BIOCATALYTIC CONVERSION OF BIOMASS TO BIOFUELS

A DISSERTATION

Submitted in partial fulfilment of the
requirements for the award of the degree
of
MASTER OF TECHNOLOGY
in
CHEMICAL ENGINEERING
(With Specialization in Computer Aided Process Plant Design)

By

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in this dissertation entitled "**BIOCATALYTIC CONVERSION OF BIOMASS TO BIOFUELS**", in partial fulfillment of the requirement for the award of the degree of **Master of Technology** in **Chemical Engineering** with specialization in "**COMPUTER AIDED PROCESS PLANT DESIGN**", submitted in the **Department of Chemical Engineering** of **Indian Institute of Technology Roorkee**, Roorkee is an authentic record of my own work carried out during the period from June 2005 to June 2006 at Indian Institute of Petroleum, Dehradun, under supervision of Dr.D.K.Adhikari, Scientist 'F', Chemical and Biotechnology Division, Dehradun, and Dr.V.K.Agarwal, Associate Professor, Department of Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this dissertation work has not been submitted for the award of any other degree.

Date: 28th June, 2006
Place: Roorkee.

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CERTIFICATE

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

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ABSTRACT

Biological conversion of biomass to biofuels has been extensively reviewed. In this experimental work sugarcane bagasse was used as biomass and ethanol was the biofuel. The biological process of ethanol production using bagasse as substrate requires: (1) Pretreatment: depolymerization of the carbohydrate polymers (cellulose and hemicellulose) to produce free sugars, and (2) Fermentation: fermentation of mixed hexose and pentose sugars to produce ethanol.

Acid hydrolysis was conducted as pretreatment of bagasse with 2% H_2SO_4 solution at residence times of 60, 90 and 120 min. 28.0125 g/L of glucose and 560.07 mg/L of furfural were obtained in 1st hydrolysis of bagasse at 120 min residence time. Several detoxification processes were used to remove / reduce the furfural concentration. Three times extraction of bagasse hydrolysate with ethyl acetate was carried out, and it removes 91.02 % of furfural with less removal of glucose (9.34%).

Batch fermentation was carried out with two strains, one was GNSA strain and another was IIPE453 strain. GNSA strain was grown in H_1 medium at 60 °C and IIPE453 strain was grown in H_2 medium at 45 °C. The highest product yield of 21.6% was obtained with IIPE453 strain at 6 hrs operation.

CONTENTS

List of Tables

List of Figures

CHAPTER-1 Introduction

The worldwide energy need has been increasing exponentially. 50 years ago the production and consumption of energy were balanced in the developed countries, but at present production in the developed countries has doubled but consumption was far more increased than production. The world major energy demand is provided from the convectional energy sources such as coal, oil, natural gas etc. The life of all the convectional fuels is limited by the present and future energy consumption of the world, e.g. the world's oil reserves are estimated to be depleted in next 50 years. Therefore, attention is being given to alternate and renewable sources, such as solar, wind, thermal, hydroelectric, biomass etc.

The world's energy requirement is being fulfilled by fossil fuels which serve as a primary energy source. Fossil fuels as the main energy sources have led to serious environmental problems, i.e. combustion of fossil fuel produces greenhouse and toxic gases, such as CO_2, SO_2, NO_x and other pollutants, causing global warming and acid rain [14]. Biomass and other renewable energy sources are receiving worldwide attention for several reasons. These include the increasing concern about potential global climate change and other environmental consequences of the growing consumption of fossil fuels, the desire to develop sustainable energy sources, decreasing dependence on foreign source of oil, improving balance of payment, and decreasing the rate of depletion of the fossil fuel reserves. Biomass energy crop production and power technology is a sustainable source of energy, and perhaps, reverse, the continued growth of fossil fuel consumption.

Though the environmental benefits of biomass and renewable technologies are well known, in the current economic environment, it is difficult for biomass energy to compete with other energy sources. Biomass combustion is the oldest and the most mature technique of biomass to energy conversion but there lies a great challenge in developing and improving new, more efficient, and environmentally more compatible small- and large-scale systems [7].

Biomass fuels constitute the major fraction of domestic energy requirement in most of the developing countries. In India itself, these fuels fulfill about 75% of the domestic energy-requirements. Biomass fuels are generally used in primitive combustion devices, which are reported to be highly energy inefficient, and release vast amounts of pollutants into the environment.

India produces annually about 600 MT (million tonnes) of agricultural residues including crop residues such as rice husk, coconut shells, groundnut shells, cotton stalks, jute sticks, baggage, etc. A rough estimate indicates that even if 30% of the agricultural residue is available, there exists a potential to generate about 30,000 MW of power at 36% overall efficiency. The total installed capacity of power plants in India in 1998 was about 90,000 MW [7].

1.1. Biomass:

Biomass can generally be defined as any hydrocarbon material which mainly consists of carbon, hydrogen, oxygen and nitrogen. Sulfur is also present in less proportion. Some biomass types also carry significant proportions of inorganic species. The concentration of the ash arises from less than 1% in softwoods to 15% in herbaceous biomass and agricultural residues.

Biomass is the name given to all the earth's living matter. It is a general term for material derived from growing plants or from animal manure. Biomass is produced by green plants converting sunlight into plant material through photosynthesis and includes all land and water based vegetation, as well as all organic wastes. The biomass resource can be considered as organic matter, in which the energy of sunlight is stored in chemical bonds. When the bonds between adjacent carbon, hydrogen and oxygen molecules are broken by digestion, combustion, or decomposition, these substances release their stored, chemical energy.

1.1.1. Composition of biomass:

Biomass is principally composed of the compounds cellulose, hemicellulose and lignin. The relative proportions of cellulose and lignin is one of the determining factors in identifying the suitability of plant species for subsequent processing as energy crop.

Cellulose, a primary component of most plant-cell walls, is made up of long chains of the 6-carbon sugar, glucose, arranged in bundles. Cellulose is a glucose polymer, consisting of linear chains of (1, 4)-D-glucopranose units, in which the units are linked 1-4 in the β-configuration, with an average molecular weight of around 100,000.

Hemicellulose is a mixture of polysaccharides, composed almost entirely of sugars such as glucose, mannose, xylose and arabinose and methlyglucuronic and galaturonic acids, with an average molecular weight of <30,000. The hemicellulose is primarily composed of the 5-carbon sugars and xylose. In the contrast to cellulose, hemicellulose is a heterogeneous branched polysaccharide that binds tightly, but non-covalently, to the surface of each cellulose micro fibril. Hemicellulose differs from cellulose, in consisting primarily of xylose and other five-carbon monosaccharides.

A typical plant cell wall is illustrated in Fig-1.1. [19]

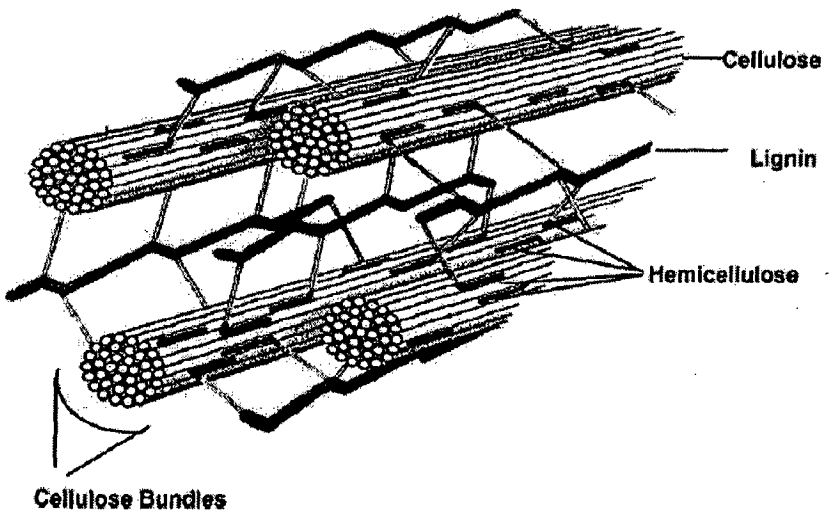


Fig-1.1. Typical plant cell wall arrangement

Another compound called lignin is also present in significant amounts and gives the plant its structural strength. Lignin is regarded as a group of amorphous, high molecular weight, chemically related compounds. The building blocks of lignin are believed to be a three-carbon chain attached to rings of six carbon atoms, called phenyl-propanes [19].

Cellulose is generally the largest fraction, representing about 40-50% of the biomass by weight, the hemicellulose portion represents about 20-40% of the material by weight.

1.1.2. Resources of Biomass:

Biomass resources include various natural and derived materials, such as woody and herbaceous species, wood wastes (e.g. sawdust, timber slash, and mill scrap), agricultural residues (e.g. bagasse from sugarcane, corn fiber, rice straw and hulls, and nutshells), industrial residues, waste paper, municipal solid waste, biosolids, grass, waste from food processing, animal wastes, aquatic plants, algae and the methane captured from landfills etc. The average majority of biomass energy is produced from wood and wood wastes (64%), followed by MSW (24%), agricultural waste (5%) and landfill gases [8].

1.1.3. Advantages of Biomass:

- Biomass is a renewable and relatively environmentally benign source of energy.
- Biomass fuels have negligible sulfur content and, therefore, do not contribute to sulfur dioxide emissions, which causes acid rain.
- The combustion of biomass produces less ash than coal combustion, and the ash produced can be used as a soil additive on farm targets.
- The combustion of agricultural and forestry residues and MSW for energy production is an effective use of waste products that reduces the significant problem of waste disposal, particularly in municipal areas.
- Biomass is a domestic resource, which is not subject to world price fluctuations or the supply uncertainties of imported fuels. In developing countries the use of

liquid biofuels, such as ethanol, reduces the economic pressures of importing petroleum products.

- Biomass provides a clean, renewable energy source that could improve our environment, economy, and energy securities.

1.2. Biofuels:

Production of energy carriers from biomass are heat, electricity and fuels for transportation or 'biofuels'. Biofuels are produced worldwide, ethanol from sugar/starch, hydrogen from sugars and methane from anaerobic digestion. Higher overall (production, distribution and use) energy conversion efficiencies and lower overall costs are the key criteria for selecting biofuels for the longer term. Various options are considered and developed that have good potentials. Key examples are ethanol produced from lignocellulosic biomass, methane and hydrogen.

A few main routes can be distinguished to produce biofuels from biomass as shown in Fig-1.2: Those are anaerobic digestion of biomass and fermentation of sugars to alcohol and hydrogen [20].

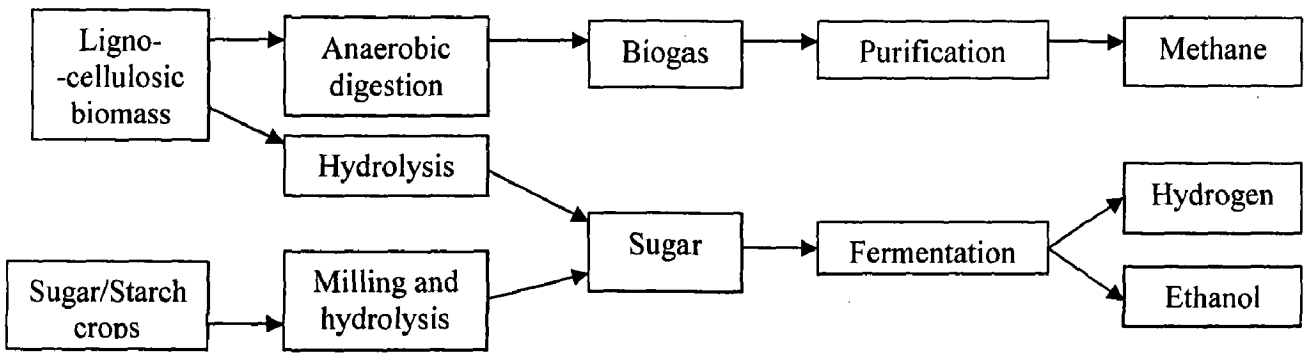


Fig-1.2. Overview of bioconversion of biomass to biofuels

Biofuels have several advantages: greater energy independence for fuel importing countries, renewable fuels, a cleaner combustion than gasoline and petrol, and no net contribution to atmospheric CO_2 due to a closed photosynthesis combustion cycle.

The net energy yield of perpetual crops, grasses and sugar cane are much higher. These crops can be grown on less valuable land. Compared with sugar, starch, and oil crops, the application of lignocellulosic biomass (e.g. wood and grasses) is more favorable and gives better economic prospects to the future of biofuels. Also, more types of feedstock are in principle suitable to produce a broader range of fuels than when applying traditional biofuels feedstock [20].

- **Ethanol:**

Ethanol (ethyl alcohol, grain alcohol, EtOH) is a clear, colorless liquid. In dilute aqueous solution, it has a somewhat sweet flavor, but in more concentrated solutions it has a burning taste. Ethanol (CH_3CH_2OH) is made up of a group of chemical compounds whose molecules contain a hydroxyl group, -OH, bonded to a carbon atom. Ethanol made from cellulosic biomass materials instead of traditional feedstocks (starch crops) is called bioethanol. Ethanol has relatively high production capacity. It is easy to transport, relatively free of impurities, has low toxicity.

Ethanol can be made synthetically from petroleum or by microbial conversion of biomass materials through fermentation. In 1995, about 93% of the ethanol in the world was produced by the fermentation method and about 7% by the synthetic method. The fermentation method generally uses three steps: (1) formation of a solution of fermentable sugars, (2) fermentation of these sugars to ethanol, and (3) separation and purification of the ethanol, usually by distillation.

Ethanol is becoming a popular biomass-derived fuel. It has the advantage of easy storage and transport. Ethanol can be used for blending with petroleum to make gasohol (mixture of gasoline and ethanol). Blends of at least 85% ethanol are considered alternative fuels. E85, a blend of 85% ethanol and 15% gasoline, is used in vehicles that are currently offered by most major auto manufacturers. In some areas, ethanol is blended with gasoline to form an E10 blend (10% ethanol and 90% gasoline). In addition to its use as a fuel or petroleum supplement, ethanol is a versatile chemical feedstock and many chemical products are derived from ethanol [24].

- **Hydrogen:**

Hydrogen is a colorless, odorless gas. Hydrogen is not primary fuel, it is already produced from fossil fuel sources such as natural gas, naphtha and coal. Hydrogen has the potential to be a clean alternative to the fossil fuels currently used in the transportation sector. This is especially true if the hydrogen is manufactured from renewable resources, primarily sunlight, wind, and biomass.

Hydrogen is generated from renewable source, namely biomass, and more particularly by the photo-biological method and dark fermentation process. Hydrogen produced from renewable resources such as water, organic wastes or biomass, either biologically or photo-biologically, is termed as "bio-hydrogen". Microorganisms are capable of producing hydrogen via either fermentation or photosynthesis. The fermentation is generally preferred, because it does not depends on the availability of light sources and the transparency of the mixed liquor [14].

Hydrogen is a high-value industrial commodity with a wide range of applications. It is an ideal fuel, producing only water upon combustion. It may be converted into electricity via fuel cells or directly used in internal combustion engines. It can also be used for the syntheses of ammonia, alcohols and aldehydes, as well as for the hydrogenation of edible oil, petroleum, coal and shale oil.

Utilization of hydrogen as an automotive fuel is encouraged by two strong driving forces in the world: (1) substitution of increasingly scarce and costly fossil fuels: and (2) abatement of air pollution. Unlike petroleum production, which is concentrated in only a few well-endowed countries, electrolytic production of hydrogen is possible in all countries as an indigenous supply of transportation fuel. Abatement of air pollution includes reduction in vehicle exhaust of both carbon dioxide (and other greenhouse gases) to the global environment and smog-forming nitrogen oxides in metropolitan air basins [18].

Various transportation means for hydrogen include trucking in gaseous form, trucking in liquefied form, trucking in metal hydride storage containers or tanks and low pressure pipeline transportation. Hydrogen may be stored in one of the following ways like compressed gas bottles, cryogenic liquid, metal hydride, carbon structures and as chemical storage. Gaseous hydrogen storage is a logical method since hydrogen is produced in gaseous form. As hydrogen is one of the lightest elements, its energy per unit volume is comparatively low. To store hydrogen as a liquid, the temperature needs to be lowered to 20◦K, which is an energy-intensive process, reducing the net energy value. The energy needed to store liquid hydrogen is equivalent to 25–30% of its energy content. Solid storage is an emerging technology that uses rechargeable metallic hydrides, these systems can store hydrogen from 1 to several wt%. However, its high capital cost process and gaseous impurities may not be tolerated [14].

- **Methane:**

Methane is a colorless, odorless gas which is lighter than air. Methane is a gas that contains molecules of methane with one atom of carbon and four atoms of hydrogen (CH_4). It is formed by the decomposition of organic carbons under oxygen poor (anaerobic) conditions and is commonly found in or near swamps and wetland areas, peat deposits, wood wastes such as hog fuel, or in the area of old landfills.

Methane is produced through anaerobic digestion of wastewater and residues including sewage sludge, animal manure and the organic fraction of municipal waste. In this process, hydrogen is an intermediary product that is, however, not available because it is rapidly taken up and converted into methane by methane-producing microorganisms.

Methane is an important fuel for electrical generation. Compared to other hydrocarbon fuels, burning methane produces less carbon dioxide for each unit of heat released. Also, methane's heat of combustion is about 802 kJ/mol, which is lower than any other hydrocarbon, but if a ratio is made with the atomic weight (16 g/mol) divided by the heat of combustion (802 kJ/mol) it is found that methane, being the simplest hydrocarbon, actually produces the most heat per gram than other complex hydrocarbons.

In many cities, methane is piped into homes for domestic heating and cooking purposes. In this context it is usually known as natural gas [25].

In the chemical industry, methane is the feedstock of choice for the production of hydrogen, methanol, acetic acid, and acetic anhydride. When methane is used to produce any of above chemicals, methane is first converted to synthesis gas, a mixture of carbon monoxide and hydrogen, by steam reforming. Less significant methane-derived chemicals include acetylene, prepared by passing methane through an electric arc, and the chloromethanes (chloromethane, dichloromethane, chloroform, and carbon tetrachloride), produced by reacting methane with chlorine gas [25].

1.2.1. Properties of biofuels:

Table-1.1: Properties of biofuels

Properties	Ethanol	Hydrogen	Methane
Formula	CH_3CH_2OH	H_2	CH_4
Molecular Weight	46.07	2.02	16.04
Density	0.789 g/ml	$0.08376*10^{-3}$ g/ml	$0.65*10^{-3}$ g/ml
M.P.	-112 °C	-259.14 °C	-183 °C
B.P.	78.3 °C	-252.87 °C	-162 °C
Heat of Combustion	29.78 KJ/g	119.93 KJ/g	50.02 KJ/g
Heat of vaporization	921.03 KJ/Kg	446.8 KJ/Kg	510 KJ/Kg
Heat of Fusion	100.87 KJ/Kg		58.68 KJ/Kg
Auto ignition Temperature	423 °C	574 °C	595 °C
C_p	0.111 KJ/mol-°K		0.035 KJ/mol-°K
Thermal Conductivity	0.018 mW/m-°K		32.18 mW/m-°K
Octane Number	108	130 +	125
Flammable Limits	4.3 – 19 vol%	4.1 – 74 vol%	5 – 15 vol%
Toxicity Limits	1000 ppm	Non Toxic	Simple Asphyxiant

1.3. Biological methods for the production of biofuels:

1.3.1. Ethanol:

Biological method for the production of ethanol from biomass is fermentation. Fig-1.3 shows the schematic diagram of the fermentation. The fermentation method generally uses three steps: (1) Hydrolysis (formation of a solution of fermentable sugars) (2) Fermentation (fermentation of these sugars to ethanol) and (3) Recovery (separation and purification of the ethanol).

