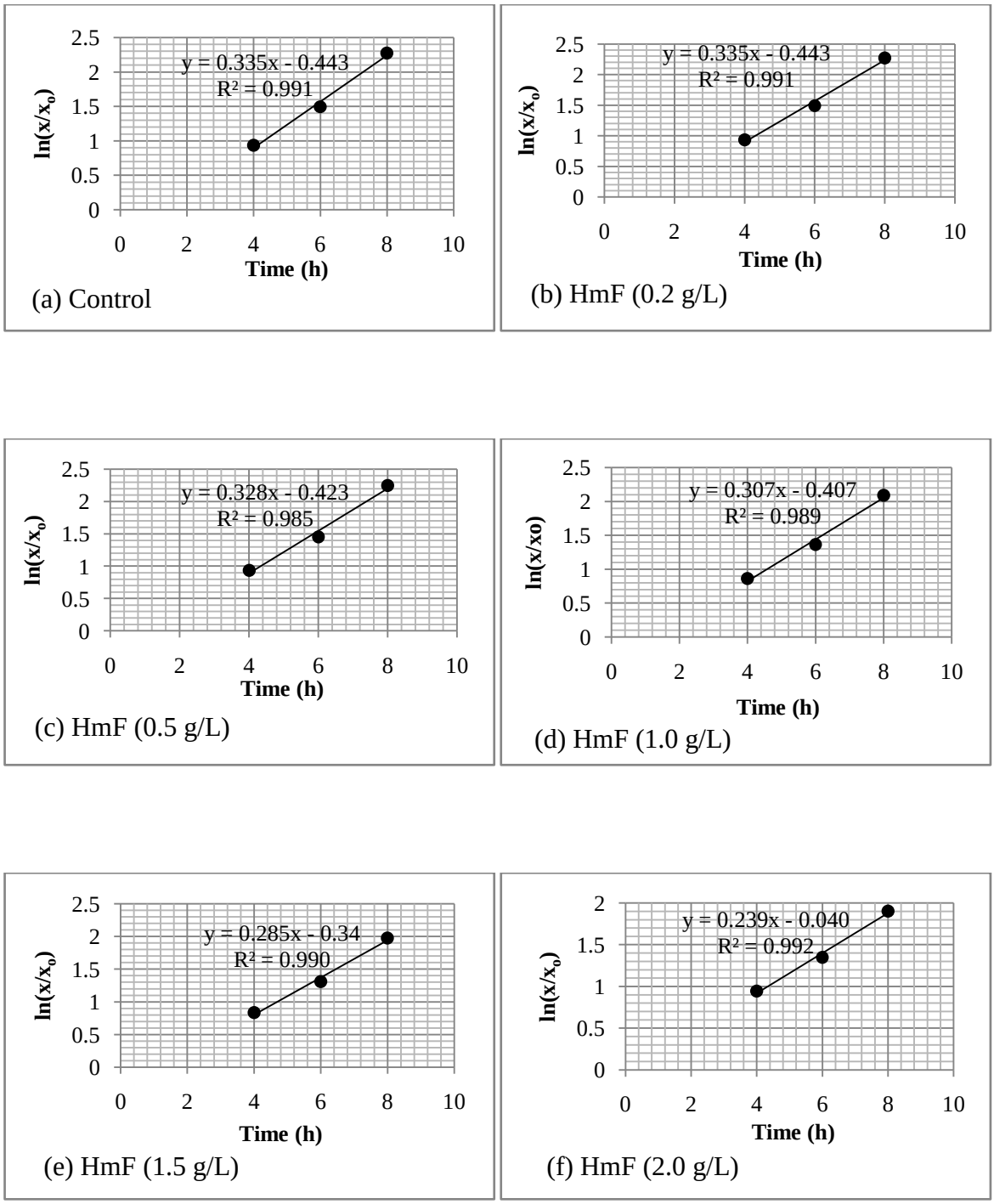


Figure 4.11 Curve for specific growth rate (μ) calculation of *Z.mobilis* with different concentration of hydroxymethyl furfural (HmF) in the growth media.

Table 4.7 Effect of HmF concentration on specific growth rate of *Z. mobilis.*

S. no.	Furfural concentration (g/L)	Specific growth rate μ (h^{-1})	Reduction in specific growth rate (%)
1	0.0	0.335	0.00
2	0.2	0.335	0.00
3	0.5	0.328	2.09
4	1.0	0.307	8.36
5	1.5	0.285	14.93
6	2.0	0.239	28.66

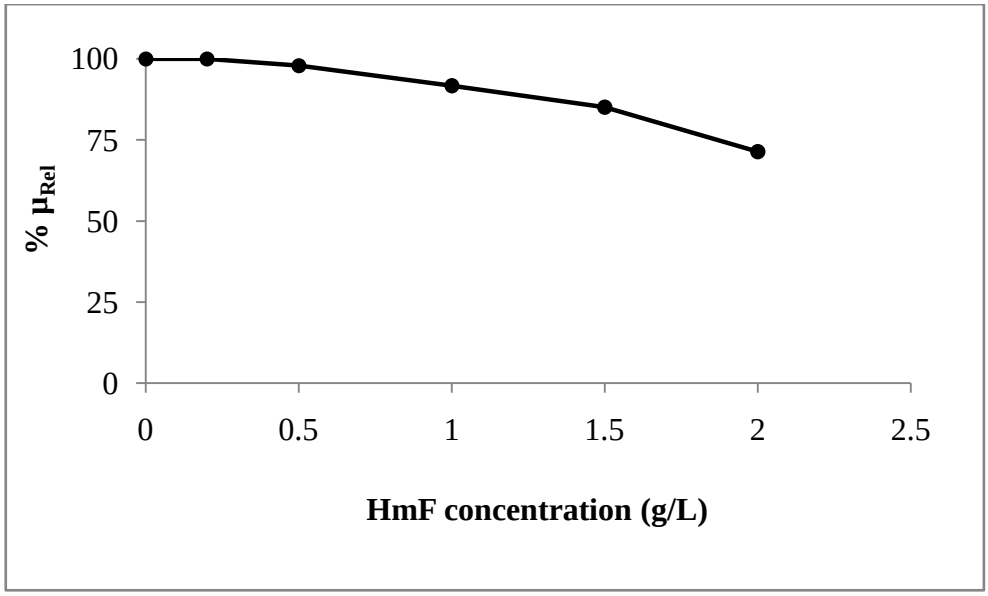


Figure 4.12 Effect of hydroxymethyl furfural (HmF) concentration on the specific growth rate of *Z. mobilis*.

4.4.4 Effect of phenolics on specific growth rate of *Zymomonas mobilis*

The lignin degradation compounds are found to be more toxic to microorganisms than furfural and HmF [Parajo et al., 1998]. Vanillin (4-hydroxy-3-methoxy benzaldehyde) was selected as the model phenolic compound to investigate its effect on the growth of *Z. mobilis*. Different concentration of vanillin was introduced in the growth media of *Z. mobilis* ranging from 0.2 to 2.0 g/L along with the control (no vanillin). The specific growth rate (μ) was calculated during the exponential growth phase of the *Z. mobilis* as the slope of the linear relationship from the curve $\ln(x/x_0)$ vs. time, shown in figure 4.13.

The effect of vanillin on specific growth rate of *Z. mobilis* is given in table 4.8. The % reduction in specific growth rate of *Z. mobilis* was calculated. It was found that the effect of vanillin on the specific growth rate of *Z. mobilis* was nominal at 0.5 g/L whereas, at 1.0 g/L or more concentration of vanillin the specific growth rate of *Z. mobilis* decreased rapidly. The relative specific growth rate (μ_{rel}) was also calculated by taking specific growth rate in control as 100% and is shown in figure 4.14. From the analysis of results it was found that ca. 7 % reduction in specific growth rate of *Z. mobilis* was observed with 0.5 g/L vanillin concentration whereas, ca. 18 % reduction (about 2.5 times more than that with 0.5 g/L concentration) was found with 1.0 g/L initial vanillin concentration. Thus 0.5 g/L vanillin concentration was marked as tolerable phenolics concentration for *Z. mobilis* growth.

The filamentous growth of *Z. mobilis* has been reported by Krug and Daugulis (1983) because plugging of column reactor was observed with an immobilized cell system of *Z. mobilis*. Fein et al. (1984) reported the minimum inhibitory concentration of phenol (a component of wood hydrolysate) was 0.29 % w/v and causative concentration for morphological disturbance of *Z. mobilis* was 0.049 to 0.088 % w/v. Delgenes et al. (1996) observed that 0.5 g/L concentration of hydroxybenzaldehyde reduced cell growth of *Z. mobilis* by 16 % and ethanol production by 21% as compared with control. It was also reported that *Z. mobilis* showed higher potential except hydroxybenzaldehyde, for both biomass growth and ethanol production than *S. cerevisiae* in the presence of tested model compounds. The tolerance of *Z. mobilis* towards lignocellulosic biomass degradation products was higher than that of *S. cerevisiae*.

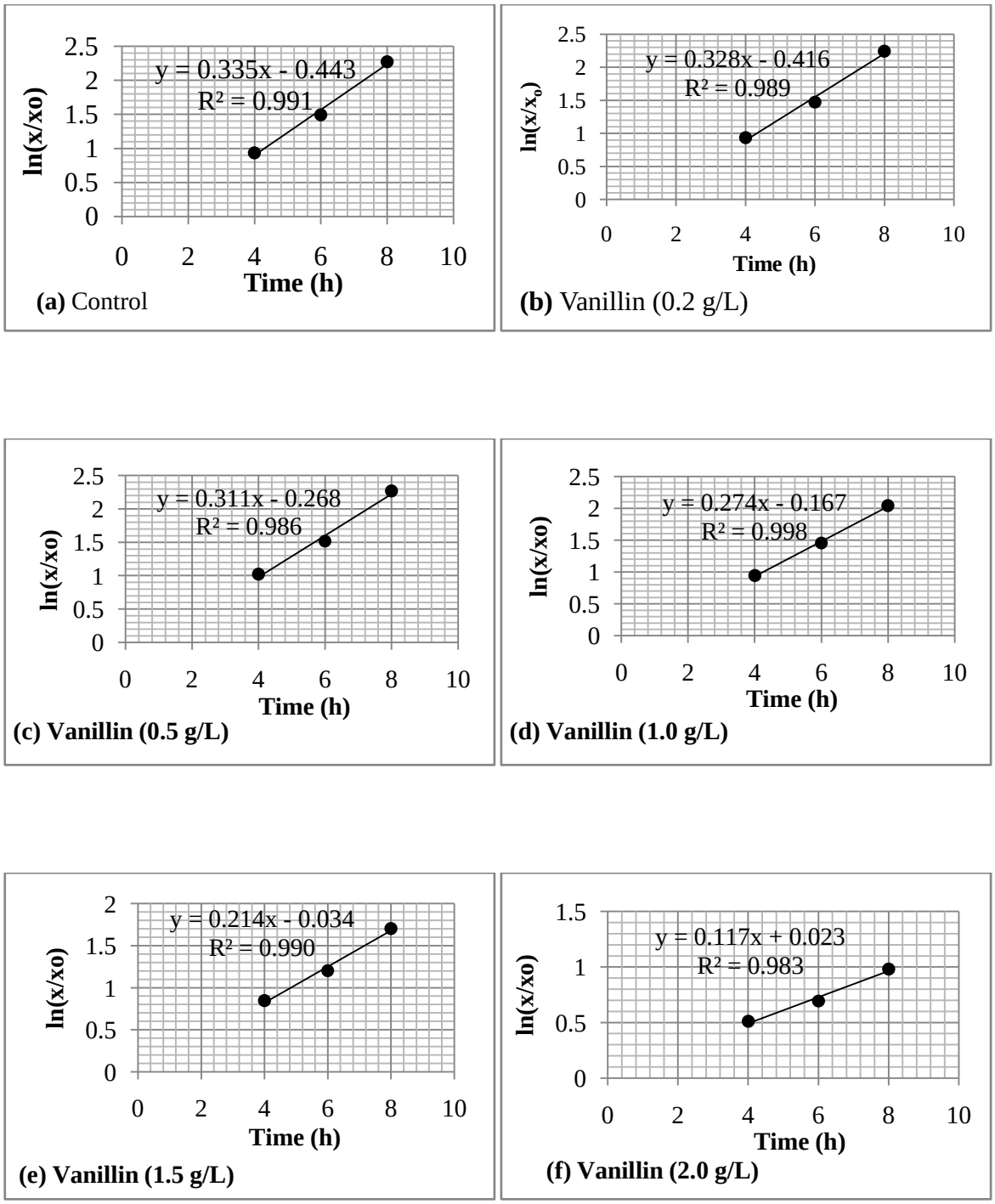


Figure 4.13 Curve for specific growth rate (μ) calculation of *Z. mobilis* with different concentration of vanillin as model phenolic compound in the growth media.

Table 4.8 Effect of vanillin concentration on specific growth rate of *Z. mobilis*.

S. no.	Vanillin concentration (g/L)	Specific growth rate μ (h^{-1})	Reduction in specific growth rate (%)
1	0.0	0.0.335	0.00
2	0.2	0.328	2.09
3	0.5	0.311	7.16
4	1.0	0.274	18.21
5	1.5	0.214	36.12
6	2.0	0.117	65.07

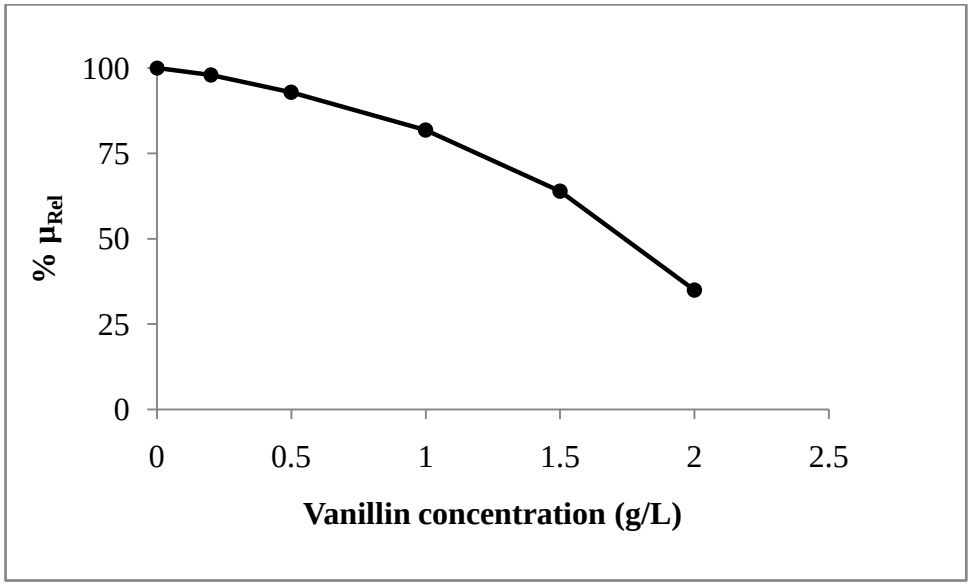


Figure 4.14 Effect of vanillin concentration on the specific growth rate of *Z. mobilis*.Vanillin used as model phenolic compound.