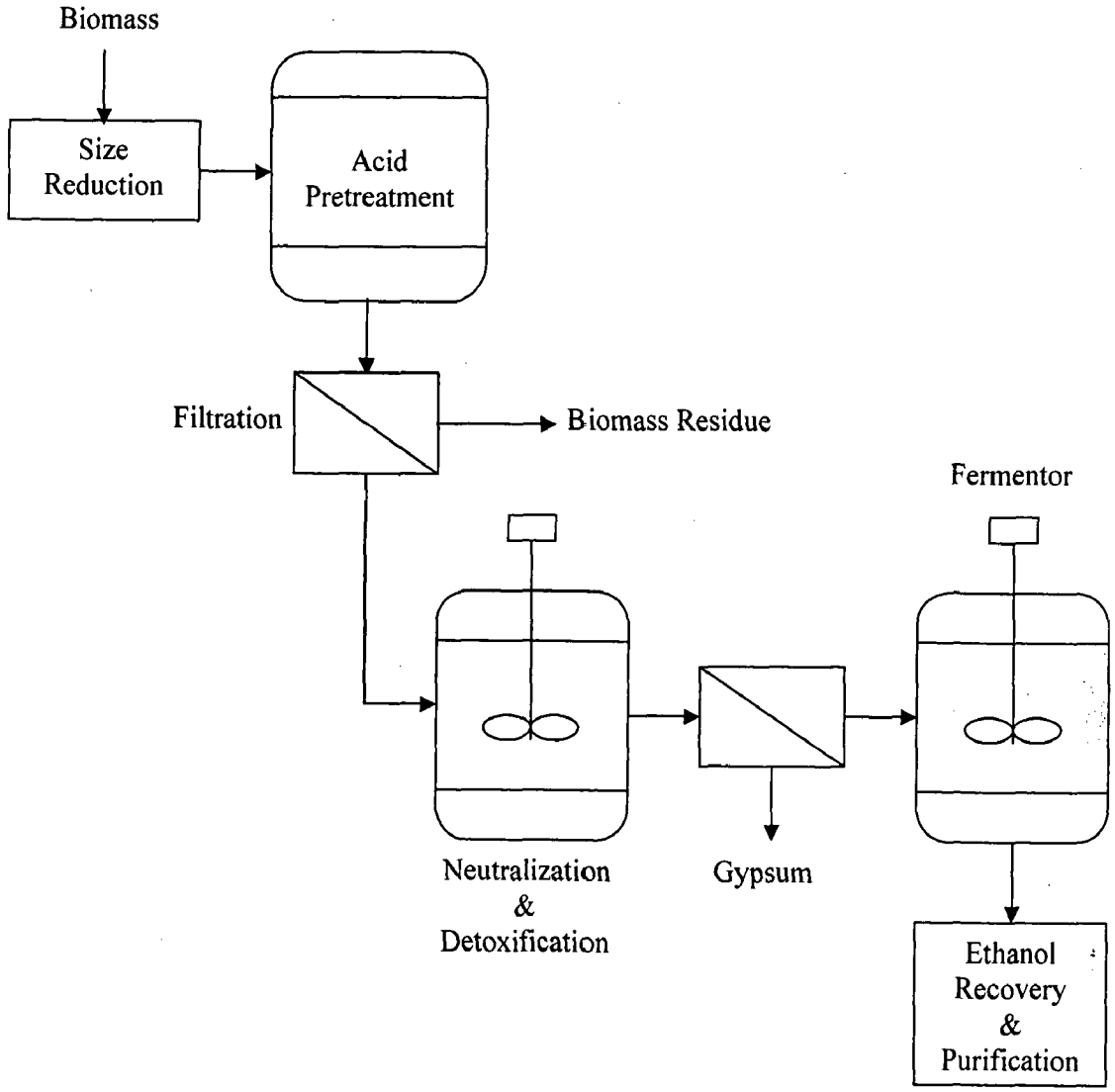


Fig-1.3. Schematic diagram of Fermentation for ethanol

- **Hydrolysis:**

Cellulose and hemicellulose are principally composed of tightly-bonded sugars. The bonds will need to be broken before fermentation to ethanol. Cellulose and hemicellulose must be broken down into the simple sugars glucose and xylose respectively. This process, as shown in Fig-1.4 for cellulose conversion, is known as hydrolysis [19].

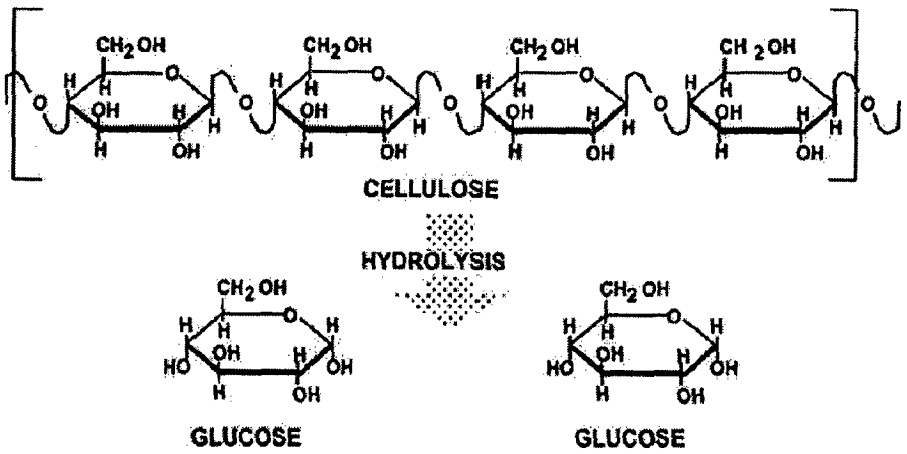


Fig-1.4. Cellulose hydrolysis

- **Fermentation:**

Once the cellulose and hemicellulose have been broken down to simple sugars, fermentation can then take place as shown in Fig-1.5 and Fig-1.6 [19].

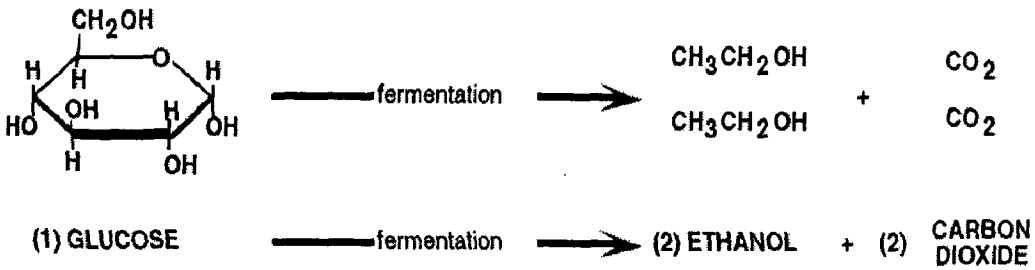


Fig-1.5. Glucose fermentation

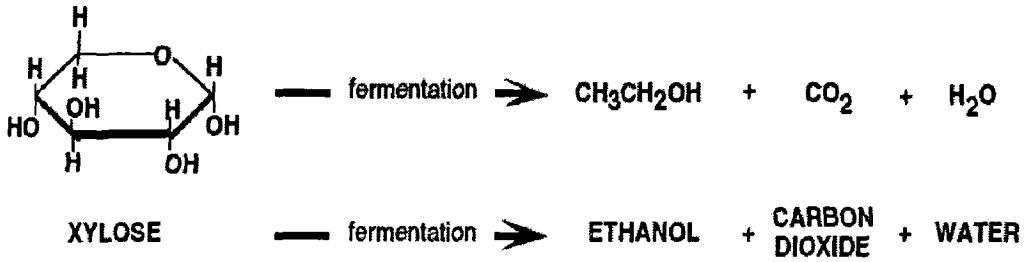


Fig-1.6. Xylose fermentation

- **Recovery:**

➢ Azeotropic Distillation:

Benzene azeotropic distillation is the best process for the recovery of ethanol from fermentation broth. In the azeotropic distillation, benzene acts as an entrainer. Water is immiscible with highly non-polar benzene and hence, the vapor pressure of water above a boiling benzene/ethanol/water liquid mixture is greatly enhanced. A rich phase (74mole% benzene decanted from the cooled distillate) is used as reflux to the head of the azeotropic column and flows downward as a liquid. The azeotropic ethanol/water mixture is fed near the middle of the column. Above the feed, the volatility of water is increased by the presence of benzene and the ethanol volatility is relatively depressed. Water is concentrated in the vapor and a water concentrated head product (24% ethanol, 54% benzene, 22% water) results. This is the ethanol/benzene/water ternary azeotrope. Below the feed tray, benzene is stripped from the ethanol to give a pure ethanol bottoms product. When the distillate of the azeotropic column is cooled and decanted, a water rich phase (35% ethanol, 4% benzene, and 61% water) is removed. This is treated in a benzene recovery column which produces the benzene concentrated ternary azeotrope as overhead product the recycle to the decanter and the dilute ethanol bottoms product (49% ethanol, 51% water) the dilute ethanol stream can be concentrated in an additional rectifying column and recycled to the primary alcohol recovery column [1].

➢ Vacuum Distillation:

The ethanol/water equilibrium is pressure sensitive, and the relative volatility of ethanol over water at the high ethanol concentration is increased at low pressure. As pressure is reduced from 1atm., the ethanol water azeotropic composition increases until, at about 1.6 Kilo Pascal (80mm Hg) absolute pressure, the azeotrope disappears. The energy required for this "one step" distillation is very high and this process is uneconomical. However, vacuum operation can be used to advantage in producing a product of the normal pressure ethanol/water azeotropic composition. Under vacuum, the required reflux may be reduced. For a 13-wt% alcohol feed producing 95 wt% alcohol products, the energy requirement for a single column distillation is reduced from $7*10^6$ J/L of product to $2.41*10^6$ J/L [1].

➢ Extractive Distillation:

Further has proposed using potassium acetate or other salts as an extracting agent for ethanol distillation. Potassium acetate greatly enhances the relative volatility of ethanol over water, completely eliminating the azeotrope and greatly reducing the number of stages and the energy required for the distillation. The column is operated with salt added to the reflux. Pure alcohol is the head product, but the bottoms water product contains salt, which must be separated for recycle. Salt recovery by evaporation of the water is impractical, but application of technology developed for ocean water desalination may make this process more attractive [1].

➢ Ethanol Dehydration by Vapor Phase water Absorption:

Dehydration of azeotropic ethanol vapor by absorbing water onto dry CaO or $K_2Cr_2O_7$ is a standard laboratory procedure. Ladisch and Dyck suggest that similar processes may be used beneficially at industrial scale. Rather than distilling to the azeotrope, distillation is conducted to produce only 85 wt% alcohol vapors. Distillation to this lower level requires only $1.73*10^6$ J/L for an 85% ethanol production. These vapors are then passed through a bed of adsorbent material to produce pure alcohol. Laboratory scale tests indicate that dry corn starch will selectively adsorb all of the remaining water

and essentially no alcohol. This process seems quiet attractive, but further work appears necessary to determine the energy requirements for regeneration of the adsorbent and to test the process at pilot plant scale [1].

➢ Solvent Extraction:

Solvent extraction offers an alternative to distillation. Myers suggests counter current extraction of ethanol from water solution into diethyl ether. The high volatility of ether and its low heat of vaporization would allow a simple low energy distillation. Separation delete factors can be increased and extraction made more efficient by the addition of salts, which increase the activity of the ethanol in the aqueous phase and help drive it into the organic solvent. Such a process has been proposed for the direct production of gasohol by the extraction of ethanol from beer into gasoline. As with extractive distillation using salts, a low energy technique must be developed for salt recovery from the aqueous phase [1].

1.3.2. Hydrogen:

Biological methods for the production of hydrogen from biomass are,

1. Photo fermentation.
2. Dark fermentation.

- **Photo fermentation:**

Purple non-sulfur bacteria evolve molecular H_2 catalyzed by nitrogenase under nitrogen-deficient conditions using light energy and reduced compounds (organic acids).

$$C_6H_{12}O_6 + 12H_2O \xrightarrow{Lightenergy} 12H_2 + 6CO_2$$

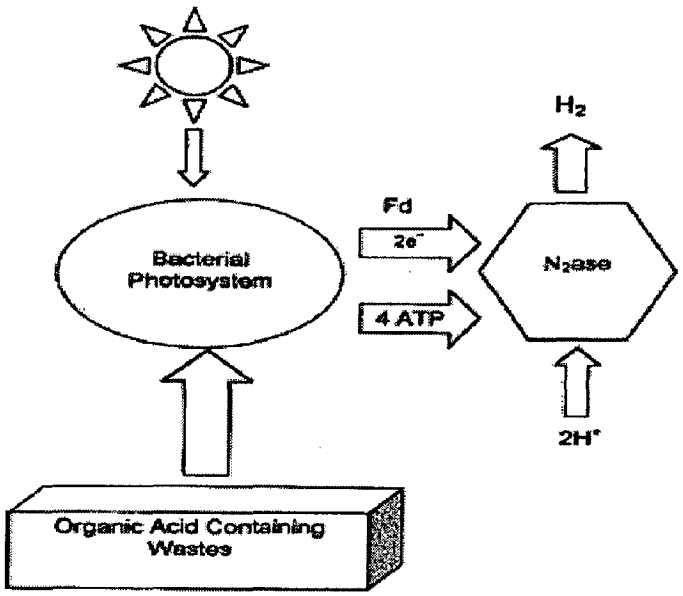


Fig-1.7. Photo fermentation

These photoheterotrophic bacteria have been investigated for their potential to convert light energy into H_2 using waste organic compounds as substrate in batch processes, continuous cultures, or cultures of bacteria immobilized in carrageenan, in agar gel, on porous glass, on activated glass, or on polyurethane foam. The rates of hydrogen production by photoheterotrophic bacteria are higher when the cells are immobilized in or on a solid matrix, than when the cells are free-living. Hydrogen production from photo fermentation was shown in Fig-1.7 [15].

The photosynthetic bacteria have been shown to produce hydrogen from various organic acids and food processing and agricultural wastes. There are some drawbacks to this type of system: (1) the low solar energy conversion efficiencies; and (2) the requirement for elaborate anaerobic photobioreactors covering large areas [9].

- **Dark fermentation:**

Hydrogen can be produced by anaerobic bacteria, grown in the dark on carbohydrate-rich substrates. Fermentation reactions can be operated at mesophilic (25–40◦C), thermophilic (40–65◦C), extreme thermophilic (65–80◦C), or hyperthermophilic

(>80◦C) temperatures. Hydrogen production from dark fermentation was shown in Fig-1.8.

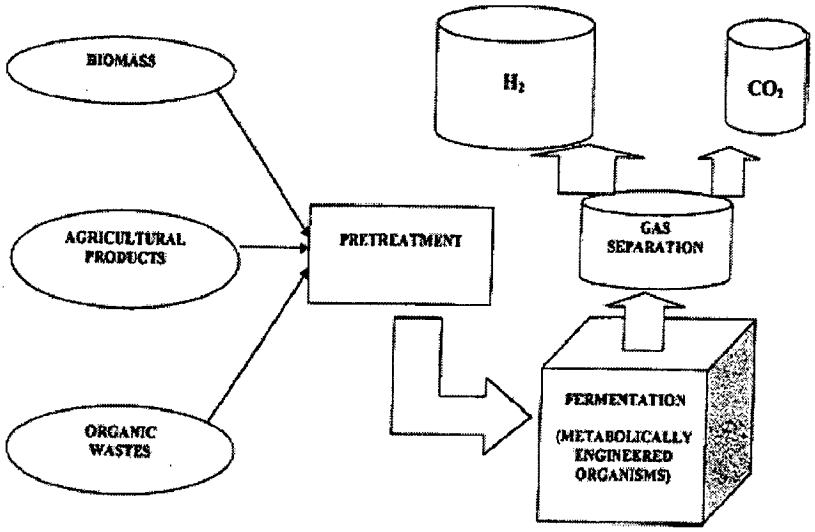


Fig-1.8. Hydrogen production through dark fermentation

Bacteria known to produce hydrogen include species of *Enterobacter*, *Bacillus*, and *Clostridium*. Carbohydrates are the preferred substrate for hydrogen-producing fermentations. Glucose, isomers of hexoses and polymers in the form of starch or cellulose yield different amounts of H_2 per mole of glucose, depending on the fermentation pathway and end-product(s). When acetic acid is the end-product, a theoretical maximum of 4 mole H_2 per mole of glucose is obtained:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$$

When butyrate is the end-product, a theoretical maximum of 2 moles H_2 per mole of glucose is obtained:

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_2CH_2CH_2COOH + 2H_2 + 2CO_2$$

Thus, the highest theoretical yields of H_2 are associated with acetate as the fermentation end-product. In practice, however, high H_2 yields are associated with a mixture of acetate and butyrate fermentation products, and low H_2 yields are associated with propionate and reduced end-products (alcohols, lactic acid). *Clostridium*

pasteurianum, *C. butyricum*, and *C. beijerinkii* are high H_2 producers, while *C. propionicum* is a poor H_2 producer. Hydrogen production by these bacteria is highly dependent on the process conditions such as pH, hydraulic retention time (HRT), and gas partial pressure, which affect metabolic balance [15].

1.3.3. Methane:

Biological method for the production of methane from biomass is anaerobic digestion. Anaerobic digestion is a biological process that produces a gas composed of methane (CH_4) and carbon dioxide (CO_2). These gases are produced from organic wastes such as livestock manure, food processing waste, etc.

Anaerobic digestion takes place in two steps: acid formation, and methane generation. Those steps are shown in the Fig-1.9. During the first step, acid formation, bacterial enzymes break down proteins, fats and sugars in the waste to simple sugars and bacteria convert the sugars to acetic acid, carbon dioxide and hydrogen. During second step, methane formation, bacteria convert the acetic acid to methane and carbon dioxide, and combine carbon dioxide and hydrogen to form methane and water [21].

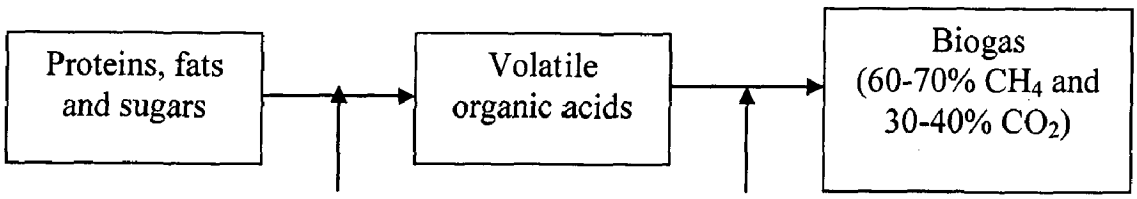


Fig-1.9. Schematic diagram of methane production

Three types of anaerobic digesters are there, those are ambient-temperature covered lagoon, complete-mix digester and plug-flow digester.

1.3.3.1. Ambient-Temperature Covered Lagoon:

Properly designed anaerobic lagoons are used to produce biogas from dilute wastes with less than 2% total solids (98% moisture) such as flushed dairy manure, dairy parlor wash water, and flushed hog manure. The lagoons are not heated and the lagoon temperature and biogas production varies with ambient temperatures. Coarse solids such as hay and silage fibers in cow manure must be separated in a pretreatment step and kept out of the lagoon. If dairy solids are not separated, they float to the top and form a crust. The crust will thicken, which will result in reduced biogas production and, eventually, infilling of the lagoon with solids. Unheated, unmixed anaerobic lagoons have been successfully fitted with floating covers for biogas recovery for dairy and hog waste [21].

1.3.3.2. Complete-Mix Digester:

Complete-mix digesters are the most flexible of all digesters as far as the variety of wastes that can be accommodated. Wastes with 2% to 10% solids are pumped into the digester and the digester contents are continuously or intermittently mixed to prevent separation. Complete-mix digesters are usually aboveground, heated, insulated round tanks. Mixing can be accomplished by gas recirculation, mechanical propellers, or circulation of liquid [21].

1.3.3.3. Plug-Flow Digester:

Plug-flow digesters are used to digest thick wastes (11% to 13% solids) from ruminant animals. Coarse solids in ruminant manure form a viscous material and limit solids separation. If the waste is less than 10% solids, a plug-flow digester is not suitable. If the collected manure is too dry, water or a liquid organic waste such as cheese whey can be added.

Plug-flow digesters consist of unmixed, heated rectangular tanks that function by horizontally displacing old material with new material. The new material is usually

pumped in, displacing an equal portion of old material, which is pushed out the other end of the digester [21].

1.4. Fermentation Modes:

1.4.1. Batch Fermentation:

Today, most ethanol is produced by the same processes developed in the beverage industry more than a hundred years ago. The general characteristics of batch systems are well known. Usually the time required to completely utilize the substrate is 36-48 hr. The temperature is held at 10-30°C and the initial pH is adjusted to 4.5. Depending upon the nature of the carbohydrate material, conversion efficiency lies in the range of 90-95% of the theoretical value with a final ethanol concentration of 10-16 % (w/v).

In an effort to increase fermentor productivity yet retain the simplicity of a batch process, cell recycle has been employed in many cases. This technique does not increase the efficiency of sugar to ethanol conversion, however, the time required for the fermentation to run to completion is reduced by as much as 60-70% over traditional batch methods. The sugar solution supplemented with yeast nutrients is added to the fermenter and the fermenter is inoculated with a rapidly growing culture of yeast from the seed tank [3].

1.4.2. Semi continuous Fermentation:

The semi continuous processes comprise the so-called outflow-inflow processes and also a wide range of battery and cyclic fermentation variants. Characteristics of the processes is the continuous feed liquid flow after addition of a nutritive worth and of a seeding culture, such as *S. cerevisiae*. The treated medium flows by gravity or is pumped as a seeding culture from the first vessel into the second, while fermentation is continued in the first. Then, in succession, the second fermentor is filled up with the nutritive worth and left for fermentation. Thereafter the third vessel is filled with the seeding culture and a prolonged nutritive inflow charge is secured. This process was continuous until all the

vessels charged. Using this system, the fermentation time is shortened because of higher yeast activity and concentration [3].

1.4.3. Continuous Fermentation:

Continuous ethanol production eliminates much of the unproductive downtime associated with batch culture. This includes striping and cleaning the apparatus, recharging with media and time required for the lag phase. In addition, since the microbe may be essentially "locked" in the exponential phase of its growth cycle, then the overall time depends on productivity of ethanol formation is increased. This allows a higher output per unit volume of equipment and as such, cost savings may be made in the construction of smaller fermenters.

Two main disadvantages in continuous systems include concern over contamination (either internal through mutation or external by an invading microbe) and problems in maintaining a high fermentation rate. Low rates of fermentation have been shown to be connected with cell death caused by a lack of oxygen. This oxygen requirement may be eliminated by the addition of certain substance (i.e. Tween 80, ergosterol, or linolenic acid) to the fermentation medium or by propagation of the yeast under aeration prior to anaerobiosis.

Specific ethanol productivity in a simple continuous fermenter is ordinarily limited by ethanol inhibition and a low cell concentration. As sugar content of the feed stream is increased, ethanol productivity decreases due to product inhibition effects. As lower concentrations of carbohydrate, inhibition is seen to decrease, however, cell mass concentration also falls off. Cell recycle has been utilized in continuous systems to overcome low cell density limitations. With much of the microbial biomass returned to the fermenter an extremely high cell concentration may be maintained [3].

1.4.4. Vacuum Fermentation:

As alcohol is toxic to cell growth, processes by which it may be removed upon formation would greatly enhance the productivity of the system. Cysewski and Wilke have taken advantage of the high volatility of ethanol by running the fermentation under sufficient vacuum to boil off the product at the temperatures conductive to yeast growth. A fermenter productivity of 82 g/L-hr. was achieved from a 33.4 % (w/v) glucose feed (12 fold that of a conventional continuous process) [3].

1.5. Objective of project report:

- The objective of present work is biocatalytic conversion of biomass (i.e., sugarcane bagasse) to biofuels (i.e., ethanol) by fermentation.
- In this work, to convert the bagasse to fermentable sugars by acid hydrolysis.
- To study the capacity of various fermentative microorganisms on fermentation of bagasse hydrolysate for maximum yield of ethanol production.

The experimental works needed for the completion of present project work is carried out at Biotechnology lab in Indian Institute of Petroleum (IIP), Dehradun.

A. Singh et al., [1984]

Production of Reducing Sugars from Bagasse and Rice Husk by Acid Hydrolysis

The agricultural residues bagasse and rice husk can be utilized for the production of reducing sugars through conc. H_2SO_4 hydrolysis. Various process conditions like solid-liquid ratio, reaction time and concentration of acid have been optimized. In the present studies, conc. H_2SO_4 was found to be a better hydrolytic agent than conc. HCl. An acid concentration of 75 % (w/w) with soaking period of 1 h, temperature 50°C, solid-liquid ratio of 1:12, primary hydrolysis and heating time of 3 h at 100 °C after dilution with 3 volumes of water, were found to be the optimum conditions for the hydrolysis of bagasse and rice husk.

Mats Larsson et al., [1997]

Recirculation of process water in the production of ethanol from soft wood

In the production of ethanol from lignocellulosics, large quantities of water are needed in various process steps. In industrial processes, recycling of process streams is necessary to minimise fresh water requirements and decrease the amount of wastewater produced. In the present study ethanol was produced from softwood in a bench-scale process development unit. The stillage stream from the distillation step was fractionated by evaporation and various evaporation fractions characterised and their inhibitory effect on fermentation investigated. It was found that the volatile fractions have no negative effect on the fermentation, while the non-volatile fraction inhibited fermentation and resulted in a decreased yield. Evaporation has been shown to be an effective way to drastically diminish the build-up of inhibitory compounds when process streams are recirculated, but the energy demand is high. Various process configurations to reduce the energy demand are suggested.

Yoshiyuki Ueno et al., [1998]

Ethanol Production by Dark Fermentation in the Marine Green Alga, *Chlorococcum littorale*

Dark fermentation in the marine green alga, *chlorococcum littorule,* was investigated with emphasis on ethanol production. In this paper state that under dark anaerobic conditions, 27% of cellular starch was consumed within 24 h at 25°C, the cellular starch decomposition being accelerated at higher temperatures. Ethanol, acetate, hydrogen and carbon dioxide were obtained as fermentation products. The maximum productivity of ethanol was 450 μmol/g-dry wt. at 30°C.

J.P. Delgenes et al., [1998]

Biological production of industrial chemicals, i.e. xylitol and ethanol, from lignocelluloses by controlled mixed culture systems

This paper describes the utilization of the mixed cultures concept for the production of ethanol from a mixture of cellulosic glucose and hemicellulosic xylose. For ethanol production, the process was studied in continuous aerated conditions with a respiratory deficient mutant of *Saccharomyces cerevisiae* CBS 1200, associated with *Pichia stipitis* NRRL 11545 and using a microfiltration membrane assisted bioreactor. Using a synthetic medium (35 g/l glucose, 15 g/l xylose), the highest fermentative performances were obtained at D=0.1 h^{-1}. Under these conditions, ethanol was produced with a yield of 0.43 g/g and a volumetric rate of 2 g/l-h. The glucose and xylose conversion yields were, respectively, 100 and 60%, giving an overall substrate conversion yield of 88%. At D=0.13 h^{-1} with the medium containing aspen wood hydrolysate as carbon source (glucose 41 g/l, xylose 9 g/l), ethanol was produced with a volumetric rate of 2.9 g/l-h, a yield of 0.46 g/g and the substrate conversion yield was 94%.

B.P. Lavarack et al., [2002]

The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products

Dilute acid hydrolysis of bagasse hemicellulose to produce xylose, arabinose, glucose, acid-soluble lignin (ASL) and furfural were conducted using a temperature-controlled digester. The reaction conditions varied were; temperature (80–200°C), mass ratio of solid to liquid (1:5–1:20), type of bagasse material (i.e. bagasse or bagacillo), concentration of acid (0.25–8 wt% of liquid), type of acid (hydrochloric or sulphuric) and reaction time (10–2000 min). Yields of up to 220 mg xylose/g solid were achieved, i.e. about 80% of the theoretical xylose available from the bagasse. The bagasse particle size was found to negligibly affect the rate of hydrolysis. Hydrochloric acid was found to be less active for the degradation of xylose compared to sulphuric acid.

R. Aguilar et al., [2002]

Kinetic study of the acid hydrolysis of sugar cane bagasse

Sugar cane bagasse is a renewable, cheap and widely available waste in tropical countries. The hydrolysis of sugar cane bagasse to obtain xylose solutions has a double consequence, the elimination of a waste and the generation of a value-added product. This paper was used to study about the xylose production from sugar cane bagasse by sulphuric acid hydrolysis at several temperatures (100, 122 and 128 °C) and concentrations of acid (2%, 4% and 6%).

The optimal conditions found were 2% H_2SO_4 at 122 °C for 24 min, which yielded a solution with 21.6 g xylose/l, 3 g glucose/l, 3.65 g acetic acid/l and 0.5 g furfural/l. In these conditions, ≈90% of the hemicelluloses was hydrolyzed.

Ria Millati et al., [2002]

Effect of pH, time and temperature of overliming on detoxification of dilute-acid hydrolyzates for fermentation by Saccharomyces cerevisiae

This paper investigated the effects of different variables in detoxification of a severely inhibiting dilute-acid hydrolyzate by overliming. Overliming was carried out by increasing the pH to 10, 11 or 12 at two different temperatures, 25 and 60°C, holding the pH and temperature at constant values for different periods of time, 0, 1, 20 and 170 h, and then adjusting the pH to 5.5. All hydrolyzates were then fermented in batch cultivation by Saccharomyces cerevisiae in shake flasks, whereupon one was then selected for continuous cultivation in a bioreactor. The most significant effect of overliming was a sharp decrease in the concentration of furfural and hydroxymethylfurfural, whereas the concentration of acetic acid remained unchanged and the decrease in the total phenolic compounds was less than 30%. Detoxification at pH 12 for more than 1 h was effective, whereas no effect was obtained at pH 10 and the hydrolyzates had to remain at pH 11 for more than 20 h to become fermentable. On the other hand, decrease in sugar concentration during overliming was a serious problem at pH 12, especially at the higher temperature, where up to 70% sugars were degraded. The fermentability of a detoxified hydrolyzate was also tested in a continuous cultivation by immobilized S. cerevisiae in Ca-alginate. The hydrolyzate was fully fermentable at different dilution rates between 0.2 and 1.0 h^{-1}.

Carlos Martin et al., [2002]

Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising *Saccharomyces cerevisiae*

Sugarcane bagasse was pretreated by steam explosion at 205 and 215°C and hydrolysed with cellulolytic enzymes. The hydrolysates were subjected to enzymatic detoxification by treatment with the phenoloxidase laccase and to chemical detoxification by overliming. Approximately 80% of the phenolic compounds were specifically removed by the laccase treatment. Overliming partially removed the phenolic

compounds, but also other fermentation inhibitors such as acetic acid, furfural and 5-hydroxy-methyl-furfural. The hydrolysates were fermented with the recombinant xylose-utilising *Saccharomyces cerevisiae* laboratory strain TMB 3001 and xylitol dehydrogenase of *Pichia stipitis*, and the *S. cerevisiae* strain ATCC 96581, isolated from a spent sulphite liquor fermentation plant. An almost two-fold increase of the specific productivity of the strain TMB 3001 in the detoxified hydrolysates compared to the undetoxified hydrolysates was observed. The ethanol yield in the fermentation of the hydrolysate detoxified by overliming was 0.18 g/g dry bagasse, whereas it reached only 0.13 g/g dry bagasse in the undetoxified hydrolysate. Partial xylose utilisation with low xylitol formation was observed.

Antonio Rodriguez-Chong et al., [2004]

Hydrolysis of sugar cane bagasse using nitric acid: a kinetic assessment

Sugar cane bagasse was hydrolyzed using nitric acid at variable concentration (2–6%), reaction time (0–300 min) and temperature (100–128°C). The concentration of sugars released (xylose, glucose and arabinose) and degradation products (acetic acid and furfural) were determined. The optimal conditions were selected from this experiment: 122°C, 6% HNO_3 and 9.3 min. Using these conditions, 18.6 g xylose/l; 2.04 g arabinose/l; 2.87 g glucose/l; 0.9 g acetic acid/l and 1.32 g furfural/l were obtained.

Larissa Canilha et al., [2004]

Eucalyptus hydrolysate detoxification with activated charcoal adsorption or ion-exchange resins for xylitol production

Eucalyptus hemicellulosic hydrolysate used for xylitol production by *Candida guilliermondii* FTI20037 was previously treated either with ion-exchange resins or with activated charcoal adsorption combined with pH adjustment, in order that acetic acid, furfural and hydroxymethylfurfural could be removed. The best results for xylitol yield factor (0.76 g/g) and volumetric productivity (0.68 g/(l h) were attained when a three-fold concentrated hydrolysate was treated with ion-exchange resins. Using activated charcoal

combined with pH adjustment for treating a three-fold concentrated hydrolysate resulted in a xylitol yield factor of 0.40 g/g and a volumetric productivity of 0.30 g/(l h). This same treatment applied to a six-fold concentrated hydrolysate resulted in a xylitol yield factor of 0.66 g/g and a volumetric productivity of 0.50 g/(l h).

Badal C. Saha et al., [2005]

Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol

Wheat straw consists of 48.57±0.30% cellulose and 27.70 ± 0.12% hemicellulose on dry solid (DS) basis. In this paper, dilute acid pretreatment at varied temperature and enzymatic saccharification were evaluated for conversion of wheat straw cellulose and hemicellulose to monomeric sugars. The maximum yield of monomeric sugars from wheat straw (7.83%, w/v, DS) by dilute H_2SO_4 (0.75%, v/v) pretreatment and enzymatic saccharification (45°C, pH 5.0, 72 h) using cellulase, b-glucosidase, xylanase and esterase was 565±10 mg/g. Under this condition, no measurable quantities of furfural and hydroxymethyl furfural were produced. The yield of ethanol (per litre) from acid pretreated enzyme saccharified wheat straw (78.3 g) hydrolyzate by recombinant Escherichia coli strain FBR5 was 19±1 g with a yield of 0.24 g/g DS. Detoxification of the acid and enzyme treated wheat straw hydrolyzate by overliming reduced the fermentation time from 118 to 39 h in the case of separate hydrolysis and fermentation (35°C, pH 6.5), and increased the ethanol yield from 13±2 to 17±0 g/l and decreased the fermentation time from 136 to 112 h in the case of simultaneous saccharification and fermentation (35°C, pH 6.0).

Sara Gamez et al., [2006]

Study of the hydrolysis of sugar cane bagasse using phosphoric acid

In the present work, samples of sugar cane bagasse were hydrolyzed with phosphoric acid under mild conditions (H_3PO_4 2–6%, time 0–300 min and 122°C) to study the feasibility of using the liquid phase as fermentation media. Solid yield, sugar

concentrations and decomposition product (inhibitor's) concentrations were measured. The optimal conditions selected were 122°C, 4% H_3PO_4 and 300 min. Using these conditions, 17.6 g of xylose/l; 2.6 g of arabinose/l; 3.0 g of glucose/l, 1.2 g furfural/l and 4.0 g acetic acid/l were obtained. The efficiency in these conditions was 4.46 g sugars/g inhibitors and the mass fraction of sugars in dissolved solids in liquid phase was superior to 55%.

3.1. Apparatus Used:

3.1.1. Rotary Shaker with incubation:

These are table shakers, which in conjunction with incubation hood can be employed as incubator shakers, the driving elements of the table shakers are electronically controlled magnetic coils. The shaking table is directly driven by means of alternatively produced magnetic fields. A digital display indicates, immediately produced magnetic field. A digital display indicates, immediately the set as well as actual temperature and also set actual speed of the rotary shaker.

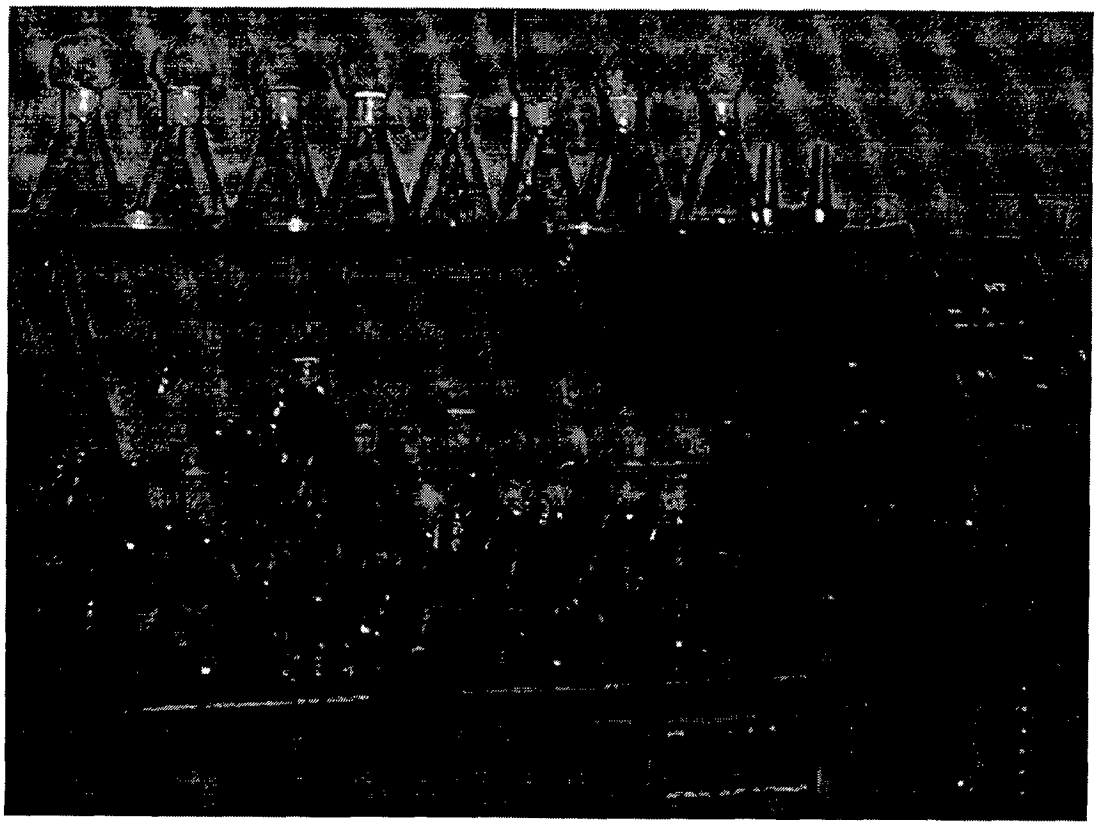


Fig-3.1. Photograph of rotary shaker incubator

3.1.2. Autoclave:

Laboratory apparatus designed to use stream under regulated pressure is called an autoclave. It is a double jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a designated temperature and pressure for any period of time. The temperature of the steam kills the microbes under pressure. The above temperature was obtained by boiling. In addition, it has advantages of rapid heating penetration which facilitates the coagulation of proteins generally. The autoclave is operated, at a pressure approximately 15 lb/in^2 (at 121C). This pressure is maintained for 15min. Then electric support supply is switched off to facilitate cooling.

3.1.3. Centrifuge:

It is used to separate heavier particles from is a suspension which settles down due to the centrifugal force.

Fig-3.2. Photograph of eppendorf centrifuge

3.1.4. Laminar flow hood:

This is instrument is used to provide bacteria free aseptic conditions for microbiological work. In this hood we get clean sterile air, high efficiency particulate. Air filters (Hepa filters) are used in laminar air flow. To maintain aseptic environment, UV light should be switched on for five minutes with the glass door of the laminar flow securely closed. UV should be put off and air flow switched on before starting the work.

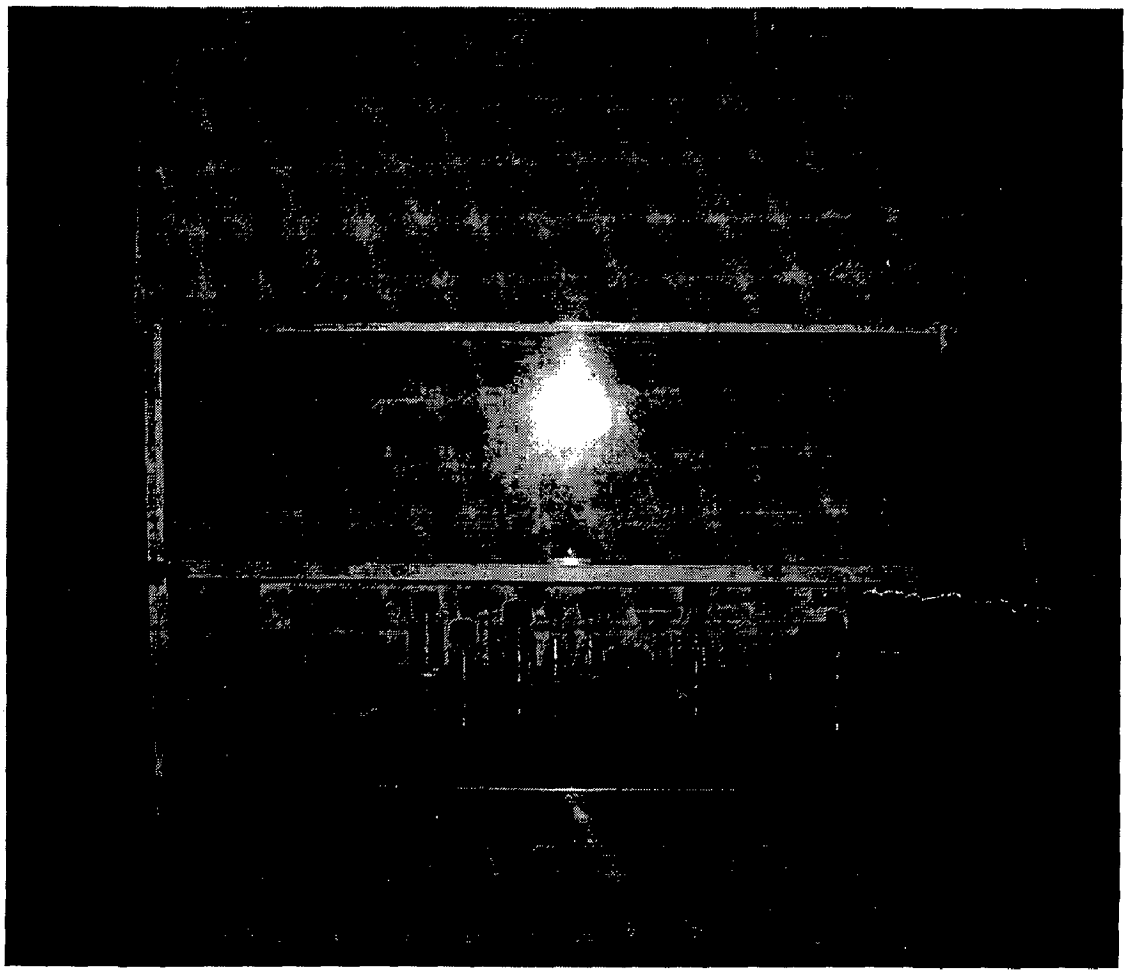


Fig-3.3. Photograph of laminar flow hood

3.1.5. Hot dry oven:

This is an electrically operated oven which is used to dry the glass wares and other materials.

3.1.6. pH meter:

To operate this instrument the temperature is set and standardization of pH meter is done with the help of pH standards 4.0, 7.0 and 9.2. The pH value of the sample solution is measured by dipping the electrode in it.

3.1.7. Vacuum oven:

In order to take the cell dry weight the sample is kept in it. The cell pellet cannot be kept in an ordinary oven to dry, because high temperature will be required for the same, which may cause charring of the cells. While in the case of vacuum ovens temperature around 80°C are sufficient to dry the cells. This is because at low pressure, the boiling point of water will also get lowered thus it will lead to vaporization of water at low temperature causing the cells to dry.

3.1.8. Hot water bath:

This is an instrument is used to maintain the constant temperature of the fermentation system. It has the facility to change the temperature to required temperature.

3.1.9. Condenser:

This is a device is used to condense the vapors formed during fermentation process.

3.1.10. Magnetic stirrer:

This is an instrument is used to mix the components and agitation in fermentation process. The speed of the agitation is adjustable. It is also used for heating purpose.

3.1.11. Jacketed three-neck flat bottom flask:

It is a device used as batch fermentor. It consists of three necks; a condenser is used at middle neck of flask to condense vapors, a thermometer is stated in one neck of flask and another neck is used for sampling. It has the jacket is used to passing the hot water to maintain constant temperature.