4.5 Effects of ethanol concentration on growth of microbes

4.5.1 Effect of ethanol concentration on specific growth rate of *Pichia stipitis*

Various studies revealed that *P. stipitis* is one of the best candidate among the yeast species for production of ethanol from hemicellulose fraction of lignocellulose biomass [Dellweg et al., 1984; Slininger et al., 1985; Agbogbo and Wenger, 2007; Lee et al, 2000]. The data on quantification of ethanol tolerance of this microorganism is limited. It is of prime importance to quantify the ethanol tolerance level for getting high ethanol concentration in the fermentation broth to facilitate further distillation process [du Preez et al., 1987]. Thus the experiment was designed to obtain critical level of ethanol concentration for *P. stipitis* growth and the model proposed by Luong (1985) was used (equation 4.4).

$$\mu_i/\mu_o = 1-(P/P_m)^{\alpha} \quad (4.4)$$

by rearranging $$\ln (1- \mu_i/\mu_o) = \alpha \ln P - \alpha \ln P_m \quad (4.5)$$

The linear relationship as developed from model (equation 4.5) was used to calculate maximum ethanol concentration above which no *P. stipitis* cell growth was observed is given by the Equation 4.5 and the graphical determination of the ethanol limit (P_m) is given in figure 4.15. Slope of the linear relationship represented the value of α and intercept - $\alpha \ln P_m$. P_m was calculated and found to be 37 g/L ethanol concentration. The result was in accordance to the studies done by du Preez et al., (1987) and Slininger et al., (1982). They found that *P. stipitis* can produce ethanol up to 33-57 g/L, however critical concentration of ethanol for *P. stipitis* was found to be 30 g/L above which no cell growth was observed at 30°C. Lee et al. (2000) have found that at 130 g/L initial xylose concentration 31.8 g/L maximum ethanol was produced at 30°C by *P. stipitis* Y-7124.

Many researchers have reported that the ethanol produced by the microbial cells via fermentation is significantly more toxic than externally added ethanol to the medium [Mota et al., 1984; Jones and Greenfield, 1985; Novak et al., 1981; Singh et al., 2011c]. This may be due to very low permeability of ethanol from outside environment to inside the cell. It is very difficult to quantify the effect of produced ethanol during xylose fermentation because oxygen is required for growth whereas, limited oxygen enhances ethanol production. Thus distinguishing the features of oxygen limitation and growth inhibition by ethanol requires very effective dissolved oxygen controlling system.

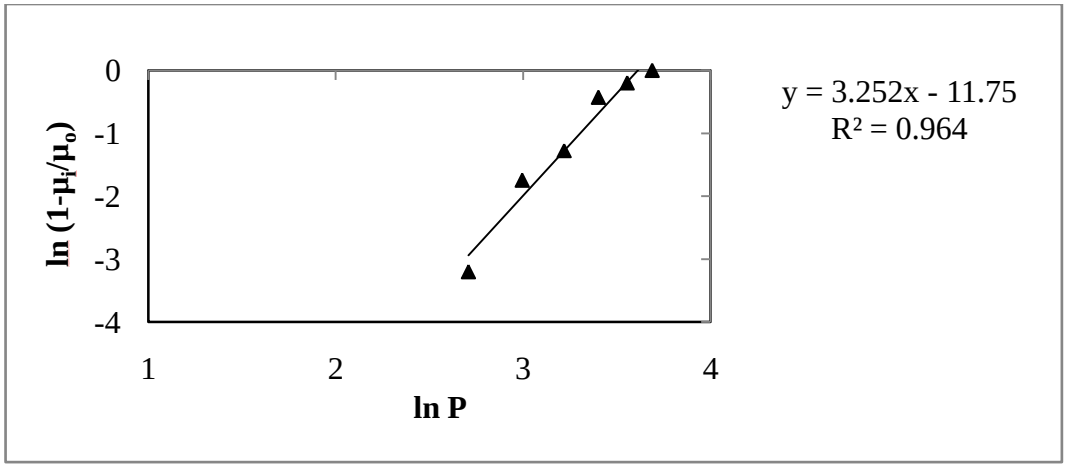


Figure 4.15 Estimation of maximum ethanol tolerance (P_m) for *P. stipitis*.

4.5.2 Effect of ethanol concentration on specific growth rate of *Zymomonas mobilis*

Zymomonas mobilis is a potential bacterium for ethanol production as described by many researchers [Gunasekaran et al., 1990; Panesar et al., 2006]. The main features of *Z. mobilis* include a high specific rate of sugar uptake, high ethanol productivity, low cell mass production, no requirement of controlled oxygen etc. The sugar conversion rate of *Z. mobilis* to produce ethanol and carbon dioxide as principal products is several folds to that of *S. cerevisiae* [Lawford et al., 1988; Rogers et al., 2007]. To quantify the exogenous ethanol tolerance level for getting high ethanol concentration in the fermentation broth, the experiment was designed to obtain critical level of ethanol concentration for *Z. mobilis* growth. The model proposed by Luong (1985) was used as described in previous section (equation 4.4 and 4.5).

The graphical determination of the ethanol limit (P_m) is given in figure 4.16. Slope of the linear relationship represented the value of α and intercept - $\alpha \ln P_m$. P_m was calculated and found to be 104 g/L ethanol concentration above which growth of *Z. mobilis* ceased. Sprenger (1996) reported that *Z. mobilis* follows homoethanol fermentation pathway and tolerates up to 120 g/L ethanol and its specific ethanol productivity is about 2.5 times higher than that of *S. cerevisiae*. Osman and Ingram (1985) have proposed the mechanism of ethanol inhibition and concluded that the increased leakage of cofactors and coenzymes through plasma membrane caused inhibition of fermentation. Various studies have different opinion about the effect of in vivo and in vitro presence of ethanol on measurable inhibition. The in vivo low concentration of ethanol (2 % w/v) created measurable inhibition, whereas concentration above 15 % w/v is required to inhibit enzymes in vitro [Millar et al., 1982; Moulin et al., 1980]. As *Z. mobilis* is easily permeable to ethanol thus intracellular concentration of ethanol do not reach to high levels responsible to inhibit key enzymes although other factors of intracellular environment may be responsible for increased sensitivity of these enzymes [Osman and Ingram, 1985].

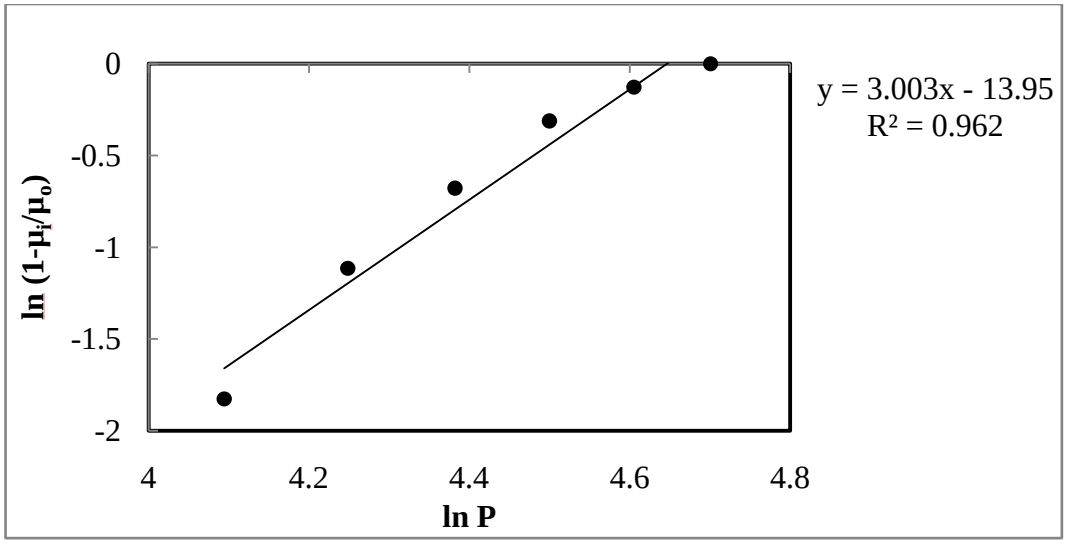


Figure 4.16 Estimation of maximum ethanol tolerance (P_m) for *Z. mobilis*.

SECTION B

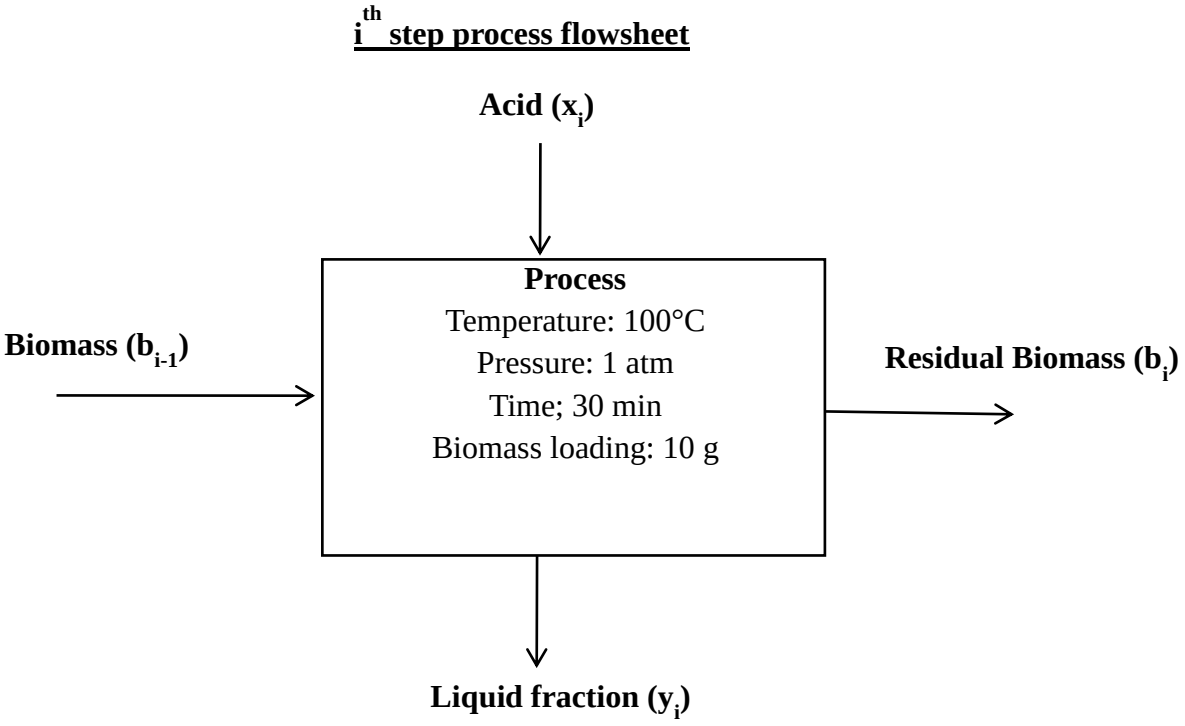
[**Patent:** "A novel fractionating hydrolysis process for production of fermentable sugars from lignocellulosic biomass of Kans grass (*Saccharum spontaneum*)" under process of filing].

4.6 Novel process for fractionating hydrolysis of lignocellulosic biomass

4.6.1 Single vessel multi-step acid hydrolysis of KGB

Effective release of protected polysaccharides from the complex lignocellulosic biomaterials to fermentable sugars is considered as prime challenge for lignocellulosic biomass to ethanol production process [Zhang et al., 2007]. The leading lignocellulose pretreatment/hydrolysis technologies suffer from low sugar yield, and/or severe reaction conditions, and/or costlier enzymes use, narrow substrate applicability, and high capital investment etc. [Eggeman and Elander, 2005]. Main objective of the present investigation was oriented towards the maximum soluble sugar extraction with minimum toxic compounds generation from KGB. A novel single vessel multi-step fractionating hydrolysis process was developed in the laboratory with the working hypothesis that concentrated acid treatment is the only method among the existing technologies to extract maximum amount of sugars (>90 % of total theoretical) from the lignocellulosic biomass and high temperature (above atmospheric pressure) during the course of hydrolysis is the main cause of toxics generation [Taherzadeh and Karimi, 2007b]. The schematic representation of the process is shown in figure 4.17.

The experimental set up as shown in figure 3.11and was used to hydrolyze the oven dried KGB. The sequential addition of increased sulfuric acid concentration from 1 to 35 % v/v (total nine steps) along with direct steam insertion at 100°C (i.e. at atmospheric pressure) to the reaction system for 30 min each step was successfully used to extract 95.3% of the total reducing sugars (TRS) available in the KGB in the form of carbohydrate polymer (0.9 x cellulosic content + 0.88 x hemicellulosic content). After each reaction time the liquid fraction was withdrawn from the reaction system, thus not allowing the hydrolysate content for long exposure towards acid or temperature. Total nine liquid hydrolysate fractions were collected in accordance to the different acid concentrations applied. The composition of various liquid fractions and the residual biomass is given in table 4.9.



Where, x = % Acid applied
y = Liquid sugar fraction
b = weight of solid biomass
Process steps: i = 1, 2, 3 ……9

Figure 4.17 The schematic representation of the fractionating hydrolysis process

Table 4.9 The composition of various fractions corresponds to the process steps of fractionating hydrolysis of KGB.

Process steps (i)	**Acid applied (% v/v) (x_i)**	**Components in the liquid fraction (% g/g dry initial KGB) (y_i)**					
		TRS	**Pentose**	**Furfural ($x10^{-2}$)**	**HmF ($x10^{-2}$)**	**Total phenolics ($x10^{-2}$)**	**Residual biomass (b_i)**
1	1	0.42	0.30	n.d.	n.d.	1.25	99.40
2	2	8.38	7.85	2.01	n.d.	2.40	89.78
3	5	11.51	10.45	8.43	2.1	3.21	76.06
4	10	3.26	2.50	4.40	2.7	4.63	72.64
5	15	4.18	n.d.	n.d.	4.3	5.13	68.22
6	20	8.28	n.d.	n.d.	8.1	5.71	58.59
7	25	13.45	n.d.	n.d.	12.2	5.98	43.20
8	30	7.16	n.d.	n.d.	13.2	6.24	35.12
9	35	1.23	n.d.	n.d.	6.2	8.60	34.21

4.6.2 TRS and pentose production

The aforementioned single vessel multi-step hydrolysis process used to fractionate KGB, generated liquid hydrolysate in the same number as different acid concentrations used (total nine steps). The concentration of TRS and pentose sugar in different liquid fractions are shown in figure 4.18. It was observed that pentose sugar was appeared in the first four fractions i.e. fraction number 1 to 4, and its amount was found very close to that of TRS. As pentose sugars (mainly xylose) are constituents of hemicellulosic fraction only, thus it was manifested that during these steps of hydrolysis hemicellulose fraction was mainly solubilized and a very little sugar was liberated from cellulosic fraction. Furthermore, cellulose degradation was started in subsequent steps of hydrolysis. In fraction number 5 to 9 no detectable pentose sugar was present. Thus it could be stated that the TRS consisted only glucose in these fractions (a monomer unit of cellulose polymer). The maximum solubilization of cellulose was occurred in the 7^{th} process step (25% acid concentration and 210 min of total elapsed time from the start of the process at 100°C).

After total elapsed time of 240 min, only 35% of the initial biomass was present and mainly consisted of lignin and other non-carbohydrate entities. The recovery of TRS from all the process steps was 95.3%, whereas up to 8^{th} step it was 93.3% on the basis of TCC present in dry KGB sample. In 9^{th} fraction, TRS was only 1.23 g/L. Thus it was concluded that most of the carbohydrate fraction of KGB was dissociated up to 8^{th} process step.

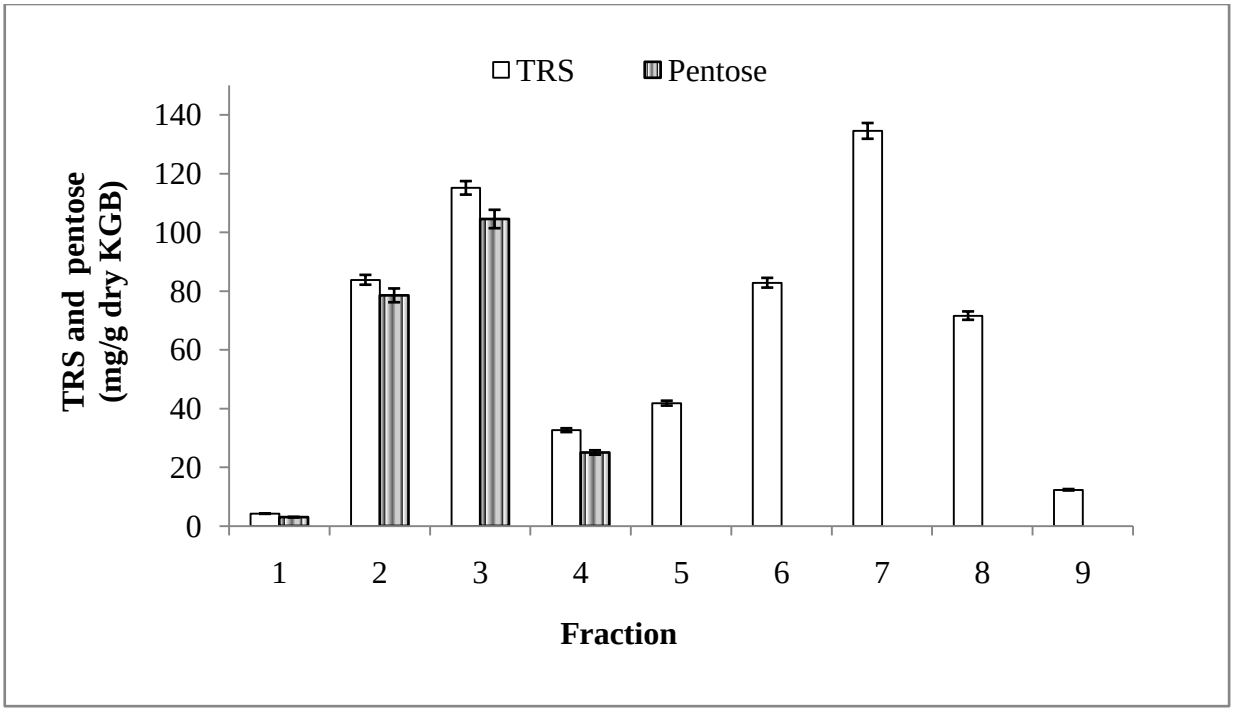


Figure 4.18 Total reducing sugar and pentose sugar released (mg/g dry KGB) in various hydrolysate fractions collected at every 30 min of reaction time with varying acid concentration. Each data point represents the mean value (n=2, SD<0.3, TRS and SD<0.2, pentose).

4.6.3 Toxics generation

The toxic compounds generation during the hydrolysis in various fractions is shown in figure 4.19. It was observed that furfural was generated only up to 4^{th} fraction. It is dehydration product of xylose and this sugar was not detected in later fractions (i.e. in fraction number 5 to 9) as shown in Figure 4.19. Thus it was again confirmed that the whole hemicellulose fraction of the KGB was hydrolyzed during first four process steps. The hydroxymethylfurfural (HmF), a dehydration product of glucose was mainly found when cellulosic fraction was started to hydrolyze. The phenolic compound generation was observed in all fractions. This reveled that lignin was degraded slowly in all process steps under the set experimental conditions.

The temperature of the hydrolysis process using the proposed reaction system was kept constant at normal boiling point of water (100°C) by using direct steam insertion at atmospheric pressure thus not much lignin was degraded. The immediate withdrawal, cooling and neutralization of liquid hydrolysate from the reaction vessel after each preferred reaction process step of 30 minutes prevents further dehydration of the soluble sugars liberated from biomass, therefore inhibitory compounds are not generated in significant amount. Many researchers have agreed on one point that low temperature and pressure minimize the degradation of sugars [Demirbas, 2005; Mussatto and Roberto, 2004; Palmqvist and Hahn-Hagerdal, 2000].

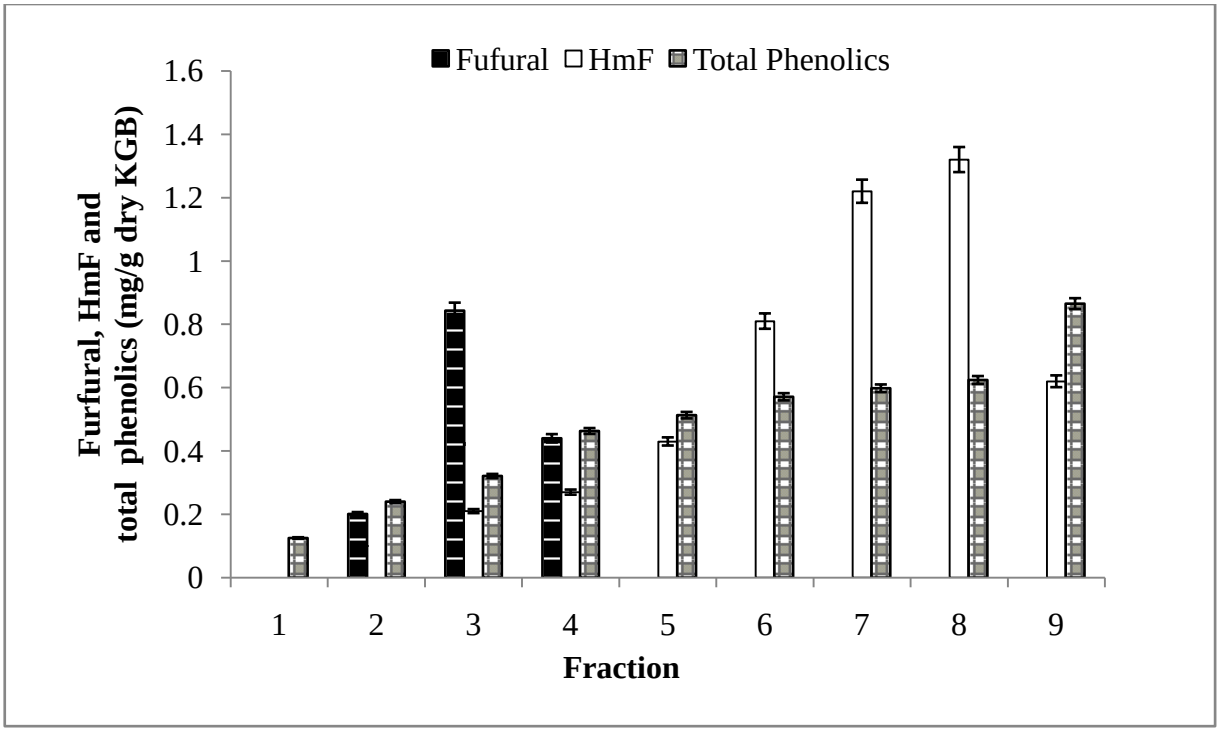


Figure 4.19 The toxic compounds generation in various process steps, collected as liquid fractions at every 30 min of reaction time with varying acid concentration. Each data point represents the mean value (n=2, SD<0.3, Furfural and HmF; SD<0.2, total phenolics).

4.6.4 Generation of two main sugar streams

After analyzing the sugars and inhibitory compounds generated during hydrolysis of KGB in all 9 fractions collected separately it was observed that the pentose sugar (xylose) was extracted only up to forth fraction and was main component in the TRS. Thus fraction 1, 2, 3 and 4 were pooled together to generate first sugar stream as xylose rich fraction (XRF). Further it was observed that in the ninth fraction collected after applying the highest acid concentration (35%), TRS concentration was 1.23 g/L which was very low, whereas phenolic concentration was at its highest value 86.5 mg/L than that were present in previous fractions. Also 62 mg/L HmF and no furfural was found in this particular fraction. The recovery of TRS from all the process steps was 95.3% whereas up to 8^{th} step it was 93.3% on the basis of TCC present in dry KGB sample. Thus it was concluded that most of the carbohydrate fraction of KGB was dissociated up to 8^{th} process step and after that increase in acid concentration was not beneficial as far as our objective was concerned. Therefore, 9^{th} liquid hydrolysate fraction was excluded from further processing steps. The rest fractions (5^{th} to 8^{th}) were the mixed together to form second sugar stream as glucose rich fraction (GRF). After setting the desired level of pH and sugar concentration using conditioning method as described in section 3.3.6 the sugar and inhibitory compound concentrations in XRF and GRF are given in table 4.10. After conditioning and ready to use for fermentation, the XRF contained 0.362 g/L furfural, 0.271 g/L total phenolics and very low concentration of HmF (0.118 g/L). The tolerable concentration of furfural and phenolics for *P. stipitis* were found as 0.4 g/L and 0.5 g/L respectively (section 4.4.1 and 4.4.2). Similarly GRF contained 0.972 g/L HmF and 0.613 g/L total phenolics which were found near to the acceptable concentration of HmF and phenolics for *Z. mobilis* cells (1.0 g/L HmF and 0.5 g/L phenolics) as mentioned in section 4.4.3 and 4.4.4.

In most of the hydrolysis processes due to severe reaction conditions, the soluble sugars liberated further converted to their respective dehydration products like furfural (from xylose) and hydroxymethyl furfural (from glucose), thus reducing the overall availability of these sugars for bioconversion to valuable products. Further these sugar dehydration products and phenolic compounds generated during the hydrolysis process steps are known to be inhibitory to the microorganisms in further fermentation thus essentially required to remove or reduce in such a concentration that are tolerable to the used microorganisms [Pienkos and Zhang, 2009]. Detoxification, an additional process step is used to remove or reduce the concentration of these inhibitory compounds. Overliming is often used [Mohagheghi et al., 2006] to detoxify the hydrolysate by adding $Ca(OH)_2$ to raise the pH 9-10, hydrated gypsum, $CaSO_4.2H_2O$ is formed and precipitates. The disposal of produced gypsum in bulk is problematic. Also 8-10% sugar

loss is reported during overliming [Martinez et al., 2001]. The significance of the present investigation revealed that the novel process of fractionating hydrolysis of KGB generated toxics in very low quantity and found to be acceptable for the microorganisms like *P. stipitis* and *Z. mobilis* for fermentation without performing any detoxification process step. Thus the above said problems are addressed successfully.

The photographs (figure 4.20) of dried residual sample of unhydrolysed KGB and acid treated KGB (8 samples) showed the effect of increasing order of acid concentration applied on KGB for hydrolysis. The colour of KGB particles were getting darker as the reaction process proceeded. The images are representing total eight stages of the hydrolysis process. The qualitative analysis of residues obtained from each process step using SEM is shown in figure 4.21. It is shown in SEM image of untreated KGB sample that the texture was compact and covered with a thin film probably of wax found in herbaceous biomaterials [Hu and Wen, 2008]. During sequential hydrolysis the layer disappeared and breaks down of the biomass started the phenomenon is clearly viewed from the images.

(a)

(b) (c)

(d) (e)

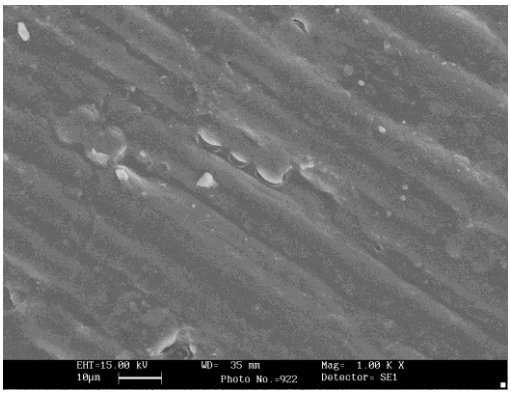
(e)

(f)

(g)

(h)

Figure 4.20 The photographs of original (a) and acid treated KGB sample (b), (c), (d), (e), (f), (g), (h), (i) showing the effect of increasing order of acid concentration applied on KGB for hydrolysis. The images are representing total eight stages of the hydrolysis process.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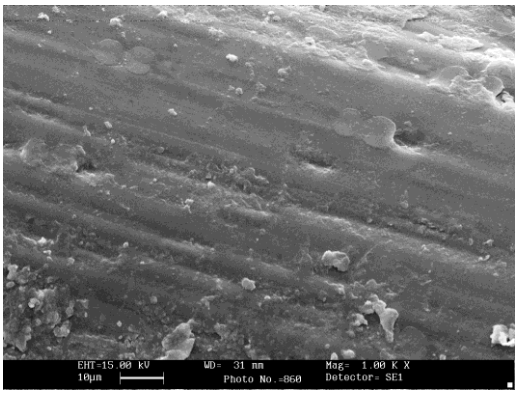
(a)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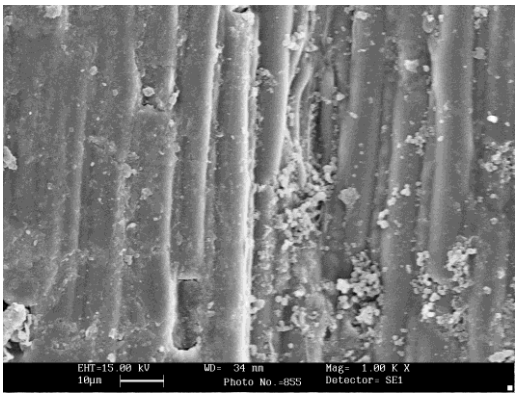
WD= 31 mm
Mag= 1.00 K X
10µm
Photo No.=860
Detector= SE1