4.1. Procedure:

Ethanol fermentation from biomass (bagasse) is two steps process. These are

1. Pretreatment
2. Fermentation

4.1.1. Pretreatment:

Pretreatment of biomass to fermentation feed stock is consists of saccharification and detoxification.

4.1.1.1. Saccharification:

Saccharification is the process to convert the biomass to fermentable sugars. Commonly hydrolysis is used as saccharification process. There are three types of hydrolysis processes. These are dilute acid hydrolysis, strong acid hydrolysis and enzymatic hydrolysis. In my experiment, I was carried out dilute acid hydrolysis.

Bagasse was taken from outside and chopped into small pieces (nearly 5mm in size). Bagasse pieces was boiled gently in warm water to remove the sugars and dried in oven. 100 g of dried bagasse was taken and mixed with 2 L of 2 % (v/v) H_2SO_4 solution (i.e., S: L = 1: 20). Bagasse and acid mixture was set a side for 30 min to soaking, and the mixture was kept in an autoclave at 15 psi and 121°C for residence time of 60 min. After 60 min the mixture was take it outside and kept for cooling. The hydrolysate from residual bagasse was separated by filtration. Hydrolysate was neutralized with $Ca(OH)_2$ and formed $CaSO_4$ was removed by filtration. Sample of hydrolysate was analyzed for estimation of glucose and furfural. Fig-4.1 was shown schematic diagram of acid hydrolysis process.

The same process was conducted with residual bagasse as the feed and fresh 2 L of 2 % (v/v) H_2SO_4. Sample of hydrolysate was analyzed for estimation of glucose and furfural.

The above procedure was repeated with fresh dried bagasse for resident times of 90 min and 120 min.

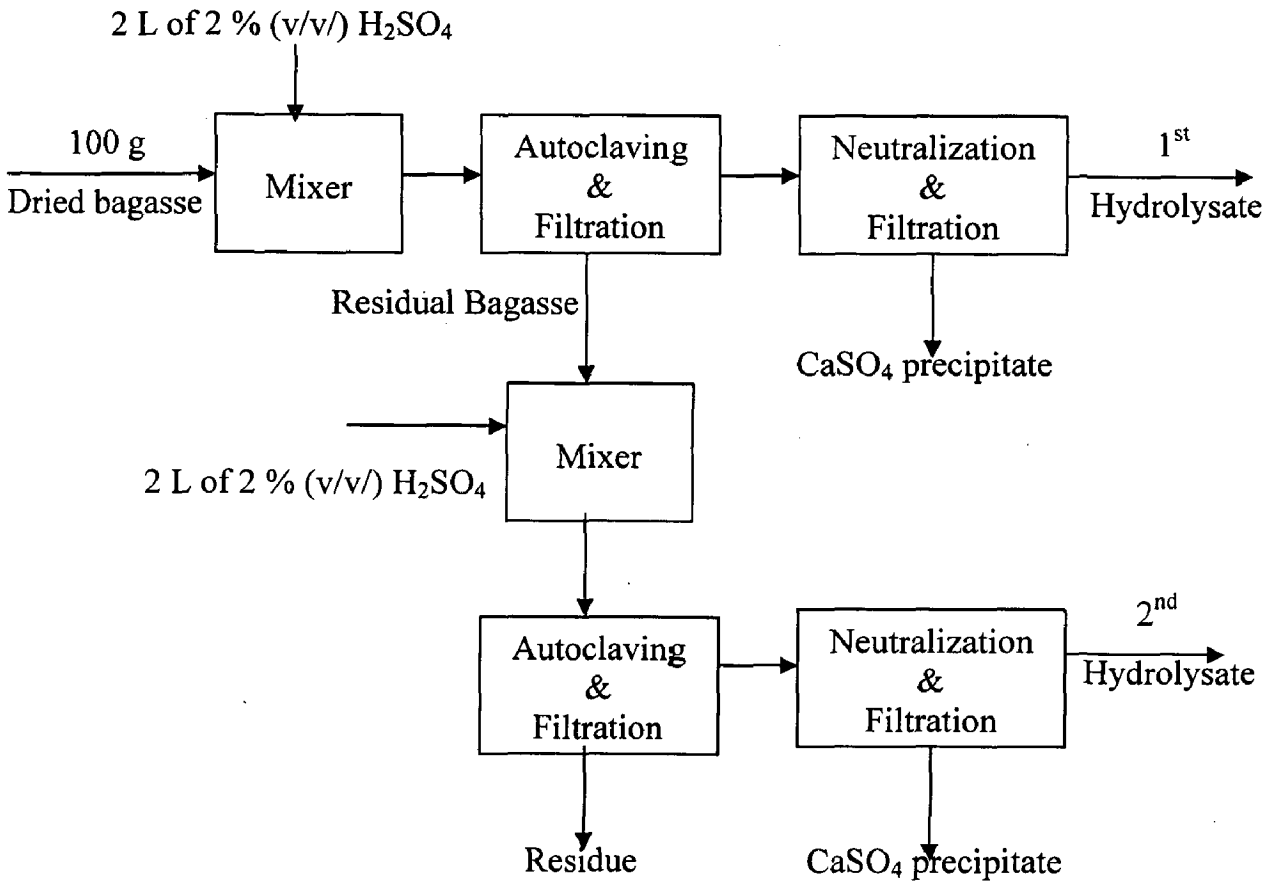


Fig-4.1. Schematic diagram of Acid hydrolysis

In this process cellulose was converted into hexoses and hemicellulose was converted into pentoses. During this process some part of hexoses and pentoses are decomposed into 5-hydroxy methyl furfural and furfural respectively. In this process furfural was the major inhibitor for fermentation.

4.1.1.2. Detoxification:

Some inhibitors formed during acid hydrolysis which is toxic for the growth of microbes, mainly furfural. The process which is removing these inhibitors is called detoxification.

In my experiment, I was conducted five types of detoxification processes individually to identify which process was better to remove furfural with less removal of fermentable sugars.

1st process (Agitation along with activated carbon), 30 ml of 1st hydrolysate was mixed with 0.3 g of activated carbon in 100 ml conical flask and agitated for 1 hr. Then activated carbon was separated from hydrolysate by filtration. Glucose and furfural concentrations were estimated in hydrolysate.

2nd process (Passing through adsorption column with activated carbon), 10 cm height and 1 cm inner diameter of the adsorption column was prepared with activated carbon. 30 ml of 1st hydrolysate was passed through the adsorption column. Glucose and furfural concentrations were estimated in adsorbent (hydrolysate).

3rd process (Passing through adsorption column with silica gel), 10 cm height and 1 cm inner diameter of the adsorption column was prepared with silica gel. 30 ml of 1st hydrolysate was passed through the adsorption column. Glucose and furfural concentrations were estimated in adsorbent (hydrolysate).

4th process (Passing through adsorption column with bauxite), 10 cm height and 1 cm inner diameter of the adsorption column was prepared with bauxite. 30 ml of 1st hydrolysate was passed through the adsorption column. Glucose and furfural concentrations were estimated in adsorbent (hydrolysate).

5th process (Extraction with ethyl acetate), 30 ml of hydrolysate and 10 ml of ethyl acetate (in the ratio of 3:1) was taken into 250 ml conical flask, and extracted by agitation for 30 min. The mixture was transferred into a separator funnel. The organic

(ethyl acetate) and aqueous (hydrolysate) phases were separated. Air stripping was conducted through extracted hydrolysate phase to remove volatile components (i.e., ethyl acetate). Distillation was conducted to ethyl acetate phase to recover the pure ethyl acetate. Glucose and furfural concentrations were estimated in hydrolysate.

I was conducted combination of two or three above mentioned processes to get the best result for the removal of furfural with less removal of fermentable sugars.

4.1.2. Fermentation:

Sub culturing of microorganisms are needed to conduct the fermentation. Two fermentation experiments were conducted by me, one with GNSA strain and another with IIPE453 strain.

600 ml of H_1 medium (medium composition was shown in appendix-B) was prepared in 1 L flask, and the medium was transferred into four 500 ml conical flasks with working volume of 150 ml each. Entire medium was sterilized in an autoclave. Sterilized medium was cooled to room temperature. GNSA strain was inoculated to medium in laminar hood chamber, and the medium was incubated in a rotating shaker at 60°C and 138 rpm for 24 hrs. Growth of GNSA strain was observed. Incubated medium was centrifuged at 10,000 rpm for 5 min to harvesting the cells.

The same procedure was conducted to IIPE453 strain with H_2 medium (medium composition was shown in appendix-B), and incubated at 45°C and 138 rpm for 24 hrs. Growth of IIPE453 strain was observed. Incubated medium was centrifuged at 10,000 rpm for 5 min to harvesting the cells.

1 Liter jacketed three-neck flat bottom flask with a working volume of 500 ml was used for fermentation. 250 ml of fermentation medium (H_3 medium), composition given in appendix-B, was prepared and transferred into jacketed flask. 0.45 g of harvested GNSA strain was added into fermentation medium. The jacketed flask was stated on magnetic stirrer, shown in Fig-4.2. The temperature was maintained by passing the water at 60°C. A condenser was used at middle neck of flask to condense vapors. The gases

produced during fermentation was passed through 0.0625M $Ba(OH)_2$ to determine CO_2. A thermometer was stated in one neck of flask and another neck was used for sampling. Samples were taken at different time intervals and estimated glucose concentration, ethanol concentration, and dry cell weight.

The above process was repeated with IIPE453 strain. 450 ml of fermentation medium (H_4 medium), composition given in appendix-B, was prepared and transferred into jacketed flask. 2.475 g of harvested IIPE453 strain was added into fermentation medium. The temperature of the process was maintained by passing the water at 45°C. Samples were taken at different time intervals and estimated glucose concentration, ethanol concentration, and dry cell weight.

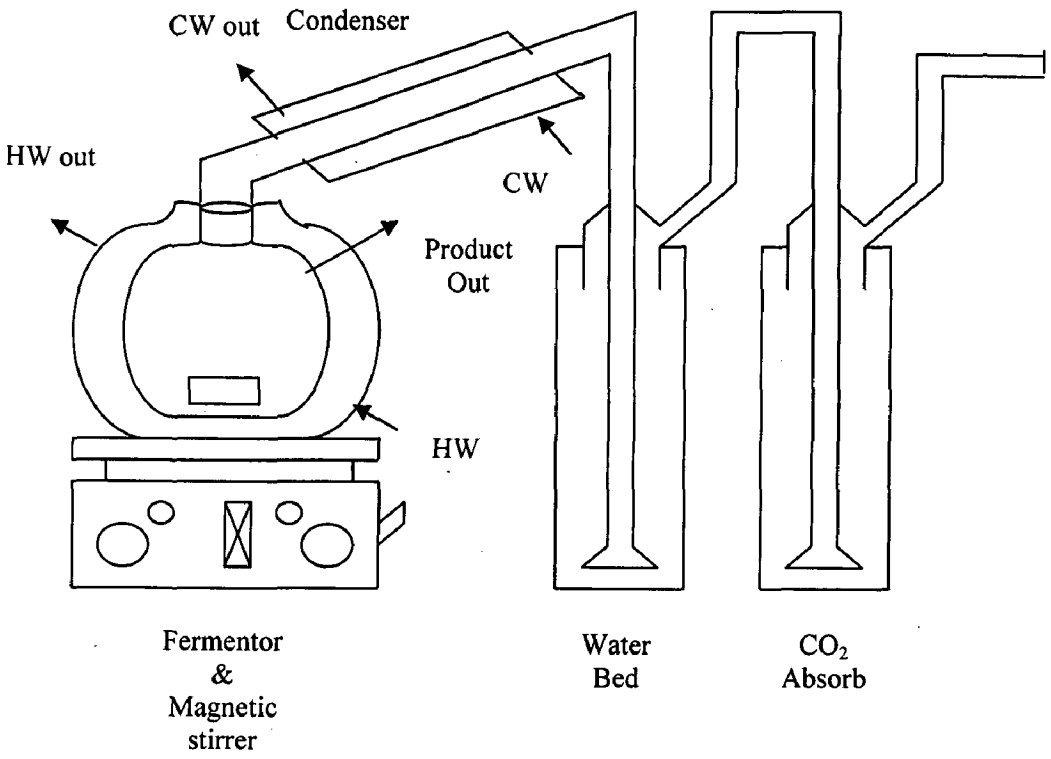


Fig-4.2. Schematic diagram of Fermentation setup

4.2. Analysis:

4.2.1. Characterization of Strains:

Hucker Gram Staining Method

Table-4.1. Composition for crystal violet staining reagent

Solution A	
Crystal violet (certified 90% dry content)	2.0 g
Ethanol 95% v/v	20 ml
Solution B	
Ammonium oxalate	0.8 g
Distilled water	80 ml

Mix A and B to obtain the crystal violet staining reagent. Store it for 24 hrs and filter it before use.

Table-4.2. Composition for mordant

Mordant	
Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml

Grind the iodine and potassium iodide in a mortar and add water slowly with continuous grinding until the iodine is dissolved. Store the mordant in amber battles.

Table-4.3. Composition for decolorizing agent and counter stain

Decolorizing Agent	
Ethanol 95% v/v	
Counter strain	
Safranin 0, as a 2.5% w/v alcoholic solution in 95% v/v ethanol	10 ml
Distilled water	100 ml

Glass slide was taken, cleaned and dried. Small amount of smear was added on the slide, spread into a thin film and dried. Smear film was flooded with crystal violet for 1 min, smear was washed in a gentle and indirect stream of tap water for 2 sec. Smear

was flooded with iodine mordant. Washed the smear in a gentle and indirect stream of tap water for 2 sec and then blotted the film dry with absorbent paper. Flooded the smear with 95 %v/v ethanol for 30 sec with agitation and then blotted the film dry with absorbent paper. Smear was flooded with safranin counter stain for 10 sec. The smear was washed with a gentle and indirect stream of tap water until no color appeared in the effluent and blotted the film dry absorbent paper.

4.2.2. Glucose estimation:

Reducing sugars in hydrolysate and fermented broth were determined by Dinitrosalicylic acid (DNS) method. Each 3 ml of distilled water was taken in test tubes, one tube was kept as blank and remaining tubes were used for dilution of samples (hydrolysate or fermented broth) which will measure the glucose concentration. After completion of known dilutions, 3 ml of 3,5-Dinitrosalicylic acid was added to each tube and boiled for 5 min in water bath. Then these tubes were immediately cooled to room temperature. Absorbance was taken in reference of blank at wavelength 575 nm by Double Beam UV-VIS Spectrophotometer 2600. The glucose concentration was determined from standard curve corresponding to that absorbance. Original glucose concentration of sample was obtained by multiplying the above glucose concentration with dilution factor.

DNS was prepared by the following method, 1.87 g 3,5-Dinitrosalicylic acid and 3.48 g sodium chloride were mixed in 250 ml distilled water, added 53.9 g potassium sodium tartrate, 1.34 ml phenol and 1.46 sodium metabisulphite.

Standard curve shown in appendix-C was prepared to determine reducing sugars in samples. Standard curve was prepared with known conc. of glucose as following procedure; stock solution of glucose concentration 1 g/L was prepared. By using this stock solution, different glucose concentrations in the range of 0.05 g/L to 0.35 g/L were prepared. 3 ml of glucose solution of each concentration was taken in test tubes and one tube contains 3 ml of distilled water used as blank. 3 ml of DNS was added to each tube. Boiled all the tubes for 5 min in water bath and immediately cooled to room temperature.

Absorbance was taken in reference of blank at wavelength 575 nm by Spectrophotometer. Graph was plotted between glucose concentration and absorbance.

4.2.3. Furfural estimation:

3 ml of distilled water was taken in each test tube and those were used for dilution of samples. After completion of known dilutions, absorbance was taken in reference of distilled water at wavelength 277 nm by Double Beam UV-VIS Spectrophotometer 2600. The furfural concentration was determined from standard curve corresponding to that absorbance. Original furfural concentration of sample was obtained by multiplying the above furfural concentration with dilution factor.

Standard curve shown in appendix-D was prepared to determine furfural concentration in samples. Standard curve was prepared with known conc. of furfural as following procedure; stock solution of furfural concentration $100*10^{-5}$ %v/v was prepared. By using this stock solution, different furfural concentrations in the range of $3.33*10^{-5}$ %v/v to $100*10^{-5}$ %v/v were prepared. 3 ml of furfural solution of each concentration was taken in test tubes. Absorbance was taken in reference of distilled water at wavelength 277 nm by Spectrophotometer. Graph was plotted between furfural concentration and absorbance.

4.2.4. Ethanol estimation:

Colorimetry method or gas chromatography was used to estimate the ethanol concentration in fermented broth.

4.2.4.1. Colorimetry Method:

To determine the ethanol conc. in fermented broth, first distilled the broth by adding equal volume of distilled water at 100°C and collected distillate half of the total volume. 1 ml of distillate was taken in a test tube, 4 ml of distilled water and 5 ml of chromic acid were added to distillate. One test tube with 5 ml of distilled water and 5 ml

of chromic acid was prepared to use as blank. Test tubes were incubated at 60°C for 20 min. Absorbance was taken in the reference of blank at 584 nm by Double Beam UV-VIS Spectrophotometer 2600. The ethanol concentration was determined from standard curve corresponding to that absorbance.

Chromic acid was used in colorimetry method, it was prepared in the following method, 34 g of Potassium dichromate was dissolved in 500 ml of distilled water and added 325 ml of concentrated sulfuric acid, made upto 1000 ml with distilled water.

Standard curve shown in appendix-E was prepared to determine ethanol concentrations. Standard curve was prepared with known concentration of ethanol as following procedure; stock solution of ethanol concentration 2.5%v/v was prepared. By using this stock solution, different ethanol concentrations in the range of 0.5 to 2.5 %v/v were prepared. 1 ml of ethanol solution of each concentration was taken in test tubes, and 4 ml of distilled water and 5 ml of chromic acid were added to each tube. One test tube with 5 ml of distilled water and 5 ml of chromic acid was prepared as a blank. All the tubes were incubated at 60°C for 20 min and cooled to room temperature. Absorbance was taken in the reference of blank at wavelength 584 nm by spectrophotometer. Graph was plotted between ethanol concentration and absorbance.

4.2.4.2. Gas Chromatography:

Ethanol concentration was also determined by using gas chromatography. The instrument used was a Chemito 8600 Refinery Gas Analyser with a 4 m long and 1/8 in diameter polar column with Chemosorb 80/60. Sample was injected using a syringe at an oven temperature 150°C and inlet and flame ionization detector temperature of 200°C using helium as a gas carrier.

4.2.5. Dry cell weight estimation:

For determination of cell dry weight, empty eppendorf tube was taken and it is dried in vacuum oven at 70°C to a constant weight. The weight of empty tube was measured. 1 ml of sample was taken in eppendorf tube and centrifuged this eppendorf tube by using Eppendorf Centrifuge 5415 C at 10000 rpm for 5 min and washed twice with distilled water. This eppendorf tube with cells was dried in vacuum oven at 70°C to a constant weight. The weight of the tube with cells was measured. The difference between these two readings was given the dry cell weight per ml.

4.2.6. Carbon dioxide estimation:

0.0625M $Ba(OH)_2$ solution was prepared. Sample of this solution was titrated with 0.05N HCl solution. The volume of HCl used for titration was measured. CO_2 produced during fermentation was estimated by passing gases through the solution of 0.0625M $Ba(OH)_2$. Some amount of $BaCO_3$ was formed in the form of precipitate. This remaining $Ba(OH)_2$ solution was titrated with 0.05N HCl and the volume of HCl used for titration was measured. CO_2 produced during fermentation was estimated as follows:

$$CO_2\ (mg) = N/2 * (Z_a - Z_b) * 44$$

Where, N- normality of HCl

Z_a- Volume of HCl used to titrate fresh $Ba(OH)_2$ solution.

Z_b- Volume of HCl used to titrate remaining $Ba(OH)_2$ solution.

Based on the procedure described in the previous chapter, experiments were conducted. On conducting the experiments, Acid hydrolysis, glucose and furfural are obtained as products. Fermentation, the ethanol and CO_2 are obtained as products.

In this chapter, I discussed about characteristics of microorganisms were used, results of acid hydrolysis, results of detoxification processes and results of fermentation process.

5.1. Characteristics of microorganisms:

In my experiment, I was used two microorganisms. One was GNSA strain, it is thermopile and grown at 60°C in H_1 medium. Another one was IIPE453 strain, it is also thermopile and grown at 45°C in salt medium.

Table-5.1. Characteristics of GNSA and IIPE453

Strain Code	Medium	Strain Characteristics
GNSA	H_1 medium	Spherical shape, gram -ve
IIPE453	H_2 medium	Large oval, budding, gram +ve

5.1.1. Growth studies on strains:

➢ Growth of GNSA was studied in H_1 medium. The absorbance at 600 nm and glucose concentration was observed with time shown in Table-5.2. Graph was plotted between glucose concentration and OD vs. time, shown in Fig-5.1.