(b)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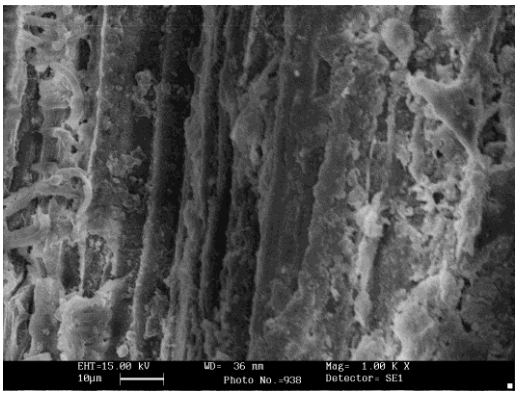
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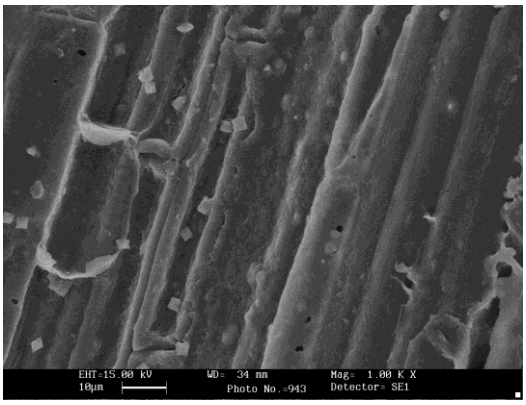
(d)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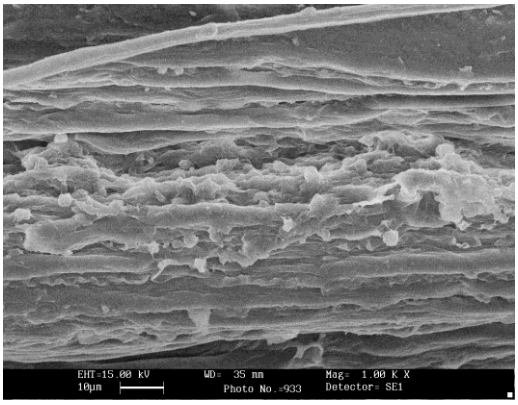
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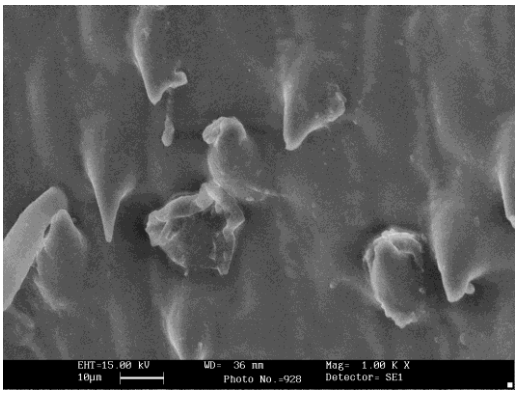

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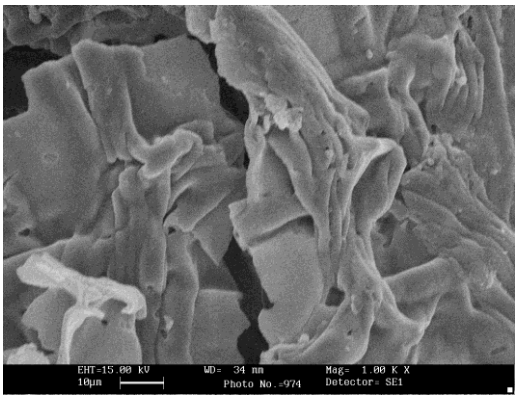

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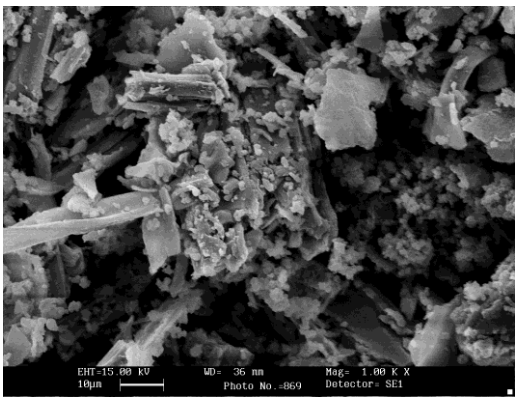


(i)

Figure 4.21 SEM images of original (a) and acid treated KGB sample (b), (c), (d), (e), (f), (g), (h), (i) showing the effect of increasing order of acid concentration applied in sequential manner on KGB for hydrolysis. The images are representing total eight stages of the hydrolysis process.

Table 4.10 The sugar and toxic compound concentrations in XRF and GRF.

Constituents	**before conditioning**		**after conditioning***	
Sugar (g/L)	**XRF**	**GRF**	**XRF**	**GRF**
TRS	5.893±0.025	8.268±0.03	58.900±0.26	98.811±0.30
Xylose	5.275±0.018	n.d.	52.608±0.20	n.d.
Toxics (g/L)				
Furfural	0.037±0.002	n.d.	0.362±0.015	n.d.
HmF	0.012±0.001	0.095±0.004	0.118±0.021	0.972±0.042
Total phenolics	0.029±0.003	0.057±0.002	0.271±0.034	0.613±0.056

*conditioning refers to process in which pH of both the fractions (XRF and GRF) were raised to 2.0 with 1N NaOH then 6.0 ± 0.2 by using $Ca(OH)_2$ and filtered to remove any precipitate. The filtrate were concentrated under vacuum at 60°C to desired level of sugar concentration and supplemented with nutrients to prepare hydrolysate ready for fermentation (section 3.3.6).

4.6.5 Ethanol production from XRF

The conditioned XRF was fermented by *P. stipitis* with 58.9 g/L TRS where xylose concentration was 53.6 g/L and rest was glucose. The XRF and corresponding synthetic media contained 60 g/L TRS with 54 g/L xylose concentration were inoculated with 5% v/v inoculum (18 h culture, $1x10^7$cells/ml) of *P. stipitis*. The concentration profile of TRS, biomass and ethanol concentration during fermentation is shown in figure 4.22(a) and (b). The comparison of kinetic parameters of XRF and synthetic media fermentation is given in table 4.11. Total sugar utilization was observed as 94.7% with 80 h of fermentation time whereas 93.4% sugar was utilized within 36 h. About three times longer lag phase of *P. stipitis* was observed in case of XRF as compared to the synthetic media. The doubling time of *P. stipitis* in XRF was calculated as 5.73 h which was also higher as found in synthetic media (4 h). This could be reduced by propagating cells in the same hydrolysate media using several adaptation steps. The specific sugar uptake rate (q_s) in XRF and synthetic media were 1.704 g/g/h and 2.662 g/g/h respectively. The specific growth rate (μ) was found as 0.121 h^{-1} in XRF and 0.173 h^{-1} in synthetic media. The ethanol yield ($Y_{p/s}$) and specific productivity (q_p) were 0.443 g/g and 1.179 g/g/h for synthetic and 0.427 g/g and 0.731 g/g/h for XRF media respectively. The other fermentation parameters are mentioned in table 4.11. The values of fermentation parameters for XRF as compared to that of synthetic media were found little lower. This may be due to the presence of inhibitory compounds in the hydrolysate.

Table 4.11 Comparison of fermentation parameters of ethanol production from xylose rich fraction (XRF) of KGB acid hydrolysate and synthetic media.

Parameter	**XRF**	**Synthetic**
Initial total reducing sugar, S_0 (g/L)	58.90	60.0
Specific growth rate, μ (h^{-1})	0.121	0.173
Biomass yield coefficient, $Y_{x/s}$ (g/g)	0.071	0.065
Maximum cell concentration, x_{max} (g/L)	4.13	3.85
Ethanol yield coefficient, $Y_{p/s}$ (g/g)	0.427	0.443
Specific ethanol productivity, q_p (g/g/h)	0.731	1.179
Maximum ethanol concentration, p_{max} (g/L)	23.8	24.8
Specific sugar uptake rate, q_s (g/g/h)	1.704	2.662
Doubling time, t_d (h)	5.73	4.00
% theoretical yield	84	87

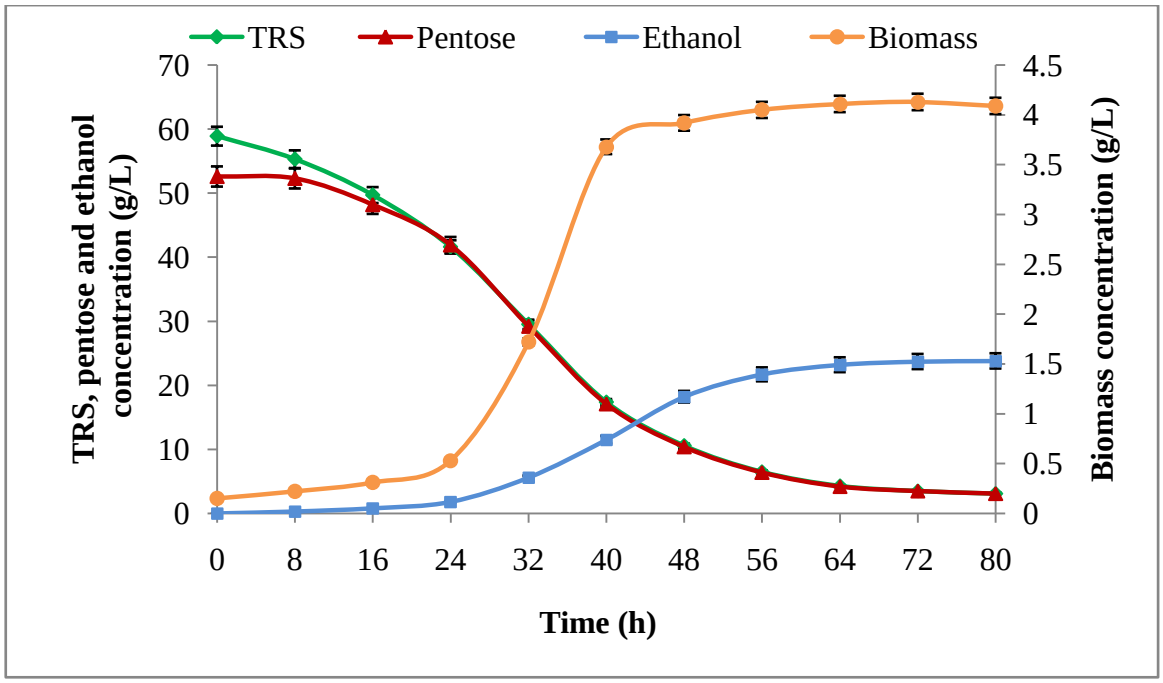


Figure 4.22(a) Concentration profile of total reducing sugar, pentose sugar, biomass and ethanol concentration during fermentation of hydrolysate (XRF) media by *P. stipitis*.

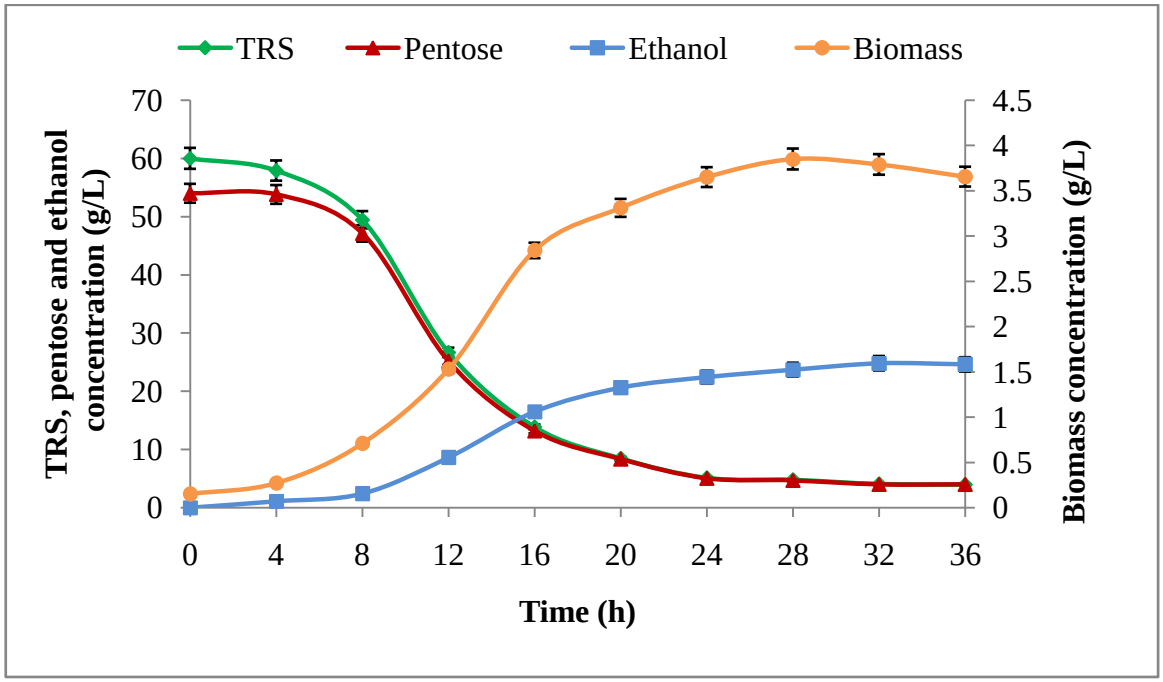


Figure 4.22(b) Concentration profile of total reducing sugar pentose sugar, biomass and ethanol concentration during fermentation of synthetic media by *P. stipitis*.

4.6.6 Ethanol Production from GRF

Initially the sugar stream GRF contained 8.268 g/L TRS. After conditioning and vacuum concentration the TRS concentration was raised to 98.8 g/L. The conditioned GRF was then fermented by *Z. mobilis* cells with 5% v/v inoculum (1 x 10^8 cells/ml). The corresponding synthetic media was also inoculated with same inoculums size and glucose was used as the only sugar with initial concentration of 100 g/L. The concentration profile of TRS, biomass and ethanol concentration during fermentation is shown in figure 4.23(a) and (b). The comparison of kinetic parameters of GRF and synthetic media fermentation is given in table 4.12. It was observed that 97.6 % sugar was utilized within 24 h of fermentation time in GRF whereas 99% sugar was utilized in the synthetic media within 16 h. The specific sugar uptake rate (q_s) in GRF and synthetic media were 7.594 g/g/h and 9.657 g/g/h respectively. The specific growth rate (μ) was found as 0.243 h^{-1} in GRF and 0.338 h^{-1} in synthetic media. The ethanol yield and ($Y_{p/s}$) and specific productivity (q_p) were 0.494 g/g and 4.805g/g/h for synthetic and 0.476 g/g and 3.587 g/g/h for GRF media respectively. The other fermentation parameters are mentioned in table 4.12. The lower values of fermentation parameters for GRF as compared to that for synthetic media were may be due to the presence of inhibitory compounds in the hydrolysate.

It is worth pointing out here that no detoxification step was used to remove or reduce the toxic compounds generated during the acid hydrolysis still comparable fermentability were obtained from GRF and XRF in comparison to the previously reported results where some detoxification steps were involved [Mohagheghi et al., 2006; Millati et al., 2002]. Thus the method applied to hydrolyze the KGB was found effective with a little production of toxic compounds which were not in significant amount to affect the fermentation and thus ethanol yield.