Table-5.2. Absorbance and glucose concentration for the study of growth curve of GNSA

Time (hr)	OD at 600 nm	Glucose concentration (g/L)
0	0.035	9.8
2	0.036	9.8
4	0.037	9.8
6	0.048	9.7
8	0.09	9.6
24	4.735	7.0
26	5.385	6.8
28	5.6593	6.5

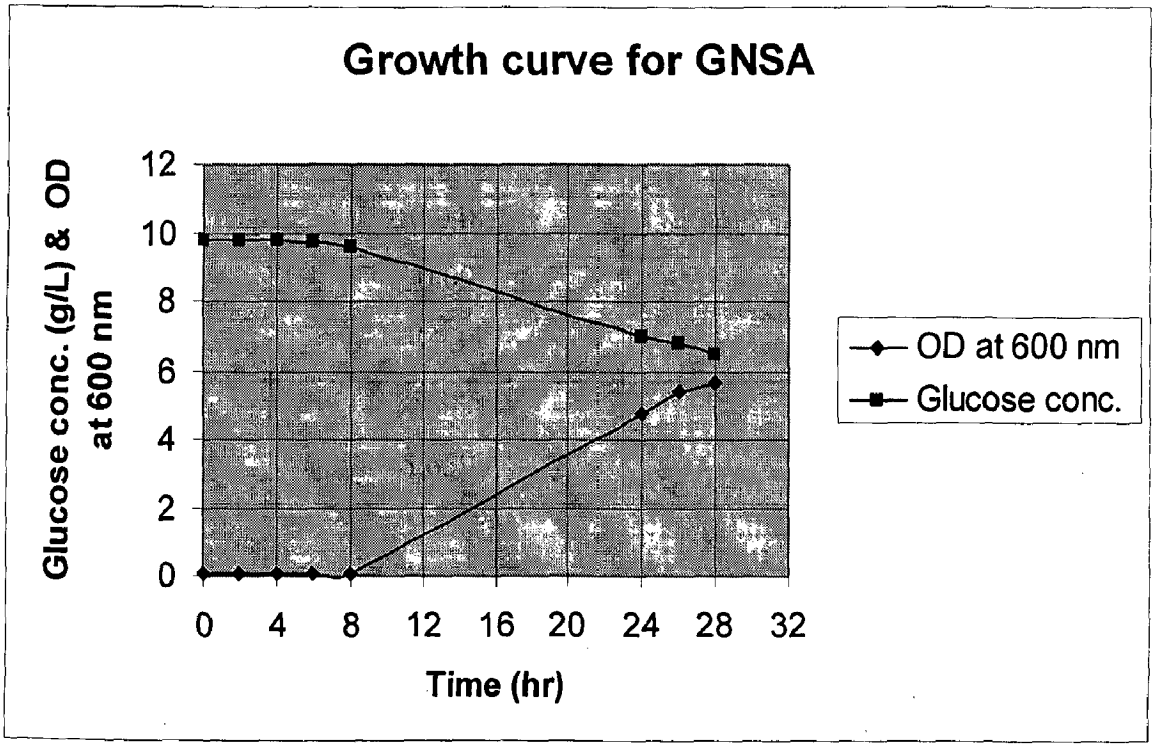


Fig-5.1. Glucose consumption and absorbance at 600 nm with time for the growth of GNSA at 60°C.

➢ Growth of IIPE453 was studied in H_2 medium. The absorbance at 600 nm and glucose concentration was observed with time shown in Table-5.3. Graph was plotted between glucose concentration and OD vs. time, shown in Fig-5.2.

Table-5.3. Absorbance and glucose concentration for the study of growth curve of IIPE453

Time (hr)	OD at 600 nm	Glucose concentration (g/L)
0	0.034	9.6
2	0.036	9.6
4	0.05	9.5
6	0.11	9.4
8	0.333	9.2
24	9.096	4.2
26	10.02	4.0
28	10.26	3.6

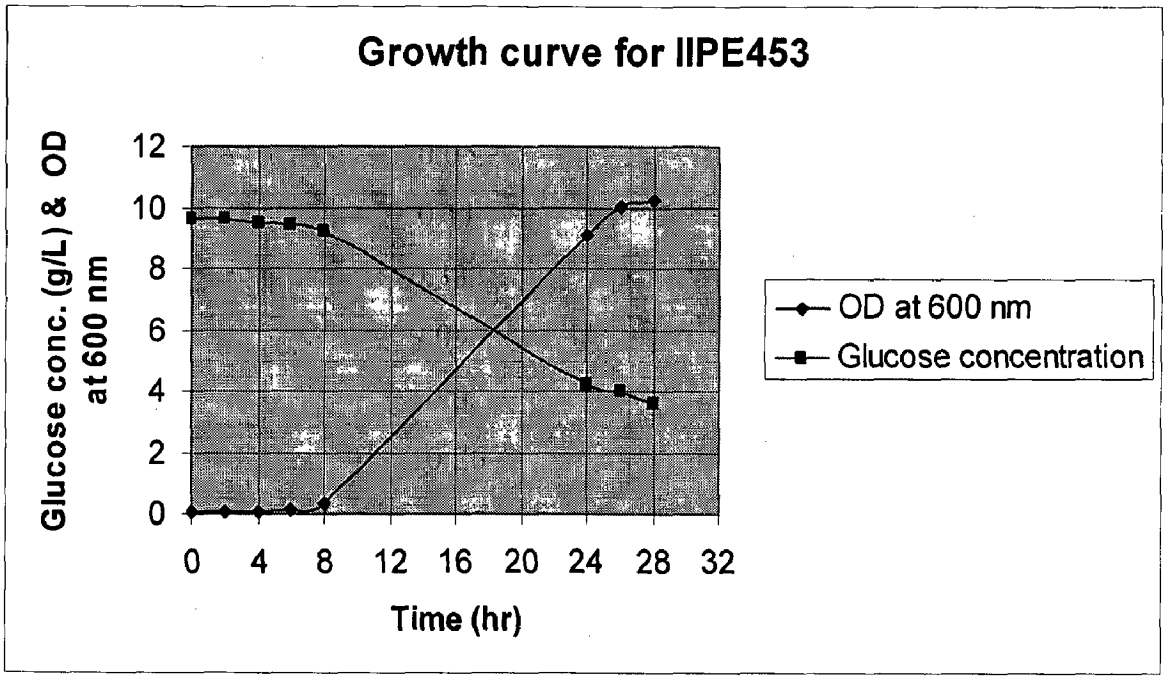


Fig-5.2. Glucose consumption and absorbance at 600 nm with time for the growth of IIPE453 at 45°C.

Strain IIPE453 was grown faster than GNSA was observed from the above two figures (i.e., Fig-5.1 and Fig-5.2).

5.2. Acid hydrolysis:

Acid hydrolysis of bagasse was carried out at three residence times. Acid hydrolysis was conducted with fresh bagasse is called 1st hydrolysis and with residual bagasse (i.e., bagasse from 1st hydrolysis) was called 2nd hydrolysis.

Observed the conversion of bagasse to glucose and furfural with residence time and type of hydrolysis was shown in Table-5.4. Graphs were plotted between glucose concentrations vs. time for both hydrolysis in Fig-5.3 and furfural concentrations vs. time for both hydrolysis in Fig-5.4.

Table-5.4. Glucose and furfural concentrations from type of hydrolysis and different residence times

Time (min)	1st Hydrolysis		2nd Hydrolysis	
	Glucose conc. (g/L)	Furfural conc. (mg/L)	Glucose conc. (g/L)	Furfural conc. (mg/L)
60	19	493.217	9.375	244.145
90	23	546.088	11.725	319.085
120	28.125	560.07	14.75	360.045

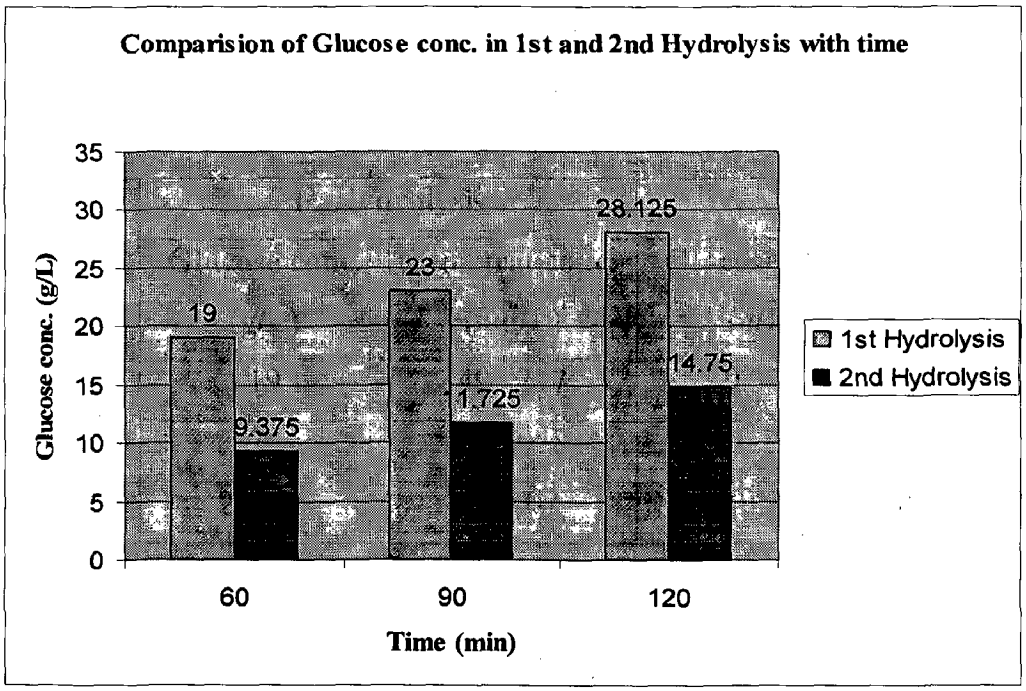


Fig-5.3. Comparision of Glucose conc. in 1st and 2nd Hydrolysis with time

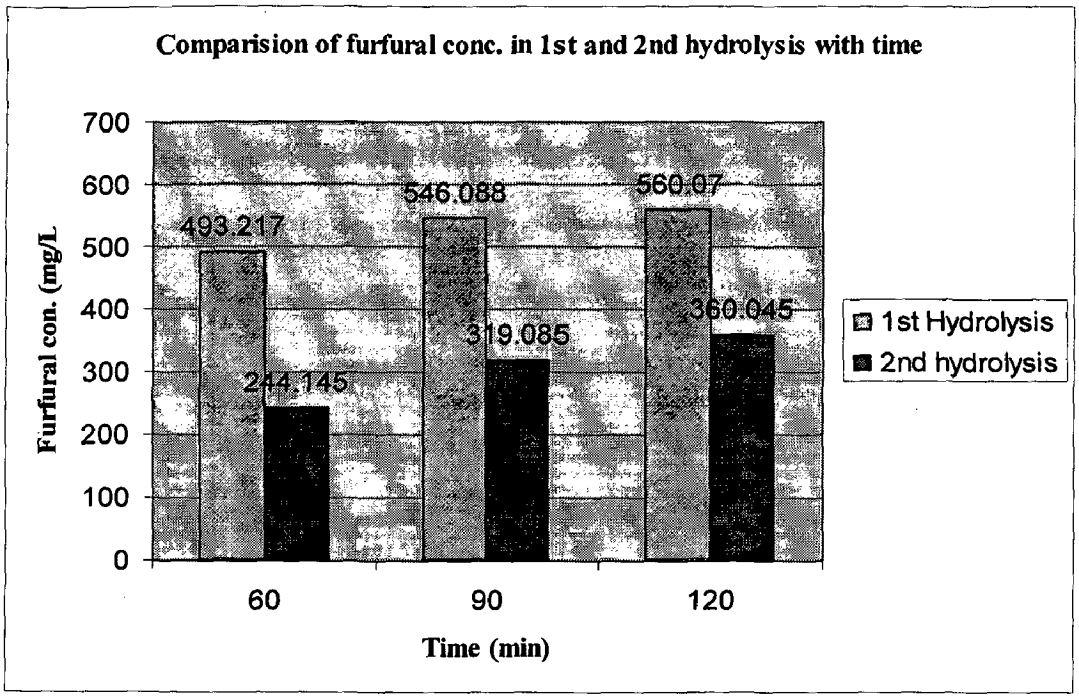


Fig-5.4. Comparision of furfural conc. in 1st and 2nd Hydrolysis with time

From the Fig-5.3, it was observed that more amount of glucose obtained from 1st hydrolysis rather than 2nd hydrolysis. In 1st hydrolysis glucose concentration was nearly twice that of 2nd hydrolysis. But furfural concentration was also nearly doubled in 1st hydrolysis rather than 2nd hydrolysis, was observed from Fig-5.4.

From the Fig-5.3, glucose concentration was increased with time of operation. Highest glucose concentration (i.e., 28.125 g/L) was obtained in the 1st acid hydrolysis and it was carried out for 120 min. This hydrolysate was used for fermentation.

5.2. Detoxification:

I was conducted five detoxification processes individually with the same amount of 1st hydrolysate was used in each process. Glucose, furfural concentrations and % removal of glucose and furfural of these processes were estimated, shown in Table-5.5.

From Table-5.5, it was observed that hydrolysate passing through adsorption column with activated carbon was removed 92.30 % of furfural. But the problem occurred from this process that it removed 82.30 % of glucose. In fermentation point of

view, we require high glucose concentration. So this process is not feasible than other processes for fermentation.

Table-5.5. Glucose and furfural concentrations along with % removal in hydrolysate after detoxification processes

Detoxification Process	Glucose conc. (g/L)	Furfural conc.(mg/L)	% Removal of glucose	% Removal of furfural
Hydrolysate before detoxification process	19	493.217	0	0
30 ml of hydrolysate was agitated with 0.3 g of activated carbon.	12.42	131.45	34.63	73.35
30 ml of hydrolysate was passed through adsorption column with activated carbon.	3.42	37.94	82.3	92.30
30 ml of hydrolysate was passed through adsorption column with silica gel.	11.64	85.725	38.74	82.62
30 ml of hydrolysate was passed through adsorption column with bauxite.	10.5	77.15	44.73	84.36
30 ml of hydrolysate was extracted with 10 ml of ethyl acetate.	18.06	120.015	4.95	75.67

Extraction with ethyl acetate was removed 75.67 % of furfural, and only 4.95 % of glucose. This process was better than other processes in fermentation point of view.

The remaining processes were removed 75 to 85 % of furfural and 35 to 45 % of glucose. These processes are not that much feasible to fermentation.

I was conducted the following detoxification processes in sequential order with 1st hydrolysate. The order of the processes and glucose, furfural concentrations were reported in Table-5.6.

Table-5.6. Glucose and furfural concentrations in hydrolysate after mixed detoxification processes

Process No.	Detoxification Process	Glucose conc. (g/L)	Furfural conc.(mg/L)	% Removal of glucose	% Removal of furfural
0	Hydrolysate before detoxification process	19	493.217	0	0
1	Hydrolysate was passed through adsorption column with bauxite.	10.5	77.15	44.74	84.36
2	Hydrolysate from bauxite bed was extracted with ethyl acetate	8.25	44.577	56.58	90.96
3	Hydrolysate from extraction was agitated with activated carbon	4.2	3.429	77.89	99.30

Three times extractions of hydrolysate were carried out with ethyl acetate. Glucose and furfural concentrations were shown in Table-5.7. Graphs were plotted between % removal of glucose vs. process no. for mixed detoxification processes and extraction processes in Fig-5.5, and % removal of furfural vs. process no. for mixed detoxification processes and extraction processes in Fig-5.6.

Table-5.7. Glucose and furfural concentrations in hydrolysate after extraction processes

Process No.	Detoxification Process	Glucose conc. (g/L)	Furfural conc.(mg/L)	% Removal of glucose	% Removal of furfural
0	Hydrolysate before detoxification process	28.125	560.07	0	0
1	1st time extraction with ethyl acetate	27.25	137.16	3.11	75.51
2	2nd time extraction with ethyl acetate	26.375	82.296	6.22	85.31
3	3rd time extraction with ethyl acetate	25.5	50.29	9.34	91.02

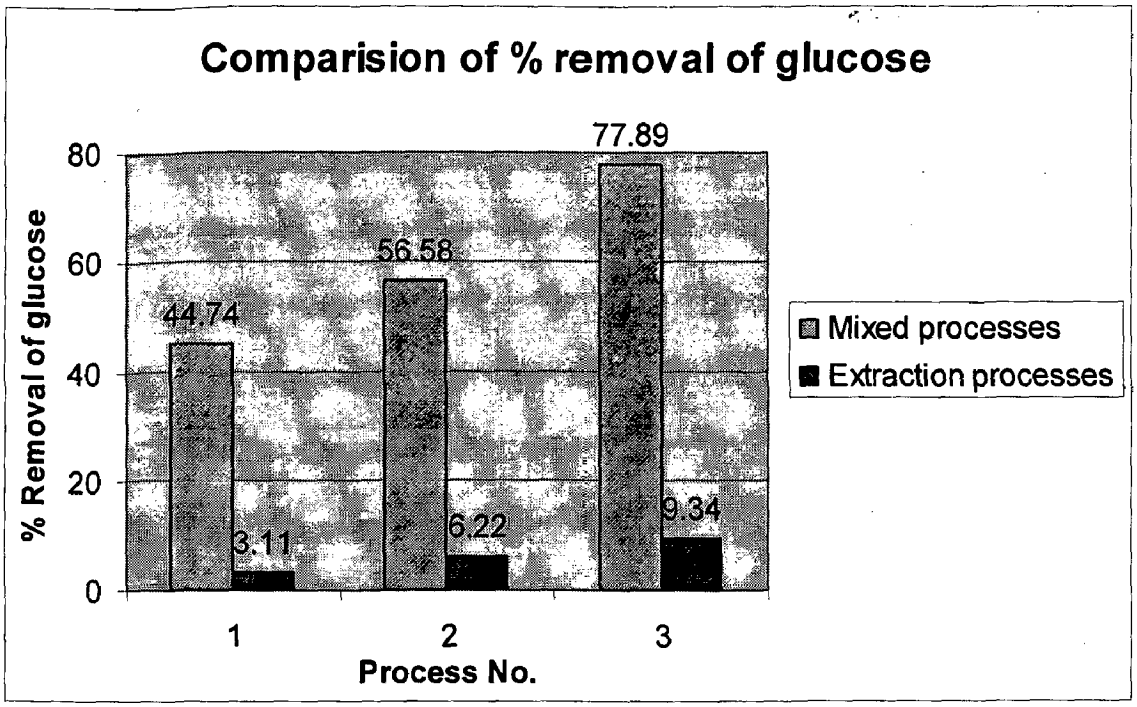


Fig-5.5. Comparision of % removal of glucose in mixed and extraction processes

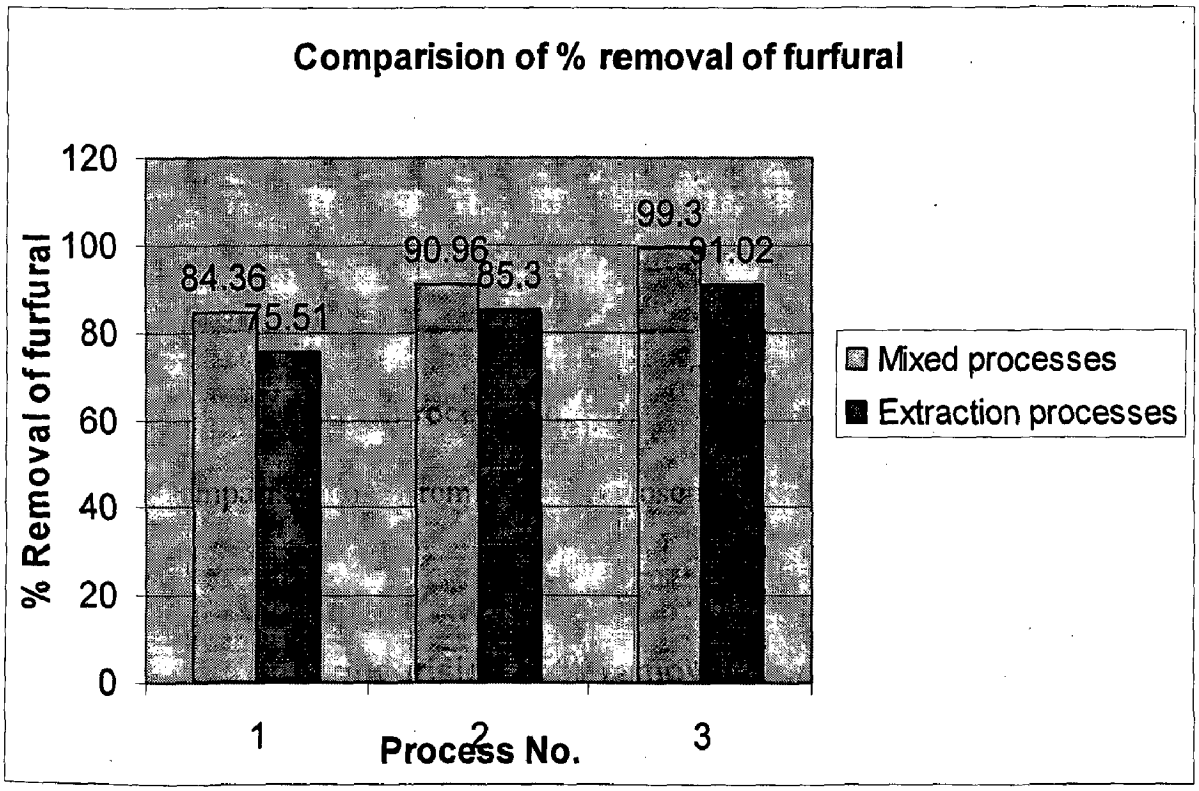


Fig-5.6. Comparision of % removal of furfural in mixed and extraction processes

From Fig-5.5, it was observed that more amount of glucose was removed in mixed detoxification processes as compared to extraction processes. From Fig-5.6, the removal of furfural was comparative. Three times extractions of hydrolysate with ethyl acetate were better process for the effective removal of furfural with less removal of glucose as compared to mixed detoxification processes. Hydrolysate was obtained from three times extractions of hydrolysate with ethyl acetate were further used for fermentation.

5.3. Fermentation:

Two fermentations for the production of ethanol were carried out with two different stains, one with GNSA strain and another with IIPE453 strain.

➢ Fermentation of ethanol with bagasse hydrolysate was performed by GNSA strain in batch mode. Glucose consumed, ethanol conc., dry cell weight, pH, product yield and productivity were observed with different time intervals, shown in Table-5.8. The process was carried out for 120 hrs at 60°C. Graph was plotted between glucose consumed, ethanol produced, pH and dry cell weight vs. time, shown in Fig-5.7.

Table-5.8. Batch fermentation with GNSA strain showing glucose consumption, ethanol production, DCW, pH, product yield and productivity with time

Time (hr)	Glucose consumed (g/L)	Ethanol produced (g/L)	Dry Cell Weight (g/L)	pH	Product Yield ($Y_{P/S}$)*100	Productivity (g/L-hr)
0	0	0	1.8	7.0	0	0
6	1.25	0	1.8	7.0	0	0
25	2.625	0.502	2.0	6.5	13.84	0.02
53	3.75	0.7868	2.2	5.5	16.56	0.0148
72	5.0	0.543	2.4	5.0	10.86	0.0075
96	5.625	0.9314	2.6	5.0	16.55	0.0097
102	6.125	0.592	2.7	5.0	9.67	0.0058
120	6.5	1.08542	2.7	5.0	16.7	0.009

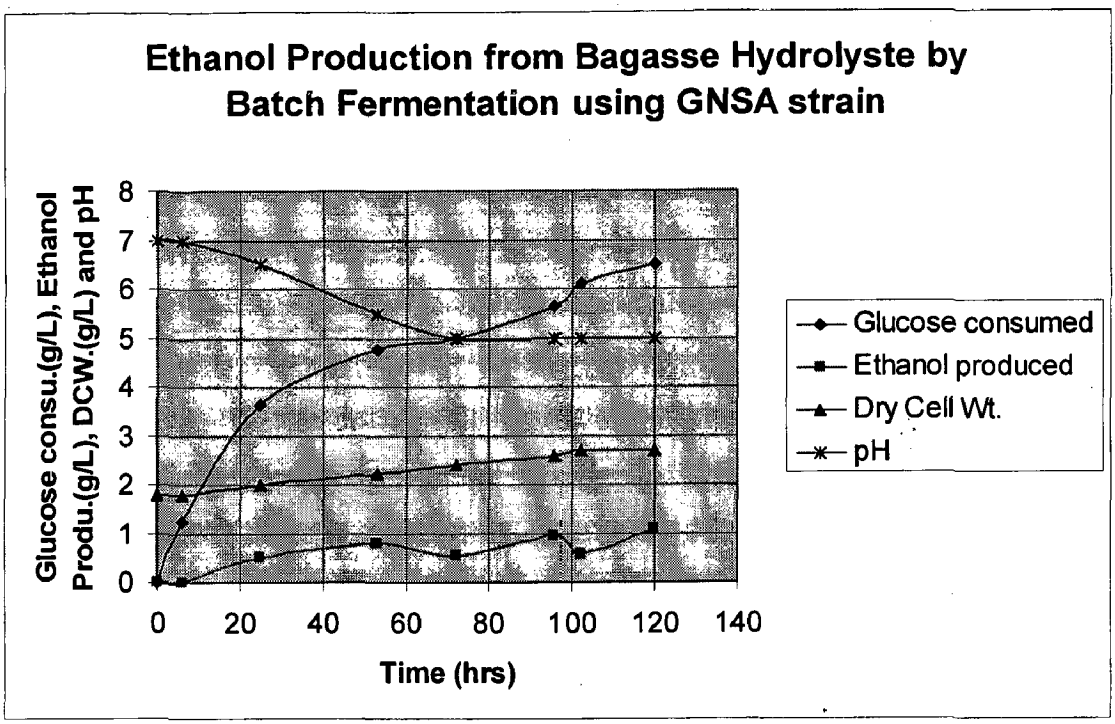


Fig-5.7. Glucose consumption, Ethanol production and Dry Cell Wt. and pH at different time intervals in Batch Fermentation with GNSA strain

➢ Fermentation of ethanol with bagasse hydrolysate was performed by IIPE453 strain in batch mode. Glucose consumed, ethanol conc., dry cell weight, pH, CO_2, product yield and productivity were observed with different time intervals, shown in Table-5.9. The process was carried out for 8 hrs at 45°C. Graph was plotted between glucose consumed, ethanol produced, pH and dry cell weight vs. time, shown in Fig-5.8.

Table-5.9. Batch fermentation with IIPE453 strain showing glucose consumption, ethanol production, DCW, pH, product yield and productivity with time

Time (hr)	Glucose consumed (g/L)	Ethanol produced (g/L)	Dry Cell Weight (g/L)	pH	CO_2 Produced (mg/L)	Product Yield ($Y_{P/S}$)*100	Productivity (g/L-hr)
0	0	0	5.5	6.0	-	0	0
2	5	1.028	5.5	5.5	-	20.56	0.514
4	11.25	2.370	5.6	5	-	21.076	0.5925
6	20.55	4.44	5.7	5	148.5	21.6	0.74
8	22.30	4.50	5.6	5	-	20.18	0.5625

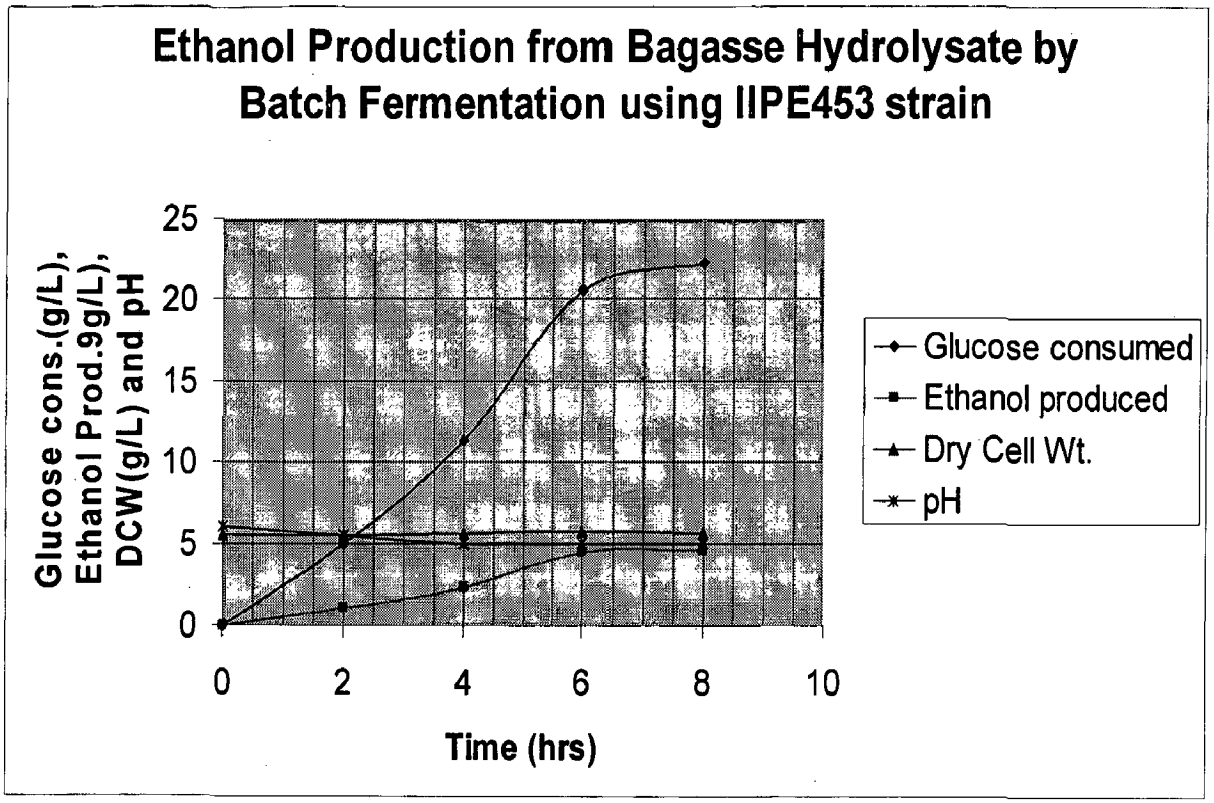


Fig-5.8. Glucose consumption, Ethanol production and Dry Cell Wt. and pH at different time intervals in Batch Fermentation with IIPE453 strain.

From the Fig-5.7, it was observed that till to 53 hrs ethanol produced gradually, but after 53 hrs the production of ethanol was cyclic (i.e., production of ethanol was gradually decreased and then increased). Batch fermentation with GNSA strain to produce ethanol from bagasse hydrolysate was taken more time to complete consumption of glucose rather than IIPE453 strain. Using IIPE453 strain, it was taken only 8 hrs to 90% consumption of glucose. From the Fig-5.8, it was observed that the production of ethanol was increased gradually.

From the above data, it was observed that strain IIPE453 was better than strain GNSA to produce ethanol from bagasse hydrolysate by batch fermentation.

CHAPTER-6 Conclusions and Recommendations

Conclusions:

Experimental study of Biocatalytic conversion of biomass to biofuels (i.e., ethanol) was carried out in two steps. In 1st step, it was acid hydrolysis was conducted with 2% H_2SO_4 solution and in 2nd step, it was fermentation was conducted in batch mode.

From the experiments it was observed that,

1. In 1st acid hydrolysis nearly double amount of glucose was obtained rather than 2nd acid hydrolysis for the same residence time.

2. In acid hydrolysis's, residence time of 120 min was better than others (i.e., residence time of 60 min and 90 min) due to high glucose concentration was obtained.

3. Maximum glucose concentration of 28.125 g/L was obtained in 1st hydrolysis with the residence time of 120 min.

4. Three times extractions of hydrolysate carried out with ethyl acetate was better detoxification process to remove furfural with less removal of glucose, because it removes 9.34% glucose and 91.02% of furfural. The remaining processes were removed more than 9.34% glucose.

5. Fermentation with IIPE453 strain was better than GNSA strain, because IIPE453 was given highest product yield of 21.6% and productivity of 0.74 g/L-hr at 6 hrs. IIPE453 was taken only 8 hrs to 90% consumption of glucose.

Recommendations:

Following are the recommendations for the future study,

- Strain DNA characterization is required to know the name of the strain.
- Acid hydrolysis can be conducted with other acid concentrations (i.e., other than 2%).
- Detoxification processes can be conducted with ion exchange resins to remove the furfural.
- Fermentation can be conducted with other strains to check the performance of those strains to produce ethanol from biomass.
- Continuous fermentation can be conducted.
- Recovery of ethanol from fermentation broth is required to get pure ethanol with high concentration by extractive distillation.
- Kinetic study of ethanol fermentation can be conducted.

- **Estimation of glucose concentration for the study of growth curve of GNSA**

Time (hrs)	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc. (g/L)
0	0.908	0.327	30	9.8
2	0.908	0.327	30	9.8
4	0.907	0.327	30	9.8
6	0.887	0.324	30	9.7
8	0.875	0.32	30	9.6
24	0.602	0.234	30	7.0
26	0.576	0.227	30	6.8
28	0.543	0.217	30	6.5

Amount of H_1 medium-1 was taken = 50 ml
Temperature = 60°C
Speed of the incubator = 138 rpm

- **Estimation of glucose concentration for the study of growth curve of IIPE453**

Time (hrs)	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc. (g/L)
0	0.875	0.32	30	9.6
2	0.874	0.32	30	9.6
4	0.863	0.317	30	9.5
6	0.851	0.313	30	9.4
8	0.827	0.307	30	9.2
24	0.312	0.14	30	4.2
26	0.277	0.1335	30	4.0
28	0.226	0.12	30	3.6

Amount of salt medium-1 was taken = 50 ml
Temperature = 45°C
Speed of the incubator = 136 rpm

- **Estimation of glucose concentrations in acid hydrolysis**

Type of hydrolysis	Time (min)	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc. (g/L)
1st hydrolysis	60	0.899	0.317	60	19
	90	1.328	0.46	50	23
	120	0.749	0.28125	100	28.125
2nd hydrolysis	60	0.376	0.1562	60	9.375
	90	0.604	0.2345	50	11.725
	120	0.327	0.1475	100	14.74

Amount of bagasse was taken = 100 g
Vol. of dilute acid solution = 2 L

- **Estimation of furfural concentrations in acid hydrolysis**

Type of hydrolysis	Time (min)	OD at 277 nm	Furfural conc. (%v/v)	Dilution Factor	Furfural conc.	
					%v/v	mg/L
1st hydrolysis	60	0.353	$14.38*10^{-5}$	300	0.04315	493.217
	90	0.362	$15.93*10^{-5}$	300	0.0478	546.088
	120	1.125	$49*10^{-5}$	100	0.049	560.07
2nd hydrolysis	60	0.162	$7.12*10^{-5}$	300	0.02136	244.145
	90	0.213	$9.3*10^{-5}$	300	0.0279	319.085
	120	0.725	$31.5*10^{-5}$	100	0.0315	360.045

Amount of bagasse was taken = 100 g
Vol. of dilute acid solution = 2 L

- **Estimation of glucose concentration in hydrolysate after detoxification process**

Detoxification Process	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc.(g/L)
Hydrolysate before detoxification process	0.899	0.317	60	19
30 ml of hydrolysate was agitated with 0.3 g of activated carbon.	0.513	0.207	60	12.42
30 ml of hydrolysate was passed through adsorption column with activated carbon.	0.201	0.108	30	3.24
30 ml of hydrolysate was passed through adsorption column with silica gel.	0.475	0.194	60	11.64
30 ml of hydrolysate was passed through adsorption column with bauxite.	0.413	0.175	60	10.5
30 ml of hydrolysate was extracted with 10 ml of ethyl acetate.	0.824	0.301	60	18.06

1st hydrolysate of 60 min residence time was used for detoxification processes.

- **Estimation of furfural concentration in hydrolysate after detoxification process**

Detoxification Process	OD at 277 nm	Furfural conc. (%v/v)	Dilution Factor	Furfural conc.	
				%v/v	mg/L
Hydrolysate before detoxification process	0.982	$43.2*10^{-5}$	100	0.04315	493.217
30 ml of hydrolysate was agitated with 0.3 g of activated carbon.	0.263	$11.5*10^{-5}$	100	0.0115	131.45
30 ml of hydrolysate was passed through adsorption column with activated carbon.	0.081	$3.32*10^{-5}$	100	0.00332	37.94
30 ml of hydrolysate was passed through adsorption column with silica gel.	0.171	$7.5*10^{-5}$	100	0.0075	85.725
30 ml of hydrolysate was passed through adsorption column with bauxite.	0.157	$6.75*10^{-5}$	100	0.00675	77.15
30 ml of hydrolysate was extracted with 10 ml of ethyl acetate.	0.240	$10.5*10^{-5}$	100	0.0105	120.015

1st hydrolysate of 60 min residence time was used for detoxification processes.

- **Estimation of glucose concentration in hydrolysate after mixed detoxification processes**

Detoxification Process	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc.(g/L)
Hydrolysate before detoxification process	0.899	0.317	60	19
Hydrolysate was passed through adsorption column with bauxite.	0.413	0.175	60	10.50
Hydrolysate from bauxite bed was extracted with ethyl acetate	0.725	0.275	30	8.25
Hydrolysate from extraction was agitated with activated carbon	0.307	0.14	30	4.2

- **Estimation of furfural concentration in hydrolysate after mixed detoxification processes**

Detoxification Process	OD at 277 nm	Furfural conc. (%v/v)	Dilution Factor	Furfural conc.	
				%v/v	mg/L
Hydrolysate before detoxification process	0.982	$43.2*10^{-5}$	100	0.04315	493.217
Hydrolysate was passed through adsorption column with bauxite.	0.157	$6.75*10^{-5}$	100	0.00675	77.15
Hydrolysate from bauxite bed was extracted with ethyl acetate	0.302	$13*10^{-5}$	30	0.0039	44.577
Hydrolysate from extraction was agitated with activated carbon	0.028	$1*10^{-5}$	30	0.003	3.429

1st hydrolysate of 60 min residence time was used for detoxification processes.

- **Estimation of glucose concentration in hydrolysate after extraction processes**

Detoxification Process	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc.(g/L)
Hydrolysate before detoxification process	0.749	0.28125	100	28.125
1st time extraction with ethyl acetate	0.725	0.2725	100	27.25
2nd time extraction with ethyl acetate	0.703	0.26375	100	26.375
3rd time extraction with ethyl acetate	0.678	0.255	100	25.5

- **Estimation of furfural concentration in hydrolysate after extraction processes**

Detoxification Process	OD at 277 nm	Furfural conc. (%v/v)	Dilution Factor	Furfural conc.	
				%v/v	mg/L
Hydrolysate before detoxification process	1.125	$49*10^{-5}$	100	0.049	560.07
1st time extraction with ethyl acetate	0.27	$12*10^{-5}$	100	0.012	137.16
2nd time extraction with ethyl acetate	0.166	$7.2*10^{-5}$	100	0.0072	82.296
3rd time extraction with ethyl acetate	0.097	$4.4*10^{-5}$	100	0.0044	50.29

1st hydrolysate of 120 min residence time was used for extraction processes.

- **Estimation of glucose concentration in fermentation broth, the fermentation was conducted with GNSA strain**

Time (hrs)	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc.(g/L)
0	0.613	0.2375	100	23.75
6	0.575	0.225	100	22.5
25	0.531	0.20125	100	20.125
53	0.465	0.19	100	19
72	0.454	0.1875	100	18.75
96	0.443	0.18125	100	18.125
102	0.801	0.29375	60	17.625
120	0.775	0.2875	60	17.25

- **Estimation of ethanol concentration in fermentation broth, the fermentation was conducted with GNSA strain**

Time (hrs)	OD at 585 nm	Ethanol concentration	
		%v/v	g/L
0	-	-	-
6	-	-	-
25	0.027	0.065	0.502
53	0.030	0.100	0.7868
72	0.028	0.06875	0.543
96	0.031	0.11875	0.9314
102	0.029	0.075	0.592
120	0.039	0.1375	1.08542

- **Estimation of Dry Cell Weight in fermentation broth, the fermentation was conducted with GNSA strain**

Time (hrs)	Wt. of empty dry eppendorf tube (g)	Wt. of eppendorf tube with dry cell mass (g)	Dry Cell Weight	
			g/ml	g/L
0	1.0499	1.0517	0.0018	1.8
6	1.0459	1.0477	0.0018	1.8
25	1.0488	1.0508	0.002	2
53	1.0429	1.0451	0.0022	2.2
72	1.0416	1.0442	0.0024	2.4
96	1.0674	1.07	0.0026	2.6
102	1.041	1.0437	0.0027	2.7
120	1.0447	1.0474	0.0027	2.7

- **Estimation of glucose concentration in fermentation broth, the fermentation was conducted with IIPE453 strain**

Time (hrs)	OD at 575 nm	Glucose conc. (g/L)	Dilution Factor	Glucose conc. (g/L)
0	0.655	0.25	100	25
2	0.488	0.20	100	20
4	0.296	0.1375	100	13.75
6	0.568	0.2225	20	4.45
8	0.719	0.26875	10	2.6875

- **Estimation of ethanol concentration in fermentation broth, the fermentation was conducted with IIPE453 strain**

Time (hrs)	OD at 584 nm	Ethanol concentration	
		%v/v	g/L
0	0	0	0
2	0.051	0.1375	1.028
4	0.10	0.3	2.37
6	0.193	0.5625	4.44
8	0.199	0.569	4.5

- **Estimation of Dry Cell Weight in fermentation broth, the fermentation was conducted with IIPE453**

Time (hrs)	Wt. of empty dry eppendorf tube (g)	Wt. of eppendorf tube with dry cell mass (g)	Dry Cell Weight	
			g/ml	g/L
0	1.0425	1.0480	0.0055	5.5
2	1.0487	1.0542	0.0055	5.5
4	1.0416	1.0472	0.0056	5.6
6	1.0462	1.0519	0.0057	5.7
8	1.4023	1.4079	0.0056	5.6

- **Estimation of CO_2, the fermentation was conducted with IIPE453**

Vol. of HCl to treat fresh $Ba(OH)_2$ solution = 71 ml

Vol. of HCl to treat $Ba(OH)_2$ solution after 6 hrs = 57.5 ml

Amount of CO_2 liberated = 0.5*(71-57.5)*44/2

= 148.5 mg

The following are the media compositions used for growth of the cells and for fermentation.

H_1 medium:

It was used for the growth of GNSA strain. Medium composition was shown in below table.

Material	Quantity
Distilled Water	1 L
Glucose	10 g
NH_4Cl	1 g
NaCl	1 g
KH_2PO_4	6 g
Trace Elements	20 ml
Yeast Extract	10 mg

H_2 medium:

It was used for the growth of IIPE453 strain. Medium composition was shown in below table.

Material	Quantity
Distilled Water	1 L
Glucose	10 g
$(NH_4)_2SO_4$	1 g
Na_2HPO_4	0.15 g
KH_2PO_4	0.15 g
Yeast Extract	1 g

H_3 medium:

It was used for fermentation with GNSA strain. Medium composition was shown in below table.

Material	Quantity
Bagasse Hydrolysate	1 L
NH_4Cl	1 g
NaCl	1 g
KH_2PO_4	6 g
Trace Elements	20 ml
Yeast extract	10 mg

H_4 medium:

It was used for fermentation with IIPE453 strain. Medium composition was shown in below table.