Table 4.12 Comparison of fermentation parameters for ethanol production from glucose rich fraction (GRF) of KGB acid hydrolysate and synthetic media.

Parameter	**GRF**	**Synthetic**
Initial total reducing sugar, S_0 (g/L)	98.8	100.0
Specific growth rate, μ (h^{-1})	0.243	0.338
Biomass yield coefficient, $Y_{x/s}$ (g/g)	0.032	0.035
Maximum cell concentration, x_{max} (g/L)	3.25	3.58
Ethanol yield coefficient, $Y_{p/s}$ (g/g)	0.476	0.494
Specific ethanol productivity, q_p (g/g/h)	3.587	4.805
Maximum ethanol concentration, p_{max} (g/L)	45.9	48.9
Specific sugar uptake rate, q_s (g/g/h)	7.594	9.657
Doubling time, t_d (h)	2.85	2.05
% theoretical yield	93	96.8

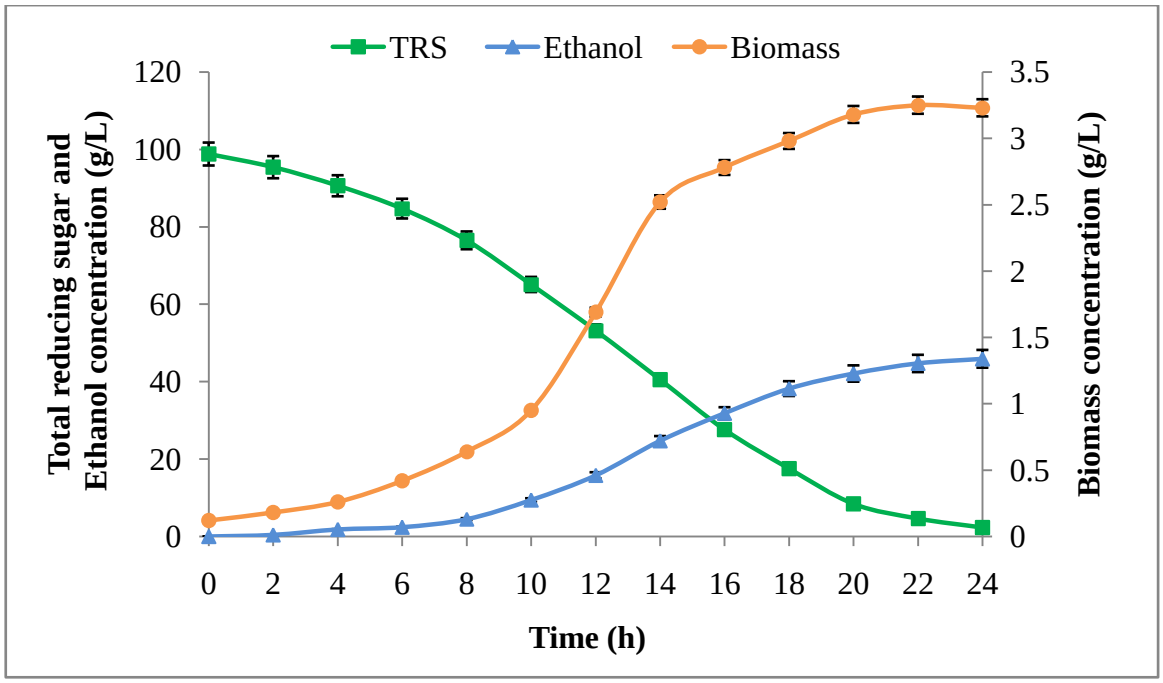


Figure 4.23(a) Concentration profile of total reducing sugar, biomass and ethanol concentration during fermentation of hydrolysate (GRF) media by *Z. mobilis*.

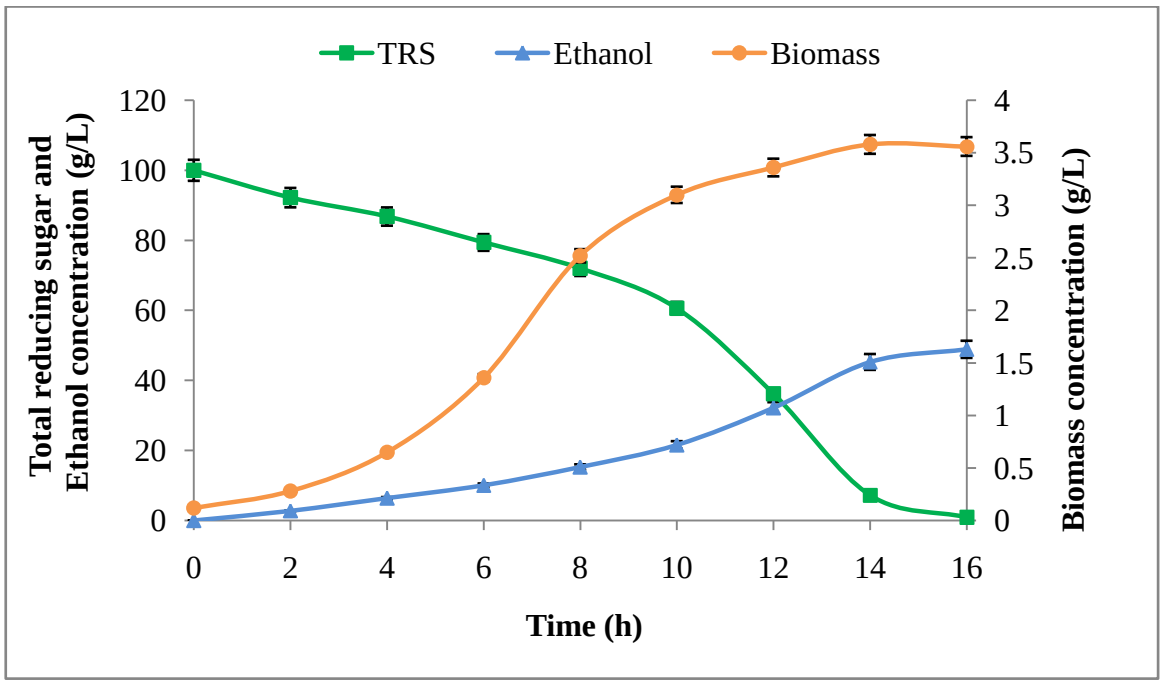


Figure 4.23(b) Concentration profile of total reducing sugar, biomass and ethanol concentration during fermentation of synthetic media by *Z. mobilis*.

SECTION C

[Singh, L. K., Majumder, C. B., Ghosh, S. Development of sequential-co-culture system (*Pichia stipitis* and *Zymomonas mobilis*) for bioethanol production from Kans grass biomass.Biochemical Engineering Journal, 82(0): 150-157 (2014)].

4.7 Development of sequential-co-culture system

The sequential-co-culture system of *P. stipitis* and *Z. mobilis* was developed at two levels based on the total working volume of fermentation media, first in flask (300 ml) then in bioreactor (4000 ml) using synthetic fermentation media. Finally the selected strategy was implemented for KGB hydrolysate (as described in section 4.6 in the form of XRF and GRF) fermentation at bioreactor level. Very limited data is available for lignocellulosic biomass to ethanol production using sequential-co-culture system particularly at bioreactor level [Chen, 2011]. Therefore, in the present investigation efforts were made to develop a novel sequential-co-culture system of high ethanol yield and volumetric productivity by utilizing sugars from KGB hydrolysate.

4.7.1 Sequential-co-culture system in flask using synthetic fermentation media

The important process conditions favorable for better ethanol productivity were examined at flask level. The sequential addition of strains and accordingly the media was based on the fact that the glucose is assimilated by *P. stipitis* preferentially over xylose as carbon source for growth and ethanol production. Furthermore, this yeast has low ethanol tolerance (<5% w/v) in comparison to the *Z. mobilis* (>10% w/v). Also high concentration of glucose can suppress xylose fermentation by *P. stipitis* due to catabolite repression, especially at the initial stage, because xylose bioconversion is completely inhibited at glucose concentration of 2.3 g/L or higher [Grootjen, 1991], thus resulting poor ethanol yield and/or delay of fermentation [Govindaswamy and Vane, 2007]. Therefore, *P. stipitis* fermentation using xylose as carbon source was conducted first followed by *Z. mobilis* fermentation using glucose as carbon source. The inhibition of xylose fermentation due to rapid formation of ethanol from glucose fermentation by *Z. mobilis* was also taken care by sequential fermentation of sugars and efficient utilization of both types of sugars for bioconversion was achieved. The difference between the two strategies was related to the oxygen availability in the media. N_2sparging at the onset of stage II and III of strategy II under static condition removed the dissolved oxygen completely from the media and provided strictly anaerobic conditions for glucose fermentation by *Z. mobilis*. The results of these two strategies of sequential-co-culture system are given in

table 4.13. The parameters such as ethanol yield ($Y_{p/s}$), volumetric ethanol productivity (r_p), substrate utilization rate (r_s) or other measures are frequently used to evaluate performance of a fermentation system. The theoretical ethanol yield for glucose or xylose fermentation is 0.51 g_p/g_s [Krishnan et al., 1999].

The kinetic parameters were calculated for each stage as well as for the whole system for both the strategies. The sugar utilization profiles for both the strategies are shown in figure 4.24. The overall % sugar utilization and sugar utilization rate were found almost same in both the strategies whereas, sugar utilization rate during stage II and III was 1.7 to 2.5 times more than that in stage I in both the strategies. The reason was that the xylose assimilation by *P. stipitis* was slow as compared to the glucose fermentation by *Z. mobilis*. The actual co-culture system exists only during second and third stages in both the strategies. The cell concentration profile in strategy I and II is presented in figure 4.25 (a) and (b). Stage I represents the mono culture batch fermentation system of *P. stipitis*, whereas stage II and III were the true co-culture system of *P. stipitis* and *Z. mobilis*. After 32 h of the fermentation time the viable cell concentration of *Z. mobilis*(69 x 10^5cfu/ml) approached to that of *P. stipitis* (65 x 10^5cfu/ml) in strategy I. After 40 h from the start of the fermentation the viable cell concentration of *P. stipitis* starts to decline at both conditions but more evident under anaerobic conditions (Table 4.13). After 40 h *P. stipitis* viable cell concentrations in the strategy I and II were found as 93 x 10^5cfu/ml and 86 x 10^5cfu/ml respectively whereas at the end of the fermentation (after 56 h) it was 24 x 10^5cfu/ml and 2.1 x 10^5cfu/ml respectively i.e. ca.10 times less in strategy II in comparison to that in strategy I. The viable cell concentration of *Z. mobilis* at the end of the fermentation was found as 82 x 10^6cfu/ml and 67 x 10^6cfu/ml in strategy I and II respectively. Thus it was concluded that the growth of *Z. mobilis* was dominating over *P. stipitis* at both conditions during co-culture fermentation but more evident under anaerobic conditions. Also about 18% less growth of *Z. mobilis* was observed at the end of fermentation in strategy II as compared to that in strategy I. This could be explained as under strictly anaerobic conditions, *Zymomonas* metabolizes glucose using the Entner-Doudoroff (ED) pathway in place of Embden-Meyerhof (EM) pathway. The ED pathway generates only half as much ATP per mol of glucose as the EM pathway consequently, produces less biomass and more carbon is moved for ethanol formation [Lin and Tanaka, 2006].

The ethanol production profiles in various stages of the two strategies are shown in figure 4.26. The pattern of ethanol production during stage I of both the strategies was same as the fermentation conditions were same, whereas a significant improvement in ethanol production

was observed during stage II and III of strategy II as compared to strategy I. The ethanol yield ($Y_{p/s}$) and volumetric ethanol productivity (r_p), was about 1.3 times more in stage II and III of strategy II as compared to that in strategy I. The average yield (Y_{av}) and overall volumetric ethanol productivity (r_{po}) were found as 0.469 g_p/g_s, 1.016 g/L/h from strategy II and 0.391 g_p/g_s, 0.760 g/L/h from strategy I respectively. Thus strategy II was found more efficient than strategy I for bioconversion of synthetic sugars (xylose and glucose) to ethanol in a single fermentation vessel using co-culture system of *P. stipitis* and *Z. mobilis*.

Table 4.13 Comparison of fermentation performance for ethanol production from two different strategies adopted at flask level during using sequential-co-culture system of *P. stipitis* and *Z. mobilis*.

Parameters	**Strategy I**			**Strategy II**		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
Sugar consumption (%)	89.9±3.2	88.2±4.1	96.2±3.9	91.2±3.1	87.2±2.7	93.5±4.1
Sugar utilization rate, r_s (g/L/h)	1.926±0.068	3.548±0.162	4.956±0.201	1.955±0.066	3.436±0.106	5.006±0.218
Ethanol yield, $Y_{p/s}$ (g_p/g_s)	0.458±0.014	0.354±0.008	0.360±0.011	0.442±0.008	0.472±0.010	0.493±0.015
Maximum ethanol production, p (g/L)	24.2±0.97	17.6±0.35	25.0±0.75	24.7±1.00	22.7±0.91	34.6±1.04
Maximum cell conc. (cfu/ml) $x10^6$						
(a) *P. stipitis*	12.1±0.9	9.3±0.6	5.8±0.4	18.1±1.5	8.6±0.5	5.1±0.3
(b) *Z. mobilis*	--	60±4	85±5	--	43±2	67±5
Volumetric ethanol productivity, r_p (g/L/h)	0.882±0.035	1.257±0.025	1.786±0.053	0.864±0.034	1.621±0.064	2.471±0.074
Overall Sugar consumption (%)	97.7±4.8			96.0±2.9		
Overall sugar utilization rate, r_{so}(g/L/h)	2.094±0.063			2.056±0.042		
Average Ethanol yield, Y_{av} (g_p/g_s)	0.391±0.015			0.469±0.018		
Overall maximum ethanol production, p_o (g/L)	46.2±2.3			56.9±1.7		
Overall Volumetric ethanol productivity, r_{po} (g/L/h)	0.760±0.030			1.016±0.041		

-- Not available; values are presented as mean value±SD, n=3.