Material	Quantity
Bagasse Hydrolysate	1 L
$(NH_4)_2SO_4$	1 g
Na_2HPO_4	0.15 g
KH_2PO_4	0.15 g
Yeast Extract	1 g

Trace Elements Composition:

Trace elements composition was shown in below table.

Material	Quantity
Distilled Water	1 L
EDTA	5 g
$MgSO_4.7H_2O$	14.15 g
$CaCl_2.2H_2O$	3.3 g
$(NH_4)_6Mo_7O_{24}.24H_2O$	9.25 mg
$FeSO_4.7H_2O$	99
Metal 44	50 ml

Metal 44 Composition:

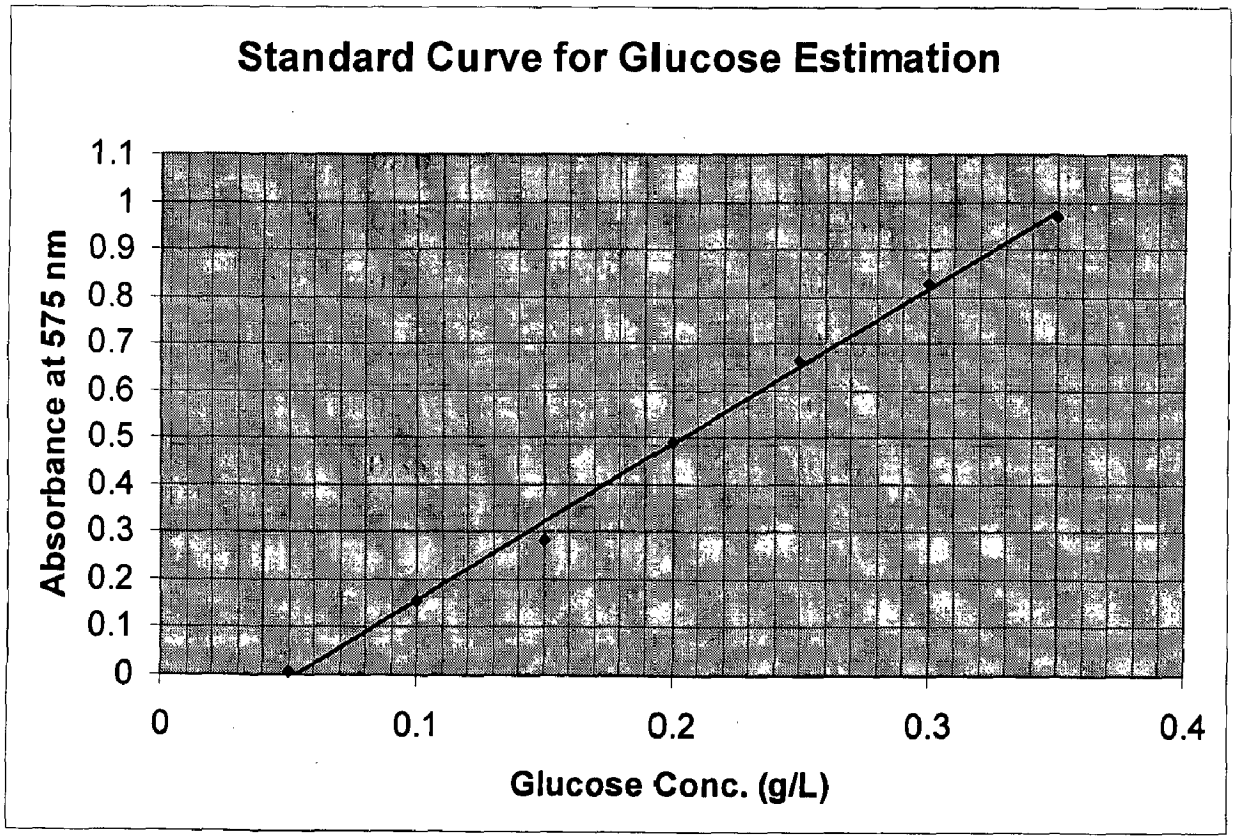
Metal 44 composition was shown in below table.

Material	Quantity
Distilled Water	1 L
EDTA	2.5 g
$ZnSO_4.7H_2O$	10.95 g
$FeSO_4.7H_2O$	5 g
$MnSO_4.H_2O$	1.54 g
$CuCl_2.5H_2O$	0.39 g
$CoCl_2.6H_2O$	0.21 g
H_3BO_3	0.11 g

Appendix-C Standard Curve for Glucose Estimation

Standard curve for glucose estimation was used to determine the reducing sugars in samples. The data for the standard curve was shown in below table.

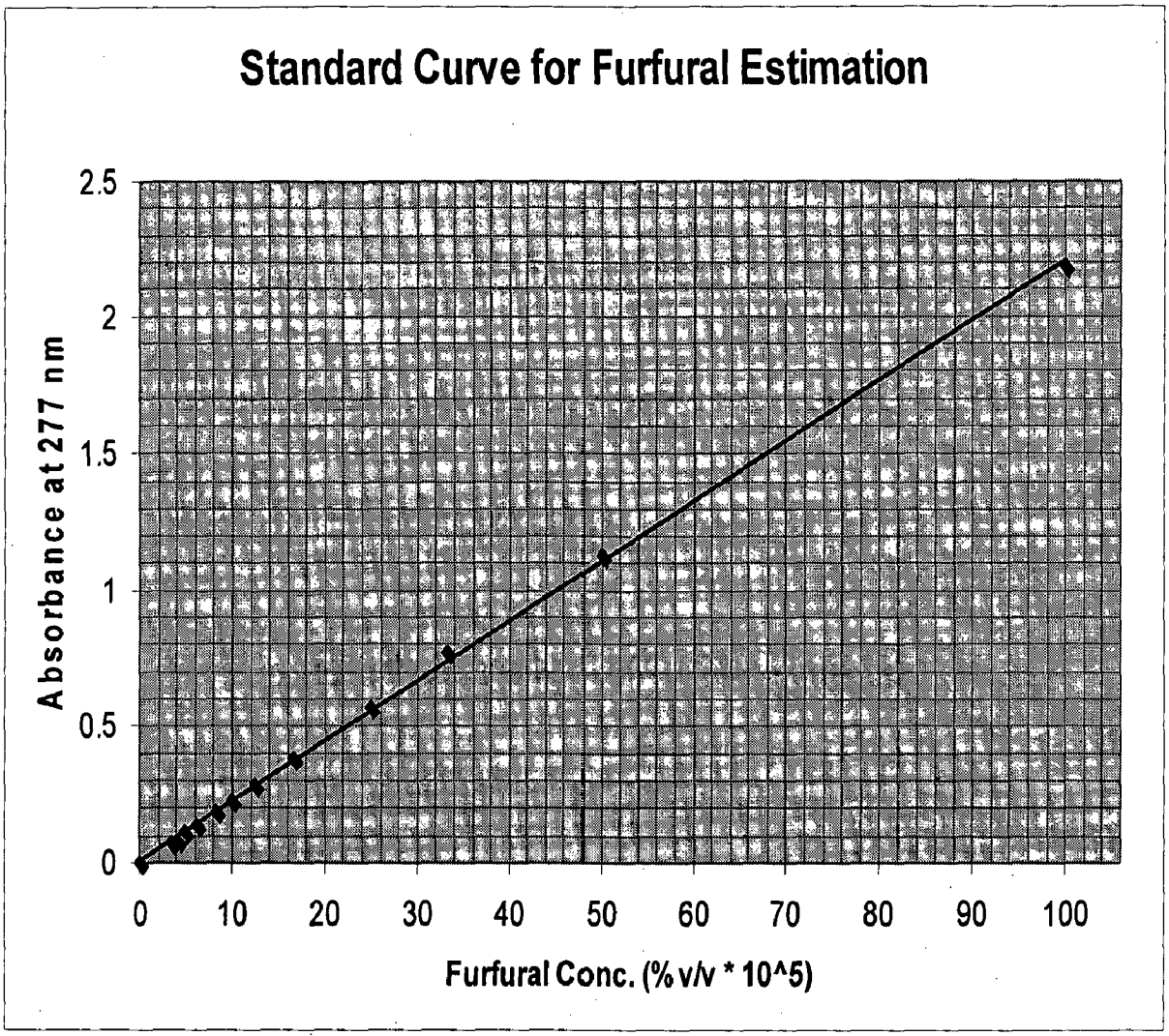
Glucose conc. (g/L)	OD at 575 nm
0.05	0.009
0.10	0.156
0.15	0.283
0.20	0.490
0.25	0.663
0.30	0.827
0.35	0.970



Appendix-D Standard Curve for Furfural Estimation

Standard curve for furfural estimation was used to determine the furfural concentrations in samples. The data for the standard curve was shown in below table.

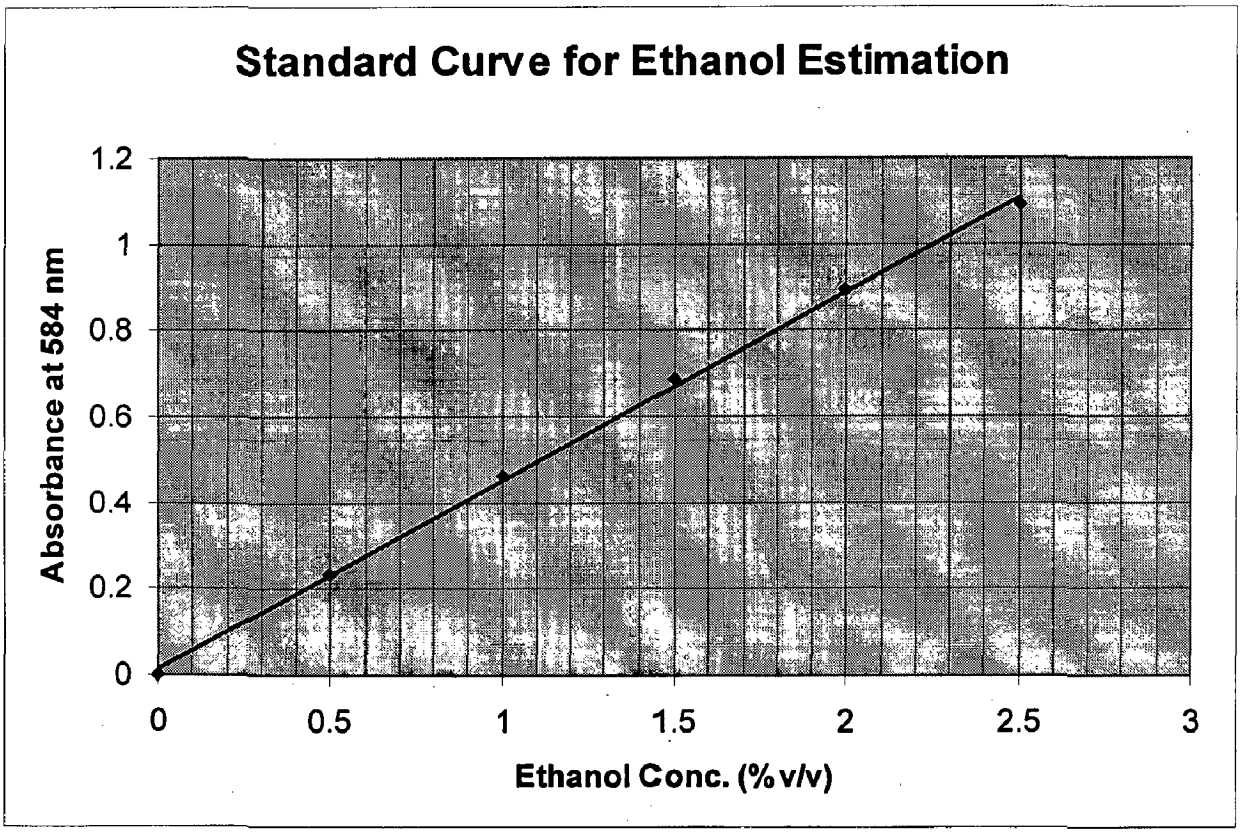
Furfural Conc. (%v/v * 10^5)	OD at 277 nm
0	0
3.33	0.074
4.17	0.085
5.0	0.115
6.25	0.142
8.33	0.188
10.0	0.228
12.5	0.285
16.67	0.381
25.0	0.571
33.33	0.768
50.0	1.131
100.0	2.183


Standard Curve for Furfural Estimation
Absorbance at 277 nm
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
100
Furfural Conc. (% v/v * 10^5)

Appendix-E Standard Curve for Ethanol Estimation

Standard curve for ethanol estimation was used to determine the ethanol concentrations in fermentation broths. The data for the standard curve was shown in below table.

Ethanol Conc. (%v/v)	OD at 584 nm
0	0
0.5	0.232
1.0	0.464
1.5	0.686
2.0	0.892
2.5	1.089



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BIOCATALYTIC CONVERSION OF BIOMASS TO BIOFUELS

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Abstract

In this experimental work sugarcane bagasse was used as biomass to get ethanol as the biofuel. The biological process of ethanol production using bagasse as substrate requires: Pretreatment and Fermentation. Acid hydrolysis was conducted as pretreatment of bagasse with 2% H_2SO_4 solution at residence times of 60, 90 and 120 min. 28.0125 g/L of glucose and 560.07 mg/L of furfural were obtained in 1st hydrolysis of bagasse at 120 min residence time. Several detoxification processes were used to reduce the furfural concentration. Three times extraction of bagasse hydrolysate with ethyl acetate was carried out, and it removes 91.02 % of furfural with less removal of glucose (9.34%). Batch fermentation was carried out with two strains, one was GNSA strain and another was IIPE453 strain. The highest product yield of 21.6% was obtained with IIPE453 strain at 6 hrs operation.

1. Introduction

The world's energy requirement is being fulfilled by fossil fuels which serve as a primary energy source. Fossil fuels as the main energy sources have led to serious environmental problems, i.e. combustion of fossil fuel produces greenhouse and toxic gases, such as CO_2, SO_2, NO_x and other pollutants, causing global warming and acid rain [4]. In the future, our energy systems will need to be renewable and sustainable, efficient and cost-effective, convenient and safe [3]. Biomass and other renewable energy sources are receiving worldwide attention for several reasons. These include the increasing concern about potential global climate change and other environmental consequences of the growing consumption of fossil fuels, the desire to develop sustainable energy sources, decreasing dependence on foreign source of oil, improving balance of payment, and decreasing the rate of depletion of the fossil fuel reserves. Biomass provides about 15% of the world's primary energy, equivalent to 25 million barrels of oil per day, and is the major fuel used in most developing countries [1]

Bagasse was taken from outside and chopped into small pieces (nearly 5mm in size). Bagasse pieces was boiled gently in warm water to remove the sugars and dried in oven. 100 g of dried bagasse was taken and mixed with 2 L of 2 % (v/v) H_2SO_4 solution (i.e., S: L = 1: 20). Bagasse and acid mixture was set a side for 30 min to soaking, and the mixture was kept in an autoclave at 15 psi and 121°C for residence time of 60 min. After 60 min the mixture was take it outside and kept for cooling. The hydrolysate from residual bagasse was separated by filtration. Hydrolysate was neutralized with $Ca(OH)_2$ and formed $CaSO_4$ was removed by filtration. Sample of hydrolysate was analyzed for estimation of glucose and furfural. The same process was conducted with residual bagasse from the above hydrolysis as the feed and fresh 2 L of 2 % (v/v) H_2SO_4. Sample of hydrolysate was analyzed for estimation of glucose and furfural. The above procedure was repeated with fresh dried bagasse for resident times of 90 min and 120 min.

2.1.2. Detoxification

Some inhibitors formed during acid hydrolysis which is toxic for the growth of microbes, mainly furfural. The process which is removing these inhibitors is called detoxification.

In this experiment, I was conducted five types of detoxification processes. *1st process (Agitation along with activated carbon)*, 30 ml of 1st hydrolysate was mixed with 0.3 g of activated carbon in 100 ml conical flask and agitated for 1 hr. Then activated carbon was separated from hydrolysate by filtration. *2nd process (Passing through adsorption column with activated carbon)*, 10 cm height and 1 cm inner diameter of the adsorption column was prepared with activated carbon. 30 ml of 1st hydrolysate was passed through the adsorption column. *3rd process (Passing through adsorption column with silica gel)*, 10 cm height and 1 cm inner diameter of the adsorption column was prepared with silica gel. 30 ml of 1st hydrolysate was passed through the adsorption column. *4th process (Passing through adsorption column with bauxite)*, 10 cm height and 1 cm inner diameter of the adsorption column was prepared with bauxite. 30 ml of 1st hydrolysate was passed through the adsorption column. *5th process (Extraction with ethyl acetate)*, 30 ml of hydrolysate and 10 ml of ethyl acetate (in the ratio of 3:1) was taken into 250 ml conical flask, and extracted by agitation for 30 min. The mixture was

transferred into a separator funnel. The organic (ethyl acetate) and aqueous (hydrolysate) phases were separated. Air stripping was conducted through extracted hydrolysate phase to remove volatile components (i.e., ethyl acetate). Distillation was conducted to ethyl acetate phase to recover the pure ethyl acetate. Glucose and furfural concentrations were estimated in hydrolysate after detoxification process. I was conducted combination of two or three above mentioned processes to get the best result for the removal of furfural with less removal of fermentable sugars.

2.2. Fermentation

2.2.1. Microorganisms and culture conditions

Two fermentation experiments were conducted, one with GNSA strain and another with IIPE453 strain. Both strains were obtained from Biotechnology lab, Indian Institute of Petroleum, Dehradun.

GNSA strain was grown in medium containing 1 L distilled water, 10 g glucose, 10 mg yeast extract, 1 g NH_4Cl, 1 g NaCl, 6 g KH_2PO_4 and 20 ml trace elements. IIPE453 strain was grown in medium containing 1 L distilled water, 10 g glucose, 0.15 g Na_2HPO_4, 0.15 g KH_2PO_4, 1 g $(NH_4)_2SO_4$ and 1 g yeast extract.

600 ml of medium for GNSA strain was prepared in 1 L flask, and the medium was transferred into four 500 ml conical flasks with working volume of 150 ml each. Entire medium was sterilized in an autoclave. Sterilized medium was cooled to room temperature. GNSA strain was inoculated to medium in laminar hood chamber, and the medium was incubated in a rotating shaker at 60°C and 138 rpm for 24 hrs. Growth of GNSA strain was observed. Incubated medium was centrifuged at 10,000 rpm for 5 min to harvesting the cells. The same procedure was conducted to IIPE453 strain with incubated temperature of 45°C and 138 rpm for 24 hrs. Growth of IIPE453 strain was observed. Incubated medium was centrifuged at 10,000 rpm for 5 min to harvesting the cells.

2.2.2. Fermentation process

1 Liter jacketed three-neck flat bottom flask with a working volume of 500 ml was used as batch fermentor. 250 ml of fermentation medium, composition is 1 L bagasse

hydrolysate, 10 mg yeast extract, 1 g NH_4Cl, 1 g NaCl, 6 g KH_2PO_4 and 20 ml trace elements, was prepared and transferred into jacketed flask. 0.45 g of harvested GNSA strain was added into fermentation medium. The jacketed flask was stated on magnetic stirrer. The temperature was maintained by passing the water at 60°C. A condenser was used at middle neck of flask to condense vapors. The gases produced during fermentation was passed through 0.0625M $Ba(OH)_2$ to determine CO_2. A thermometer was stated in one neck of flask and another neck was used for sampling. Samples were taken at different time intervals and estimated glucose concentration, ethanol concentration, and dry cell weight.

The above process was repeated with IIPE453 strain. 450 ml of fermentation medium, composition is 1 L bagasse hydrolysate, 0.15 g Na_2HPO_4, 0.15 g KH_2PO_4, 1 g $(NH_4)_2SO_4$ and 1 g yeast extract, was prepared and transferred into jacketed flask. 2.475 g of harvested IIPE453 strain was added into fermentation medium. The temperature of the process was maintained by passing the water at 45°C. Samples were taken at different time intervals and estimated glucose concentration, ethanol concentration, and dry cell weight.

2.3. Analysis

2.3.1. Characterization of strains

Glass slide was taken, cleaned and dried. Small amount of smear was added on the slide, spread into a thin film and dried. Smear film was flooded with crystal violet for 1 min, smear was washed in a gentle and indirect stream of tap water for 2 sec. Smear was flooded with iodine mordant. Washed the smear in a gentle and indirect stream of tap water for 2 sec and then blotted the film dry with absorbent paper. Flooded the smear with 95 %v/v ethanol for 30 sec with agitation and then blotted the film dry with absorbent paper. Smear was flooded with safranin counter stain for 10 sec. The smear was washed with a gentle and indirect stream of tap water until no color appeared in the effluent and blotted the film dry absorbent paper.

2.3.2. Glucose estimation

Reducing sugars in hydrolysate and fermented broth were determined by Dinitrosalicylic acid (DNS) method. Each 3 ml of distilled water was taken in test tubes, one tube was kept as blank and remaining tubes were used for dilution of samples (hydrolysate or fermented broth) which will measure the glucose concentration. After completion of known dilutions, 3 ml of 3,5-Dinitrosalicylic acid was added to each tube and boiled for 5 min in water bath. Then these tubes were immediately cooled to room temperature. Absorbance was taken in reference of blank at wavelength 575 nm by Double Beam UV-VIS Spectrophotometer 2600. The glucose concentration was determined from standard curve corresponding to that absorbance. Original glucose concentration of sample was obtained by multiplying the above glucose concentration with dilution factor.