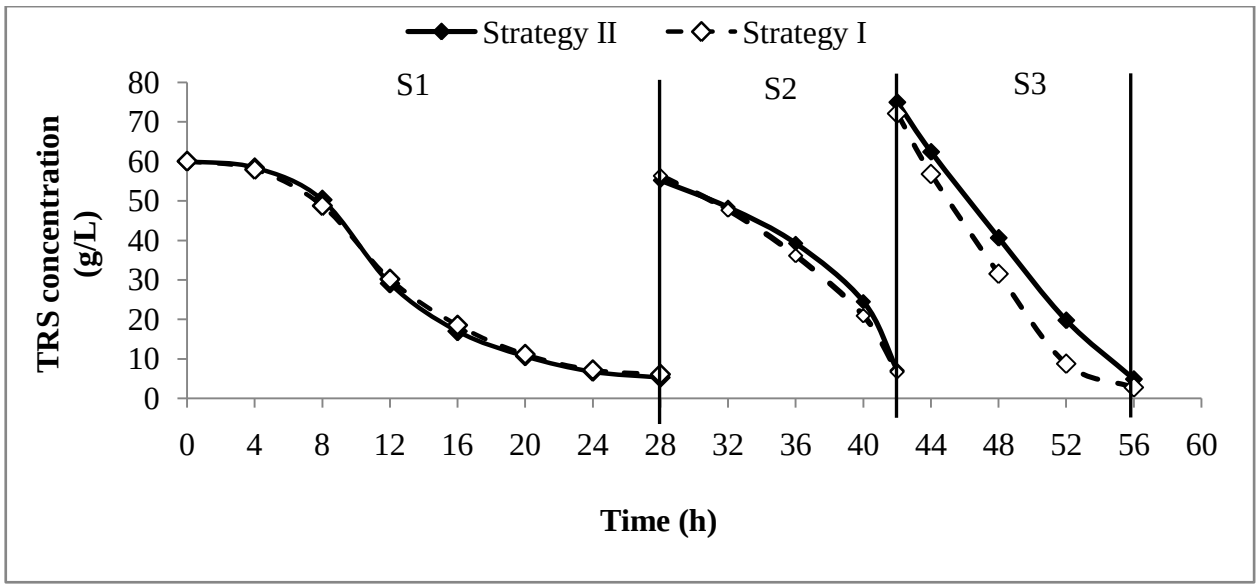
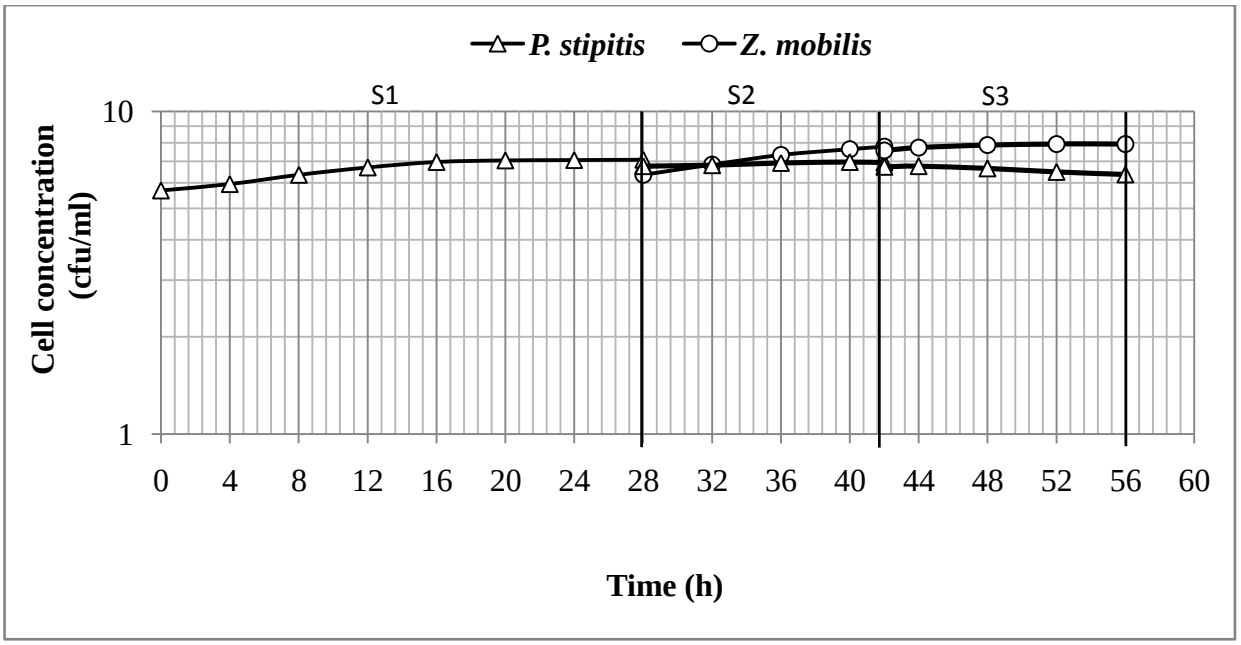
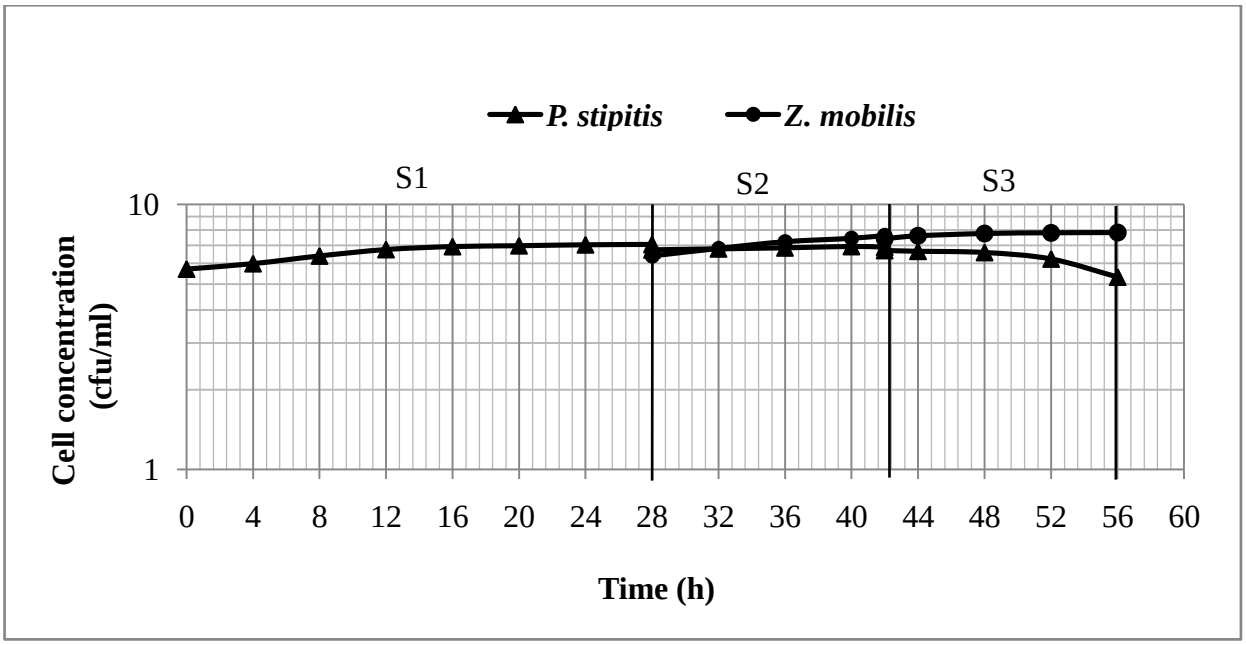


Figure 4.24 Sugar consumption profiles during three stages sequential-co-culture system of *P. stipitis* and *Z. mobilis* in culture bottles, wherein stage 1 (S1) consisting only *P. stipitis* cells and stages 2 (S2) and 3 (S3) both type of cells were present. In strategy I N_2 was not sparged at any stage; in strategy II, N_2 was sparged at the onset of stage 2 and 3 and cultured under static condition during stages 2 and 3.



(a)



(b)

Figure 4.25 Cell growth profiles during three stage sequential-co-culture system of *P. stipitis* and *Z. mobilis* in culture bottles, wherein stage 1 (S1) consisting only *P. stipitis* cells and stages 2 (S2) and 3 (S3) both type of cells were present (a) Strategy I: no N_2 was sparged at any stage and (b) Strategy II: N_2 was sparged at the onset of stage 2 and 3. Cultures were kept under static condition during stages 2 and 3.

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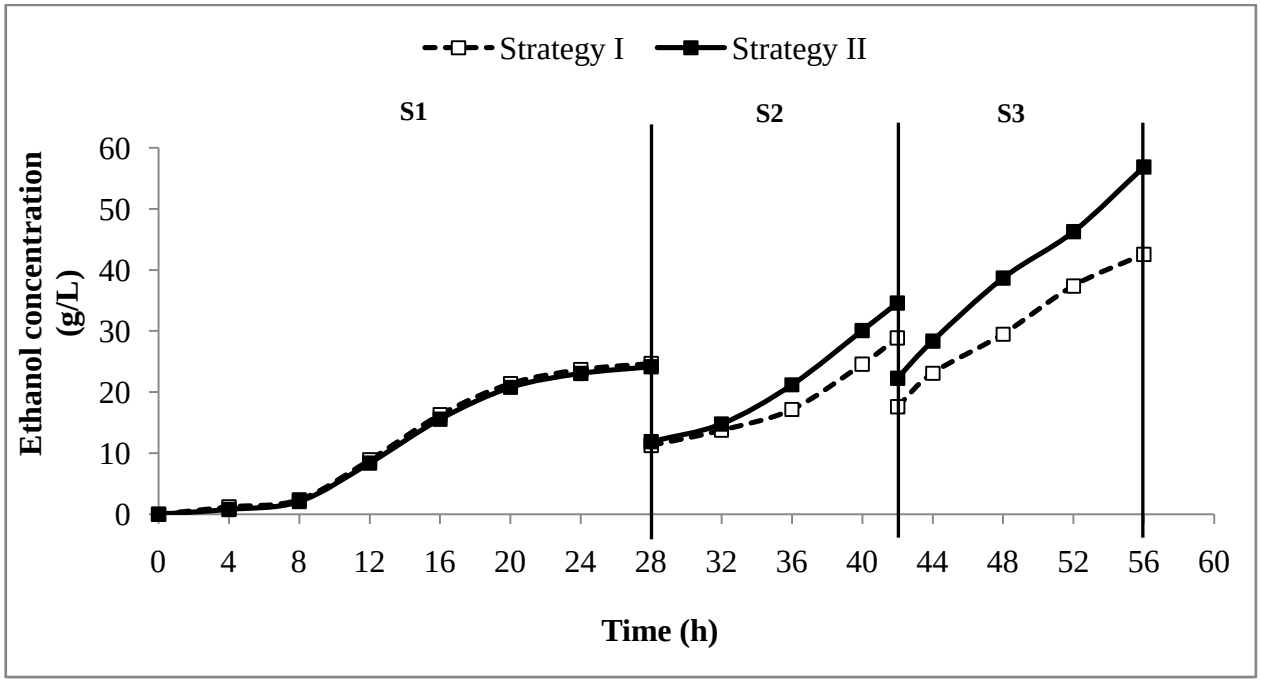

Figure 4.26 Ethanol production profiles during three stage sequential-co-culture system of *P. stipitis* and *Z. mobilis* in culture bottles, wherein stage 1 (S1) consisting only *P. stipitis* cells and stages 2 (S2) and 3 (S3) both type of cells were present. In strategy I N_2 was not sparged at any stage; in strategy II, N_2 was sparged at the onset of stage 2 and 3 and cultured under static condition during stages 2 and 3.

4.7.2 Sequential-co-culture system in bioreactor using synthetic fermentation media

The selected strategy II at flask level (300 ml working volume) for sequential-co-culture system development for bioconversion of synthetic fermentation media containing xylose and glucose to ethanol was further applied at higher level to validate this by using a bioreactor to provide better controlled environment. The working volume was enhanced to 4000 ml (>10 x). The only change made was that in place of three stages, only two stages were used to evaluate the system performance. First stage represented mono-culture of *P. stipitis* for xylose fermentation and second stage was the actual co-culture system of *P. stipitis* and *Z. mobilis*. The fermentation profiles of a two stage sequential-co-culture system in the bioreactor are shown in figure 4.27. Thesame pattern of viable cell concentration, ethanol production and residual sugar concentration was obtained as it was shown at flask level. The controlled fermentation conditions like pH and dissolved oxygen availability (microaerobic condition at stage I and strictly anaerobic condition at stage II by sparging N_2), resulted better fermentation. The results of these two strategies of sequential-co-culture system are given in table 4.14. The average ethanol yield ($Y_{p/s}$) and overall volumetric ethanol productivity (r_{po}) were found as 0.474 g_p/g_s and 1.416 g/L/h respectively. The average ethanol yield (Y_{av}) was almost same but overall volumetric ethanol productivity (r_{po}) was about 1.4 times of that in flask level system (strategy II).

Some selected results are given in comparative table 4.15. Very few studies have been reported at bioreactor level using co-culture system for ethanol production from mixture of xylose and glucose. Taniguchi et al. (1997) used two bioreactors (total working volume 2 L) with microfiltration modules, the yield and productivity of ethanol were 0.44 g_p/g_s and 0.59 g/L/h in fermentor A (inoculated with *P. stipitis*), and 0.45 g_p/g_s and 0.60 g/L/h in fermentor B (inoculated with *S. cerevisiae* no.7) respectively. Recently Fu et al. (2009) demonstrated a modified fermentor in which the best result was obtained by using immobilized *Z. mobilis* cells (1/2 batch) and free cells of *P. stipitis* (50% inoculum size), total sugar concentration 75 g/L (45 g/L glucose and 30 g/L xylose) showed ethanol yield 0.46 g_p/g_s and volumetric ethanol productivity 0.6-0.8 g/L/h. Very high inoculum size was the major drawback of the study, considering ethanol production at large volumes. Fu and Parisi (2008) reported that the co-fermentation of mixed sugar by successive inoculation of *Z. mobilis* and *P. tannophilus* improved the xylose to ethanol conversion yield (0.17 g_p/g_s) with overall ethanol yield 0.33 g_p/g_s. These results were obtained when the fermentation medium was autoclaved after the *Z. mobilis* fermentation (to inactivate *Z. mobilis* cells) and then inoculated with *P. tannophilus* for

xylose fermentation. The loss of ethanol during autoclaving was not discussed in the article which may be in significant amount.

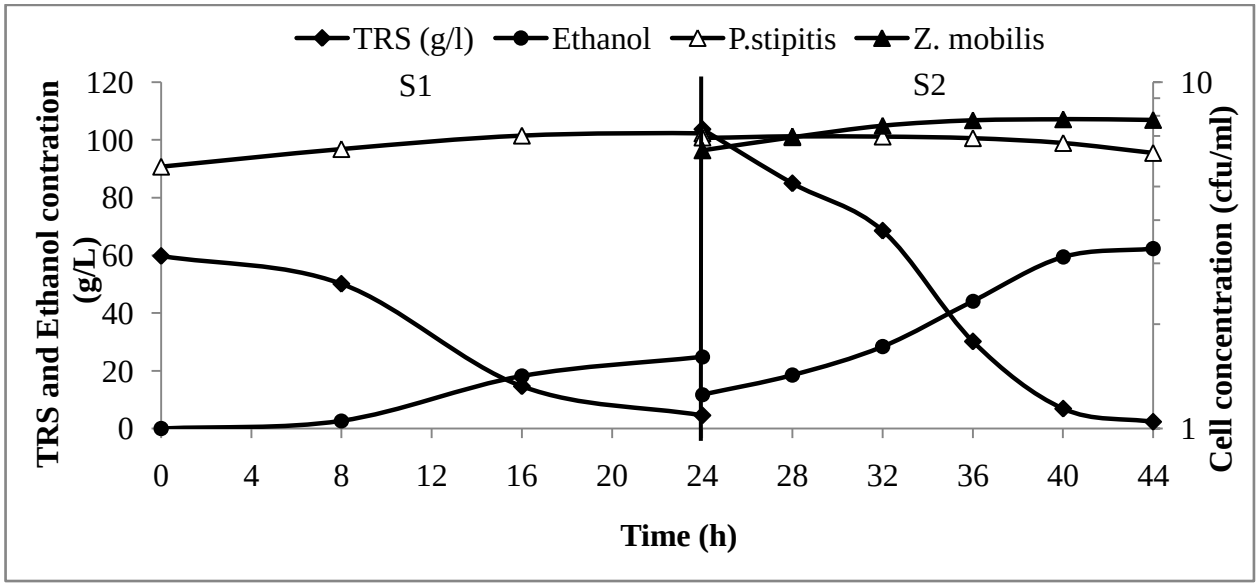

Figure 4.27 Fermentation profiles during two stage sequential-co-culture system of *P. stipitis* and *Z. mobilis* for ethanol production in bioreactor using synthetic media, wherein stage 1 (S1) consisting only *P. stipitis* cells and stage 2 (S2) both type of cells were present, N_2 was sparged at the onset of stage 2 and cultured under static condition.

Table 4.14 Comparison of fermentation performance for ethanol production using synthetic media and KGB hydrolysate media in bioreactor using sequential-co-culture system (Strategy II, two stages) of *P. stipitis* and *Z. mobilis*.

Parameters	**Synthetic**		**Hydrolysate**	
	Stage 1	**Stage 2**	**Stage 1**	**Stage 2**
Sugar consumption (%)	92.4±3.5	97.7±2.9	94.0±3.7	96.5±2.8
Sugar utilization rate, r_s (g/L/h)	2.303±0.087	5.067±0.152	0.987±0.039	4.123±0.121
Ethanol yield, $Y_{p/s}$ (g_p/g_s)	0.450±0.018	0.498±0.015	0.438±0.022	0.468±0.012
Maximum ethanol production, p (g/L)	24.9±1.5	50.5±2.4	24.2±0.9	46.3±1.2
Maximum cell conc. (cfu/ml) $x10^6$ (a) *P. stipitis* (b) *Z. mobilis*	 13.0±1.1 --	 9.5±0.7 66±3	 14.6±0.9 --	 9.1±0.5 62±2
Volumetric ethanol productivity, r_p (g/L/h)	1.037±0.062	2.525±0.126	0.432±0.016	1.929±0.050
Overall Sugar consumption (%)	98.2±3.4		97.2±1.9	
Overall sugar utilization rate r_{so}(g/L/h)	2.901±0.087		1.580±0.033	
Average Ethanol yield, $Y_{p/s}$ (g_p/g_s)	0.474±0.018		0.453±0.013	
Overall maximum ethanol production, p_o (g/L)	62.3±1.3		57.8±1.7	
Overall Volumetric ethanol productivity, r_{po} (g/L/h)	1.416±0.042		0.723±0.015	

-- Not available; values are presented as mean value±SD, n=2.

Table 4.15 Comparison of various co-culture systems using synthetic sugar media for ethanol production.

Co-culture system	**Substrate**	**Fermentation conditions**	***Fermentation parameters**	**Reference**
Z. mobilis + *P. stipitis*	Three step sugar addition; in first step 60 g/L xylose, in second 100 g/L glucose and in third 200 g/L glucose	Total working volume 300 ml; 5% v/v inoculum ($1.0x10^7$ cells/ml) of *P. stipitis* was added first for xylose fermentation and then 5% v/v inoculum ($1.0x10^8$ cells/ml) of *Z. mobilis* was added; N_2 was sparged after first stage; Flask study.	$Y_{p/s}$ (average): 0.469 r_p (overall): 1.016 p_o: 56.9	Present study
Z. mobilis + *P. stipitis*	Two step sugar addition; in first step 60 g/L xylose, in second 200 g/L glucose	Total working volume 4L; 5% v/v inoculum ($1.0x10^7$ cells/ml) of *P. stipitis* was added first for xylose fermentation and then 5% v/v inoculum ($1.0x10^8$ cells/ml) of *Z. mobilis* was added; N_2 was sparged after first stage; Bioreactor study.	$Y_{p/s}$ (average): 0.474 r_p (overall): 1.416 p_o: 62.3	Present study
Z. mobilis + *P. stipitis*	Glucose/xylose mixture (30 g/L glucose + 20g/L xylose)	Immobilized *Z. mobilis* + free *P. stipitis*30°C, 150 rpm, 80 cm^3/min air flow rate, 800 ml working volume; Bioreactor	$Y_{p/s}$: 0.49-0.50; r_p: 1.277	[Fu et al., 2009]

		study.		
Z. mobilis + *P. tannophilus*	Glucose/xylose mixture (60 g/L glucose + 40 g/L xylose)	Successive inoculation, firstly *Z. mobilis* and after glucose exhaustion *P. tannophilus* was inoculated; 30°C, 900 ml working volume, no aeration at glucose fermentation stage and aeration <1 mmol/L/h at xylose fermentation stage; Bioreactor study.	$Y_{p/s}$: 0.33; r_p: 2.32	[Fu, and Peiris, 2008]
P. stipitis + *K. marxianus*	Sugar mixture (30 g/L glucose + 30 g/L xylose + 12 g/L mannose + 8 g/Lgalactose)	pH 4.5, 100 ml working volume, 100 rpm; Flask study.	$Y_{p/s}$: 0.36 r_p:1.08 p_o: 31.87	[Rouhollah et al., 2007]
Z. mobilis + *Saccharomyces sp.*	Sucrose medium (200 g/L g/L reducing sugars)	10 ml *Z. mobilis* +10 ml *Saccharomyces sp.* Mixed inoculums; 0.5 L working volume; Bioreactor study.	$Y_{p/s}$: 0.50 r_p: 1.5	[Abate et al., 1996]

* $Y_{p/s}$: Ethanol yield (g_p/g_s); r_p: Ethanol productivity (g/L/h); p_o: Maximum ethanol production (g/L).

4.7.3 Sequential-co-culture system in bioreactor using KGB hydrolysate media

The aforementioned findings were extended for ethanol production from the KGB hydrolysate fermentation media (XRF and GRF media). In sequential-co-culture fermentation process, the XRF medium was first fermented by *P. stipitis* to eliminate catabolite repression of xylose by glucose and avoid the ethanol inhibition. After XRF fermentation, GRF media was allowed to ferment by *Z. mobilis* under strictly anaerobic conditions. The fermentation profiles during the two stage sequential-co-culture system are shown in figure 4.28 Total fermentation time was 80 h (0-56 h stage I and 56-80 h stage II) in this case, whereas, with synthetic sugars it was 44 h (0-24 h stage I and 24-44 h stage II). The longer total fermentation time of KGB hydrolysate was mainly contributed by *P. stipitis* fermentation (stage I) due to its longer adaptation period. This may be reduced by using adaptive cells [Agrawal et al., 2011; Martin et al., 2007; Qian et al., 2006]. The sugar consumed during stage I and stage II were 94 and 96.5% respectively whereas, overall sugar consumption of the sequential-co-culture system was 97.2%. The higher overall sugar consumption indicated that around 3-4% xylose was utilized during stage II. The average ethanol yield ($Y_{p/s}$) and overall volumetric ethanol productivity (r_{po}) were found as 0.453 g_p/g_s and 0.7225 g/L/h respectively. The maximum ethanol concentration achieved was 57.8 g/L which was about 7-8% less in comparison to that obtained from synthetic fermentation media. Also about 12% less biomass of *P. stipitis* at the end of stage I and about 6% less *Z. mobilis* cells were found during stage II, while utilizing KGB acid hydrolysate media. The possible reason could be the presence of some toxic chemicals in the hydrolysate media [Palmqvist and Hahn-Hagerdal, 2000].

A definite disturbance or irregular pattern was observed in the values of substrate utilization rate (r_s) while using synthetic media during 28 to 32 h. During this period, concentration of *Z. mobilis* cells (cfu/ml) were approached and crossed over to that of *P. stipitis* cells. This disturbance was not prominent in case of KGB hydrolysate fermentation. The pattern of ethanol production rate (r_p) was same in both the cases with the difference that it was flatter during stage I fermentation of KGB hydrolysate to that in synthetic media fermentation. Not much difference was observed during stage II fermentation. This may be due to longer fermentation period of *P. stipitis* while using KGB hydrolysate.

The generation of two sugar streams as XRF and GRF from KGB hydrolysis enables (a) fermentation of the xylose first and thus eliminating the problem that glucose is the preferred carbon source for *P. stipitis*, (b) to maintain the micro-aerobic condition during xylose fermentation (stage-I) and strictly anaerobic condition for *Zymomonas* fermentation in stage-II

and (c) the use of a high-ethanol tolerant *Zymomonas* strain for the glucose fermentation in stage-II as a single reactor system was used. Recently Chandel et al. (2011) have shown that the co-culture of *P. stipitis* and *S. cerevisiae*-VS_3 produced 15 g/L ethanol with 0.48 g_p/g_s ethanol yield and 0.208 g/L/h volumetric ethanol productivity from acid hydrolysate. The initial sugar concentration taken was low (32.84 g/L) in comparison to other studies done in the area of lignocellulosic biomass to ethanol like Patle and Lal (2007). The number of research paper describing the ethanol production from lignocellulosic biomass using co-culture technique in bioreactor is very few, although the important studies at flask level are given in the comparison table 4.16.

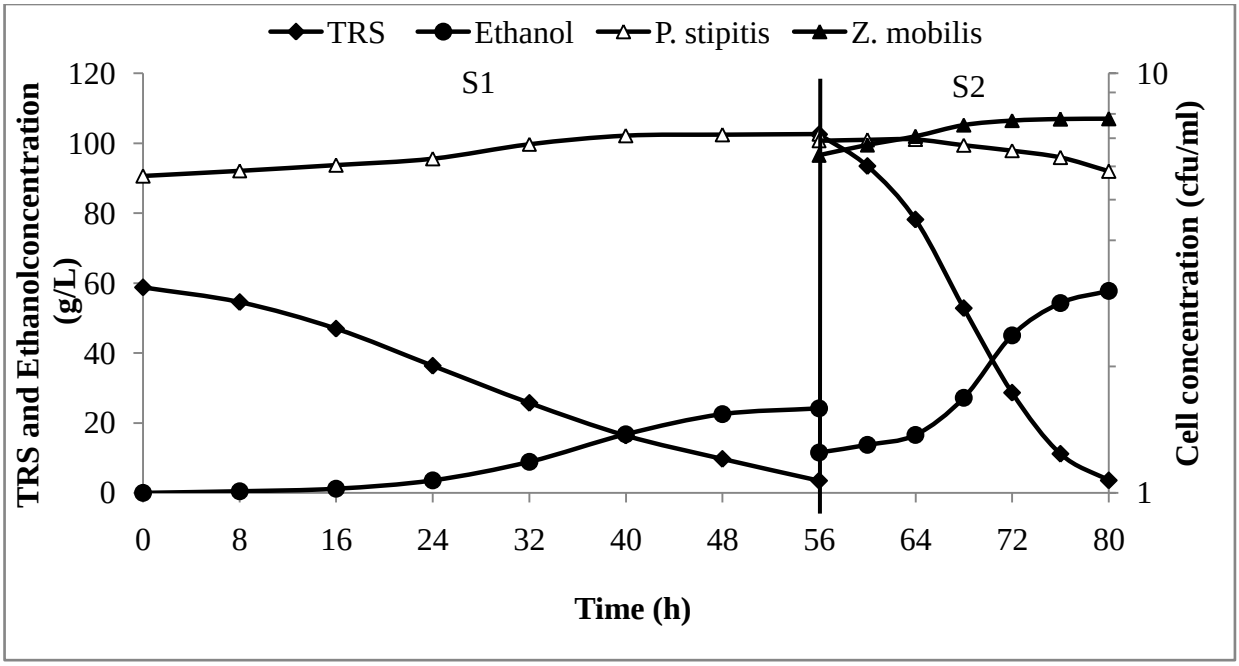

Figure 4.28 Fermentation profiles during two stage sequential-co-culture system of *P. stipitis* and *Z. mobilis* for ethanol production in bioreactor using KGB hydrolysate media, wherein stage 1 (S1) consisting only *P. stipitis* cells and stage 2 (S2) both type of cells were present, N_2 was sparged at the onset of stage 2 and cultured under static condition.

Table 4.16 Comparison of various co-culture systems for lignocellulosic hydrolysate media fermentation.

Co-culture system	**Substrate**	**Fermentation conditions**	***Fermentation parameters**	**Reference**
Z. mobilis + *P. stipitis*	Two step sugar addition; in first step 60 g/L XRFM, in second 200 g/L GRFM	Bioreactor study; total working volume 4L; 5% v/v inoculum ($1.0x10^7$ cells/ml) of *P. stipitis* was added first for xylose fermentation and then 5% v/v inoculum ($1.0x10^8$ cells/ml) of *Z. mobilis* was added; N_2 was sparged at the onset of second stage.	$Y_{p/s}$ (average): 0.453 r_p (overall): 0.723 p_o: 57.8	Present study
P. stipitis + *S. cerevisiae*	Rice straw hydrolysate (30 g/L glucose and xylose mixture)	Flask study; 75 ml detoxified hydrolysate; pH 5.5, 30°C, 150 rpm for first 18 h then static till end of fermentation.	$Y_{p/s}$: 0.40 r_p : 0.33 p_o: 12	[Yadav et al., 2011]
S. cerevisiae + recombinant *E. coli*	Treated softwood hydrolysate	Flask study; 150 ml hydrolysate; 50 ml inoculum of co-cultures; 30°C; pH 7.0	$Y_{p/s}$: 0.45 r_p : 0.71; p_o: 17.1	[Qian et al., 2006]
E. coli KO11 + *S. cerevisiae* TJ1	Waste house wood hydrolysate (27 g/L glucose +17 g/L xylose +1% corn steep liquor)	Working volume 200 ml; inoculum: 0.2 g dry cell weight/L *E. coli* and 0.02 g cell dry weight/L *S. cerevisiae*; 35°C; pH 6.0; shaking 80 rpm	$Y_{p/s}$: 0.43 r_p : 0.4 p_o: 30.3	[Okuda et al., 2008]
S. cerevisiae + *C. tropicalis*	200 g/L corn cob; alkali pretreated (2% NaOH)	Flask study; Simultaneous saccharification and fermentation (*C. thermophile* enzymes and yeast co-culture); 37°C; 150 rpm	$Y_{p/s}$: 0.19 r_p : 0.22 p_o: 21	[Latif, and Rajoka, 2001]

* $Y_{p/s}$: Ethanol yield (g_p/g_s); r_p: Ethanol productivity (g/L/h); p_o: Maximum ethanol production (g/L).

CHAPTER 5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

5.1 Concluding remarks

The main outcome of the study is a concept of ethanol production from lignocellulosic biomass. The study is focused on the extraction of fermentable sugars from a very cheap and abundantly available C4 plant, Kans grass and efficient utilization of these sugars for fermentation to ethanol. The concept provides an opportunity for the development of a proficient technology for ethanol production from lignocellulosic biomass.

The lacunae in the prior art includes the effective releaseoflocked polysaccharides from recalcitrant lignocellulose to fermentable sugars, to cop up with inhibitory compounds present in the hydrolysate and a fermentation system of microorganism for the efficient utilization of C5 and C6 sugars. One embodiment of the present study is directed to develop a robust, simple and efficient process for hydrolysis of lignocellulosic biomass to produce fermentable sugars. The novel single vessel multi-step process was successfully used for hydrolysis of Kans grass biomass to produce fermentable sugars. By the use of novel technique called fractional hydrolysis, it is possible to get C5 and C6 sugar rich fractions separately with negligible amount of toxics generation. The mixed sugar fermentation problem was addressed successfully. The key factor was the temperature and reaction time for a particular process step, which was not enough to generate toxic compounds in considerable amount. The significance of the study was that no detoxification process was used prior to fermentation. Thus it was possible to cut down the number of steps i.e pretreatment and hydrolysis could be achieved in a single stage.

The other embodiment is directed to utilize both types of sugars (C5 and C6) present in the lignocellulosic hydrolysate. The control of oxygen levels in the fermentation media duringthe development of sequential-co-culture system of *P. stipitis*and*Z. mobilis*was the key factor to achieve higher yield andproduction rate of ethanol with maximum utilization of sugars(xylose and glucose in hydrolysate). The lower ethanol tolerance of xylose fermenting yeastand catabolite repression of xylose metabolism by higher glucose concentration was successfully addressed. The single vesseloperation using this type of co-culture system definitely reducesthe process costs.

5.2Future perspectives

- The current work is only a part of efforts to find the bestalternativesfor overcoming society's fuel problems. The continuity of present work isnecessary for development of suitable and cost effective ethanol production process as well as the utilization oflignocellulosic materials. Therefore, a number of suggestions may be useful forcontinuing the work.
- The proposed hydrolysis process for fractionating lignocellulosic biomass to the soluble sugars with low level of toxics generation creates further opportunity for the process integration of different chemical and biological processes for complete utilization of lignocellulosic biomasses and should lead to the development of big "BIOREFINERIES" that allow the production of large amounts of fuel ethanol and other valuable co-products, improving the overall economical effectiveness of the conversion of a given material.
- Scaling up of fractional hydrolysis process to produce low cost pentose and hexose sugars in huge quantity may open new avenues for utilization of renewable lignocellulosic biomasses. These sugars may be used for many other purposes e.g high valued sweeteners, enzymes etc.
- Further studies of metabolic processes of the microbial consortium of *Z. mobilis* and *P. stipitis* could be useful for understanding the mechanism of cell interaction to develop a robust co-culture system for ethanol production.
- Scaling up of co-fermentation process is highly recommended to study for achieving higher productivity.
- The strategies to be adopted to operate the reactor with higher initial sugar concentrations and thereby, high ethanol concentration without inhibition could be produced.
- High cell density culture with continuous removal of ethanol must be studied to achieve high ethanol productivity.

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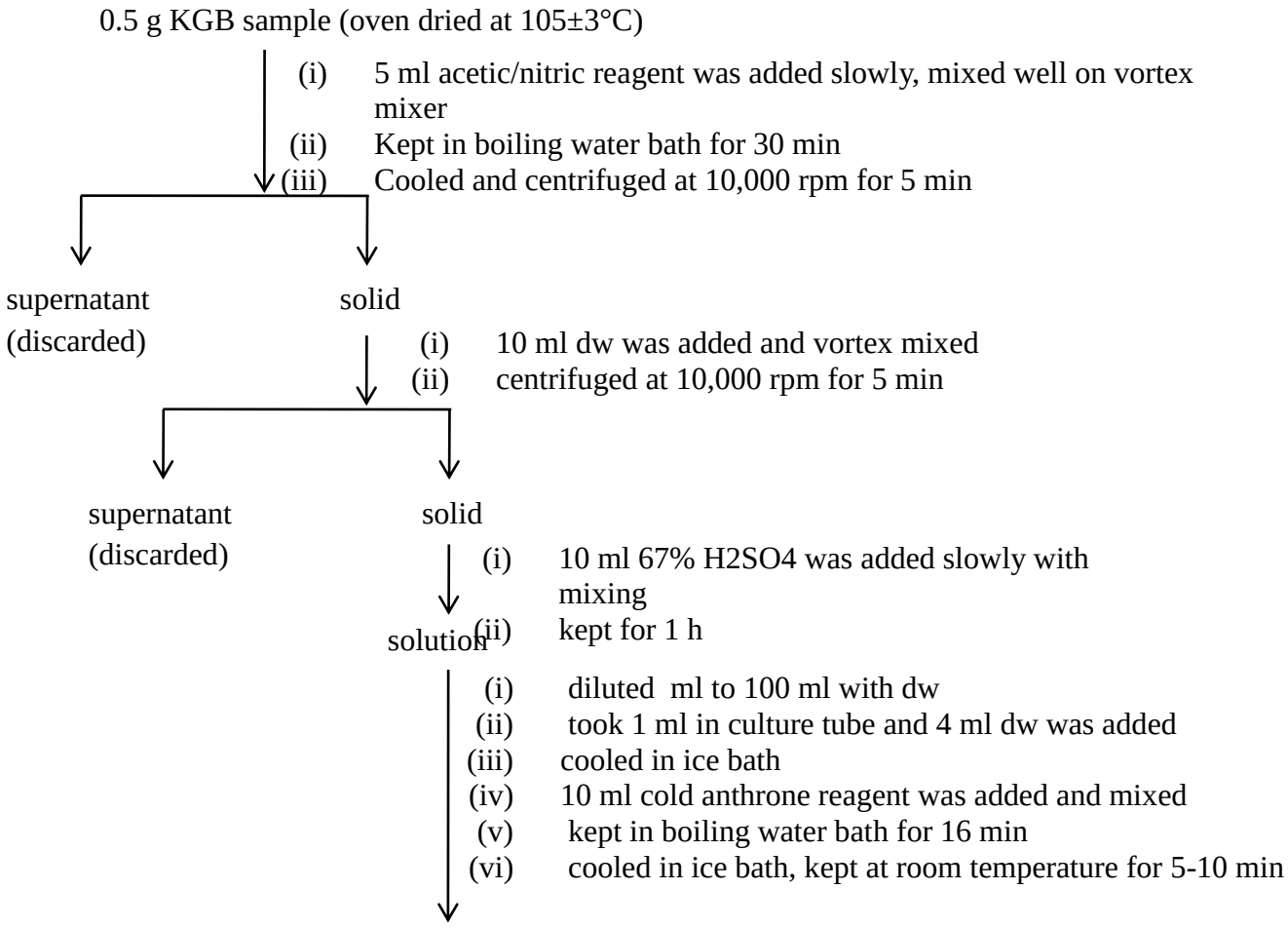
APPENDIX – A1

Process flow sheet for cellulose estimation



APPENDIX – A2

Process flow sheet for neutral detergent fiber (NDF) estimation

1.0 g KGB sample (oven dried at 55±1°C) in refluxing flask

↓ (i) 10 ml cold NDS was added
(ii) 2 ml decahydronaphthalene and 0.5 g sodium sulfite were added

refluxed

↓ (i) boiled for 60 min

filtered (through sintered glass crucible, hot water used)

↓

washed (with acetone)

↓

dried (at 100°C, 8 h)

↓

Cooled in desiccator and weighed

Process flow sheet for Acid detergent fiber (ADF) estimation

1.0 g KGB sample (oven dried at 55±1°C) in refluxing flask

↓ (i) 100 ml ADS was added at room temperature

refluxed

↓ (i) boiled for 60 min

filtered (through sintered glass crucible, boiling water used)

↓

rinsed (twice with 40 ml acetone)

↓

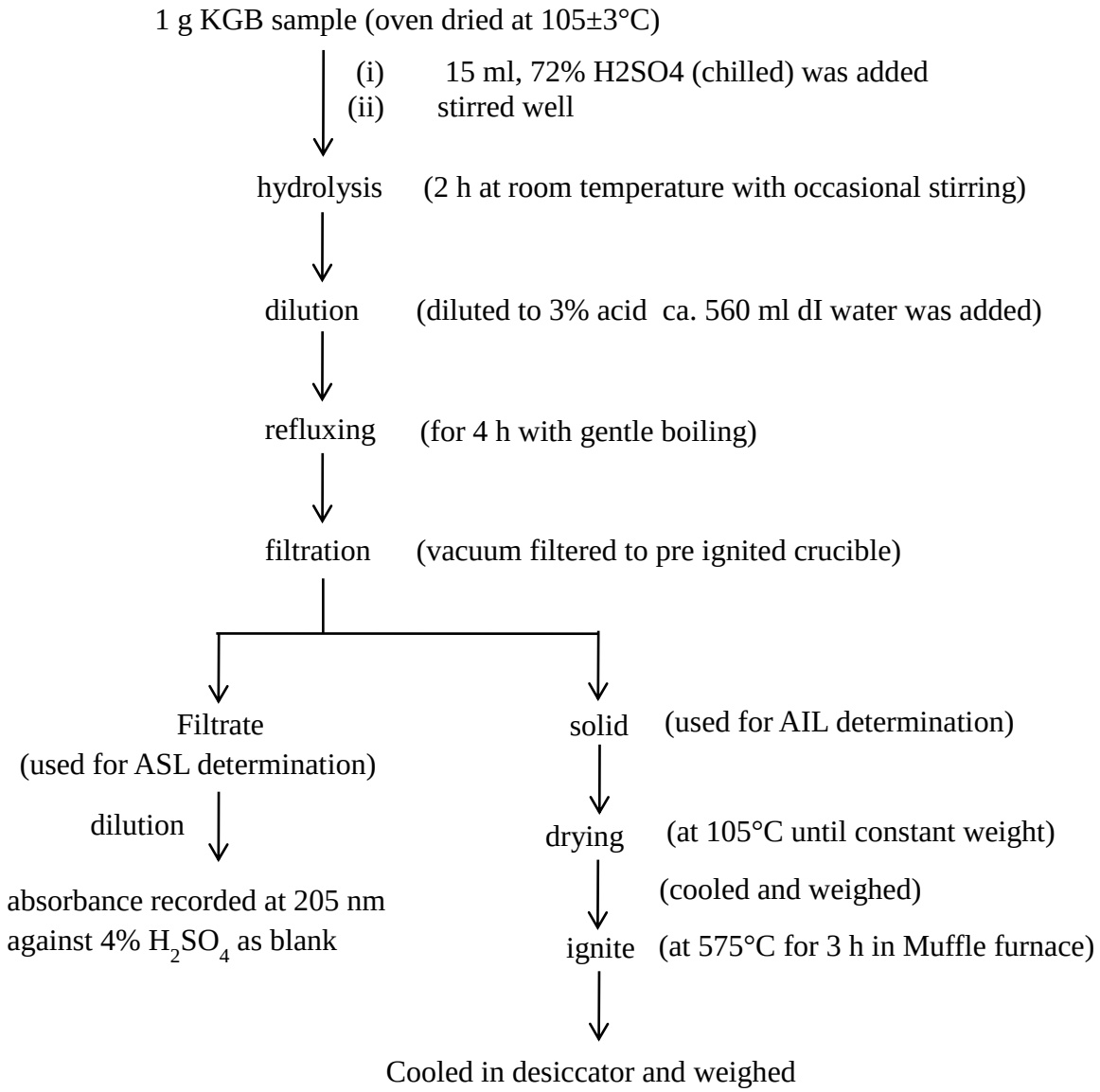
dried (at 100°C, 3 h)

↓

Cooled in desiccator and weighed

APPENDIX – A3

Process flow sheet for lignin estimation



APPENDIX – A4

Process flow sheet for ash estimation

1.0 g KGB sample (oven dried at 105±3°C and placed in pre weighed crucible)

↓

ignition (at 575°C for 3 h in Muffle furnace)

↓

cooled (in desiccator at room temperature)

↓

weighed (until constant weight)

PUBLICATIONS

(a) Patent

"A novel fractionating hydrolysis process for production of fermentable sugar from lignocellulosic biomass of Kans grass (*Saccharum spontaneum*)" permission has been granted by the IPR Cell, IITR, Roorkee and is under process of filing.

(b) In Peer Reviewed International Journals

1. Singh, L. K., Chaudhary, G., Majumder, C.B., Ghosh, S. Explore the perennial Kans grass (*Saccharum spontaneum*) biomass for releasing reducing sugars and its optimization. Der Chemica Sinica. 2 (3): 154-163 (2011).

2. Singh, L. K., Chaudhary, G., Majumder, C. B., Ghosh, S. Utilization of hemicellulosic fraction of lignocellulosic biomaterial for bioethanol production. Advances in Applied Science Research. 2 (5): 508-521 (2011).

3. Singh, L. K., Majumder, C. B., Ghosh, S. Bioconversion of Hemicellulosic Fraction of Perennial Kans Grass (*Saccharum spontaneum*) Biomass to Ethanol by Pichia stipitis: A Kinetic Study. International Journal of Green Energy. 9(5): 409-420 (2012).
4. Singh, L. K., Majumder, C. B., Ghosh, S. Development of sequential-co-culture system (*Pichia stipitis* and *Zymomonas mobilis*) for bioethanol production from Kans grass biomass. Biochemical Engineering Journal. 82(0): 150-157 (2014).http://dx.doi.org/10.1016/j.bej.2013.10.023

5. "A novel fractionating hydrolysis process for production of fermentable sugar from lignocellulosic biomass of Kans grass (*Saccharum spontaneum*) for bioethanol production." Under preparation.

6. Chaudhary, G., Singh, L. K., Ghosh, S. Alkaline pretreatment methods followed by acid hydrolysis of *Saccharum spontaneum* for bioethanol production. Bioresource Technology, 124(0), 111-118 (2012).

(c) In Conferences

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2. Lalit Kumar Singh, Gaurav Chaudhary, Rashmi Kataria and Sanjoy Ghosh. "Production of reducing sugars from lignocellulosic biomass for fuel ethanol production" **National Conference** on "Water, Energy and Biodiversity" Organized by The Institution of Engineers (India) Tripura State Centre and National Institute of Hydrology Roorkee. August 20-22, 2011, pp 209-221.

3. L. K. Singh, G. Chaudhary, C.B. Majumder, S. Ghosh." Process integration in for ethanol production from lignocellulosic biomaterials" **World Congress for Man and Nature**", Global Climate Change & Biodiversity Conservation, held at Gurukul Kangari University, Haridwar, Nov. 11-13, 2011, pp 282.

4. Lalit K. Singh, C. B. Majumder and Sanjoy Ghosh. " Bioethanol production from Kans grass using separate hydrolysis and fermentation." **4th International Congress** of Environmental Research, held at SVNIT, Surat, Dec. 15-17, 2011.