2.3.3. Furfural estimation

3 ml of distilled water was taken in each test tube and those were used for dilution of samples. After completion of known dilutions, absorbance was taken in reference of distilled water at wavelength 277 nm by Double Beam UV-VIS Spectrophotometer 2600. The furfural concentration was determined from standard curve corresponding to that absorbance. Original furfural concentration of sample was obtained by multiplying the above furfural concentration with dilution factor.

2.3.4. Ethanol estimation

Colorimetry method was used to estimate the ethanol concentration in fermented broth, first distilled the broth by adding equal volume of distilled water at 100°C and collected distillate half of the total volume. 1 ml of distillate was taken in a test tube, 4 ml of distilled water and 5 ml of chromic acid were added to distillate. One test tube with 5 ml of distilled water and 5 ml of chromic acid was prepared to use as blank. Test tubes were incubated at 60°C for 20 min. Absorbance was taken in the reference of blank at 584 nm by Double Beam UV-VIS Spectrophotometer 2600. The ethanol concentration was determined from standard curve corresponding to that absorbance.

Ethanol concentration was also determined by using gas chromatography. The instrument used was a Chemito 8600 Refinery Gas Analyser with a 4 m long and 1/8 in

diameter polar column with Chemosorb 80/60. Sample was injected using a syringe at an oven temperature 150°C and inlet and flame ionization detector temperature of 200°C using helium as a gas carrier.

2.3.5. Dry cell weight estimation

For determination of cell dry weight, empty eppendorf tube was taken and it is dried in vacuum oven at 70°C to a constant weight. The weight of empty tube was measured. 1 ml of sample was taken in eppendorf tube and centrifuged this eppendorf tube by using Eppendorf Centrifuge 5415 C at 10000 rpm for 5 min and washed twice with distilled water. This eppendorf tube with cells was dried in vacuum oven at 70°C to a constant weight. The weight of the tube with cells was measured. The difference between these two readings was given the dry cell weight per ml.

2.3.6. Carbon dioxide estimation

0.0625M $Ba(OH)_2$ solution was prepared. Sample of this solution was titrated with 0.05N HCl solution. The volume of HCl used for titration was measured. CO_2 produced during fermentation was estimated by passing gases through the solution of 0.0625M $Ba(OH)_2$. Some amount of $BaCO_3$ was formed in the form of precipitate. This remaining $Ba(OH)_2$ solution was titrated with 0.05N HCl and the volume of HCl used for titration was measured. CO_2 produced during fermentation was estimated as follows:

$$CO_2 \text{ (mg)} = N/2 * (Z_a - Z_b) * 44$$

Where, N- normality of HCl

Z_a- Volume of HCl used to titrate fresh $Ba(OH)_2$ solution.

Z_b- Volume of HCl used to titrate remaining $Ba(OH)_2$ solution.

3. Results and discussion

3.1. Characteristics of microorganisms

In my experiment, I was used two microorganisms. One was GNSA strain, it is thermopile, spherical shape and gram -ve and is grown at 60°C. Another one was IIPE453 strain, it is also thermopile, large oval, gram +ve and is grown at 45°C.

3.1.1. Growth studies on strains

Growth of GNSA and IIPE453 were studied. The absorbances at 600 nm and glucose concentrations were observed with time shown in table-1 and table-2 respectively. Graphs were plotted between glucose concentration and OD vs. time, shown in fig-1 and fig-2 respectively.

Table-1: Absorbance and glucose concentration for the study of growth curve of GNSA

Time (hr)	OD at 600 nm	Glucose concentration (g/L)
0	0.035	9.8
2	0.036	9.8
4	0.037	9.8
6	0.048	9.7
8	0.09	9.6
24	4.735	7.0
26	5.385	6.8
28	5.6593	6.5

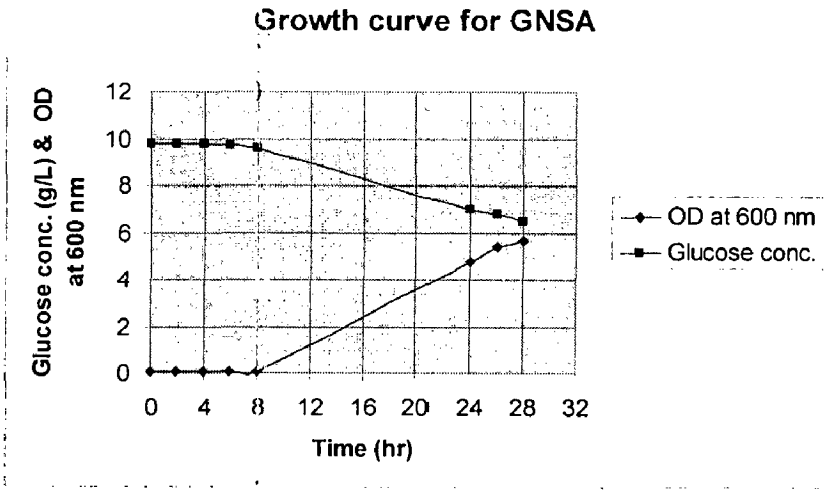

Fig-1: Glucose consumption and absorbance at 600 nm with time for the growth of GNSA at 60°C.

Table-2: Absorbance and glucose concentration for the study of growth curve of IIPE453

Time (hr)	OD at 600 nm	Glucose concentration (g/L)
0	0.034	9.6
2	0.036	9.6
4	0.05	9.5
6	0.11	9.4
8	0.333	9.2
24	9.096	4.2
26	10.02	4.0
28	10.26	3.6

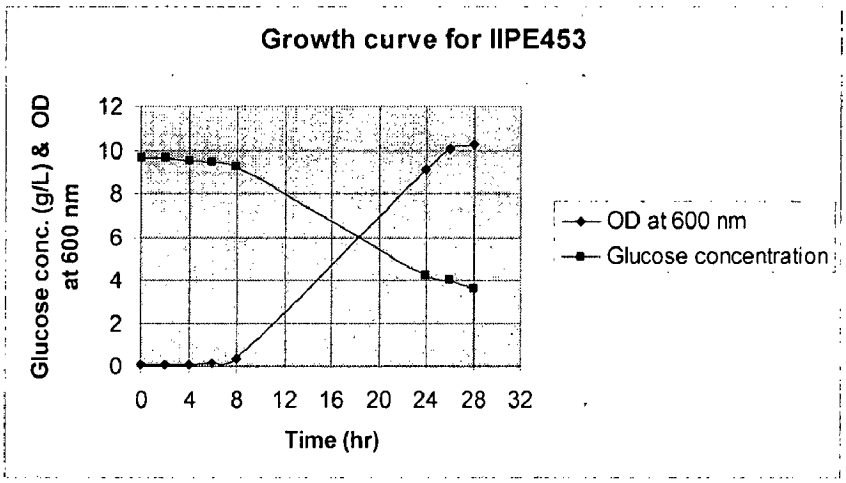

Fig-2: Glucose consumption and absorbance at 600 nm with time for the growth of IIPE453 at 45°C.

Strain IIPE453 was grown faster than GNSA was observed from the above two figures (i.e., Fig-1 and Fig2).

3.2. Acid hydrolysis

Acid hydrolysis of bagasse was carried out at three residence times. Acid hydrolysis was conducted with fresh bagasse is called 1st hydrolysis and with residual bagasse (i.e., bagasse from 1st hydrolysis) was called 2nd hydrolysis.

Observed the conversion of bagasse to glucose and furfural with residence time and type of hydrolysis was shown in table-3. Graphs were plotted between glucose concentrations vs. time for both hydrolysis in fig-3 and furfural concentrations vs. time for both hydrolysis in fig-4.

Table-3: Glucose and furfural concentrations from type of hydrolysis and at different residence times

Time (min)	1st Hydrolysis		2nd Hydrolysis	
	Glucose conc. (g/L)	Furfural conc. (mg/L)	Glucose conc. (g/L)	Furfural conc. (mg/L)
60	19	493.217	9.375	244.145
90	23	546.088	11.725	319.085
120	28.125	560.07	14.75	360.045

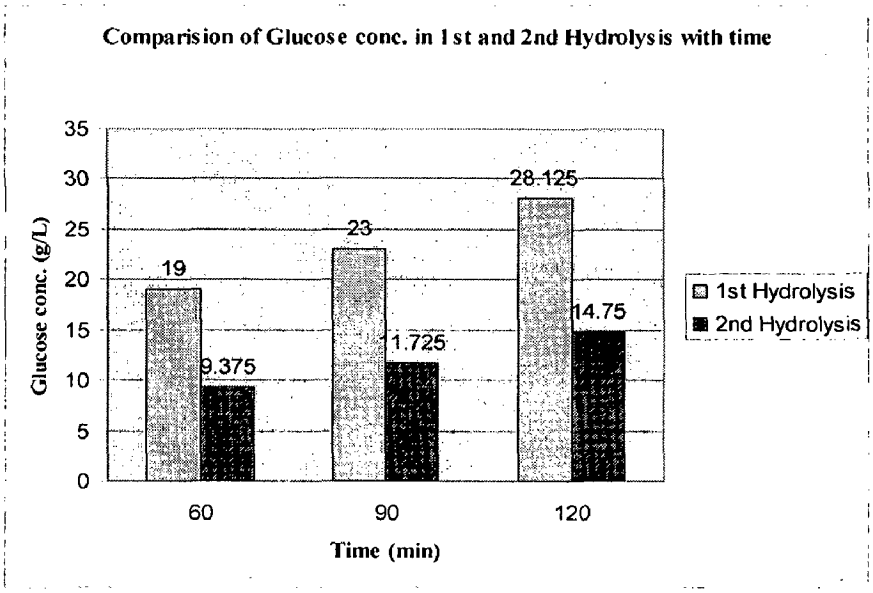

Fig-3: Comparision of Glucose conc. in 1st and 2nd Hydrolysis with time

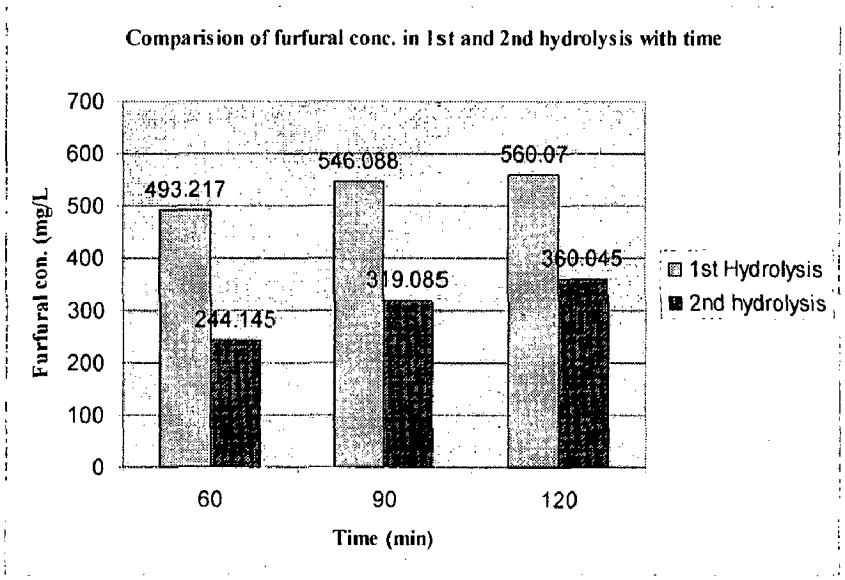

Fig-4: Comparision of furfural conc. in 1st and 2nd Hydrolysis with time

From the fig-3, it was observed that more amount of glucose obtained from 1st hydrolysis rather than 2nd hydrolysis. In 1st hydrolysis glucose concentration was nearly twice that of 2nd hydrolysis. But furfural concentration was also nearly doubled in 1st hydrolysis rather than 2nd hydrolysis, was observed from fig-4.

From the fig-3, glucose concentration was increased with time of operation. Highest glucose concentration (i.e., 28.125 g/L) was obtained in the 1st acid hydrolysis and it was carried out for 120 min. This hydrolysate was used for fermentation.

3.3. Detoxification

I was conducted five detoxification processes individually with the same amount of 1st hydrolysate was used in each process. Glucose, furfural concentrations and % removal of glucose and furfural of these processes were estimated, shown in table-4.

Table-4: Glucose and furfural concentrations along with % removal in hydrolysate after detoxification processes

Detoxification Process	Glucose conc. (g/L)	Furfural conc.(mg/L)	% Removal of glucose	% Removal of furfural
Hydrolysate before detoxification process	19	493.217	0	0
30 ml of hydrolysate was agitated with 0.3 g of activated carbon.	12.42	131.45	34.63	73.35
30 ml of hydrolysate was passed through adsorption column with activated carbon.	3.42	37.94	82.3	92.30
30 ml of hydrolysate was passed through adsorption column with silica gel.	11.64	85.725	38.74	82.62
30 ml of hydrolysate was passed through adsorption column with bauxite.	10.5	77.15	44.73	84.36
30 ml of hydrolysate was extracted with 10 ml of ethyl acetate.	18.06	120.015	4.95	75.67

From table-4, it was observed that hydrolysate passing through adsorption column with activated carbon was removed 92.30% of furfural. But the problem occurred from this process that it removed 82.30% of glucose. In fermentation point of view, we require high glucose concentration. So this process is not feasible than other processes for fermentation. Extraction with ethyl acetate was removed 75.67 % of furfural, and only 4.95 % of glucose. This process was better than other processes in fermentation point of

view. The remaining processes were removed 75 to 85 % of furfural and 35 to 45 % of glucose. These processes are not that much feasible to fermentation.

I was conducted the following detoxification processes in sequential order with 1st hydrolysate. The order of the processes and glucose, furfural concentrations were reported in table-5.

Table-5: Glucose and furfural concentrations in hydrolysate after mixed detoxification processes

Process No.	Detoxification Process	Glucose conc. (g/L)	Furfural conc.(mg/L)	% Removal of glucose	% Removal of furfural
0	Hydrolysate before detoxification process	19	493.217	0	0
1	Hydrolysate was passed through adsorption column with bauxite.	10.5	77.15	44.74	84.36
2	Hydrolysate from bauxite bed was extracted with ethyl acetate	8.25	44.577	56.58	90.96
3	Hydrolysate from extraction was agitated with activated carbon	4.2	3.429	77.89	99.30

Three times extractions of hydrolysate were carried out with ethyl acetate. Glucose and furfural concentrations were shown in table-6. Graphs were plotted between % removal of glucose vs. process no. for mixed detoxification processes and extraction processes in fig-5, and % removal of furfural vs. process no. for mixed detoxification processes and extraction processes in fig-6.

Table-6: Glucose and furfural concentrations in hydrolysate after extraction processes

Process No.	Detoxification Process	Glucose conc. (g/L)	Furfural conc.(mg/L)	% Removal of glucose	% Removal of furfural
0	Hydrolysate before detoxification process	28.125	560.07	0	0
1	1st time extraction with ethyl acetate	27.25	137.16	3.11	75.51
2	2nd time extraction with ethyl acetate	26.375	82.296	6.22	85.31
3	3rd time extraction with ethyl acetate	25.5	50.29	9.34	91.02

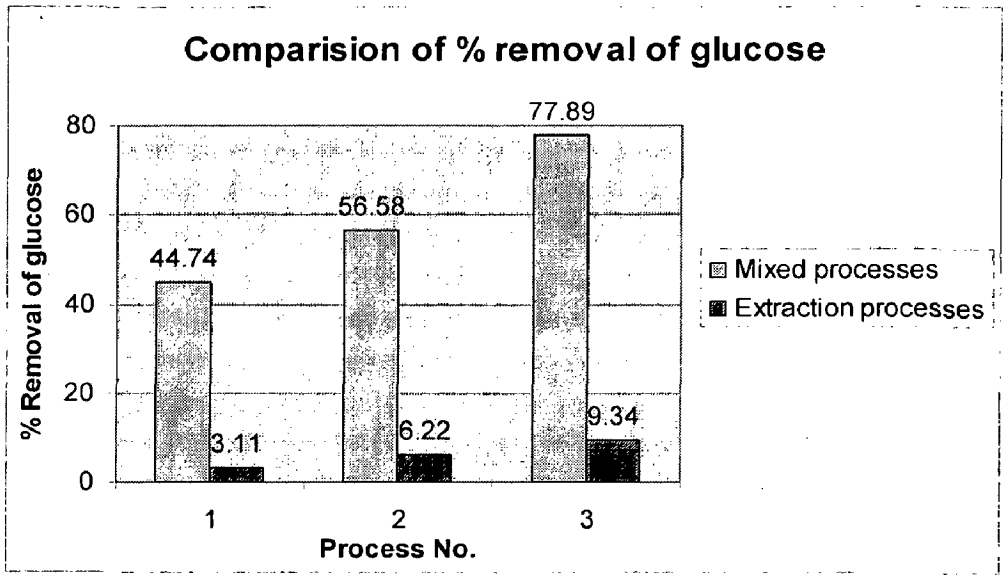

Fig-5: Comparision of % removal of glucose in mixed and extraction processes

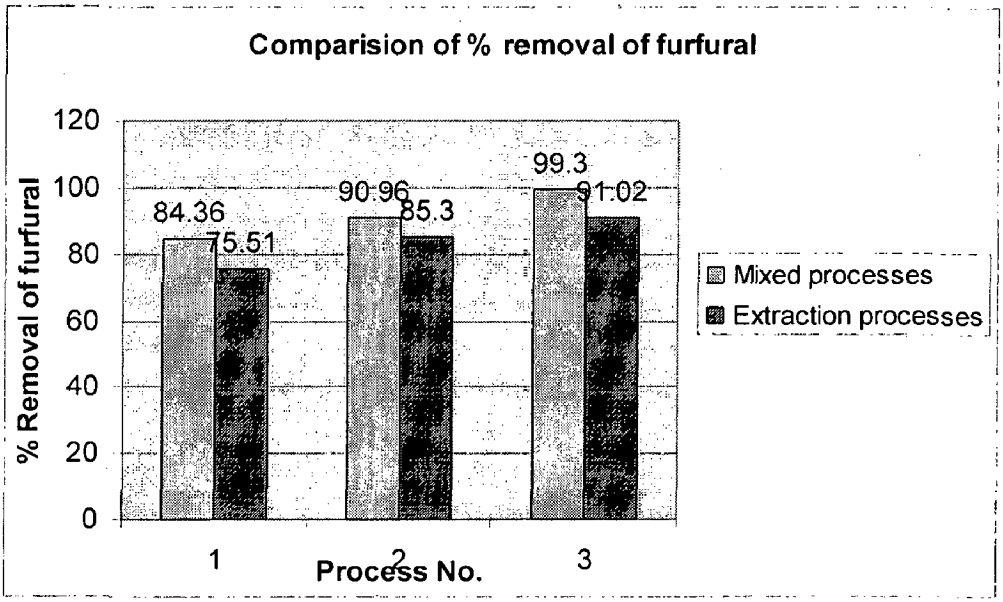

Fig-6: Comparision of % removal of furfural in mixed and extraction processes

From fig-5, it was observed that more amount of glucose was removed in mixed detoxification processes as compared to extraction processes. From fig-6, the removal of furfural was comparative. Three times extractions of hydrolysate with ethyl acetate were better process for the effective removal of furfural with less removal of glucose as compared to mixed detoxification processes. Hydrolysate was obtained from three times extractions of hydrolysate with ethyl acetate were further used for fermentation.

3.4. Fermentation

Two fermentations for the production of ethanol were carried out with two different stains, one with GNSA strain and another with IIPE453 strain. Batch fermentations were carried out with bagasse hydrolysate using GNSA and IIPE453 strains separately. Fermentation with GNSA strain was carried out for 120 hrs at 60°C and fermentation with IIPE453 strain was carried out for 8 hrs at 45°C. Glucose consumed, ethanol conc., dry cell weight, pH, product yield and productivity were observed with different time intervals, shown in table-7 for GNSA strain and table-8 for IIPE453 strain. Graphs were plotted between glucose consumed, ethanol produced, pH and dry cell weight vs. time, shown in fig-7 for GNSA strain and fig-8 for IIPE453 strain.

Table-7: Batch fermentation with GNSA strain showing glucose consumption, ethanol production, DCW, pH, product yield and productivity with time

Time (hr)	Glucose consumed (g/L)	Ethanol produced (g/L)	Dry Cell Weight (g/L)	pH	Product Yield ($Y_{P/S}$)*100	Productivity (g/L-hr)
0	0	0	1.8	7.0	0	0
6	1.25	0	1.8	7.0	0	0
25	2.625	0.502	2.0	6.5	13.84	0.02
53	3.75	0.7868	2.2	5.5	16.56	0.0148
72	5.0	0.543	2.4	5.0	10.86	0.0075
96	5.625	0.9314	2.6	5.0	16.55	0.0097
102	6.125	0.592	2.7	5.0	9.67	0.0058
120	6.5	1.08542	2.7	5.0	16.7	0.009

Table-8: Batch fermentation with IIPE453 strain showing glucose consumption, ethanol production, DCW, pH, product yield and productivity with time

Time (hr)	Glucose consumed (g/L)	Ethanol produced (g/L)	Dry Cell Weight (g/L)	pH	CO_2 Produced (mg/L)	Product Yield ($Y_{P/S}$)*100	Productivity (g/L-hr)
0	0	0	5.5	6.0	-	0	0
2	5	1.028	5.5	5.5	-	20.56	0.514
4	11.25	2.370	5.6	5	-	21.076	0.5925
6	20.55	4.44	5.7	5	148.5	21.6	0.74
8	22.30	4.50	5.6	5	-	20.18	0.5625

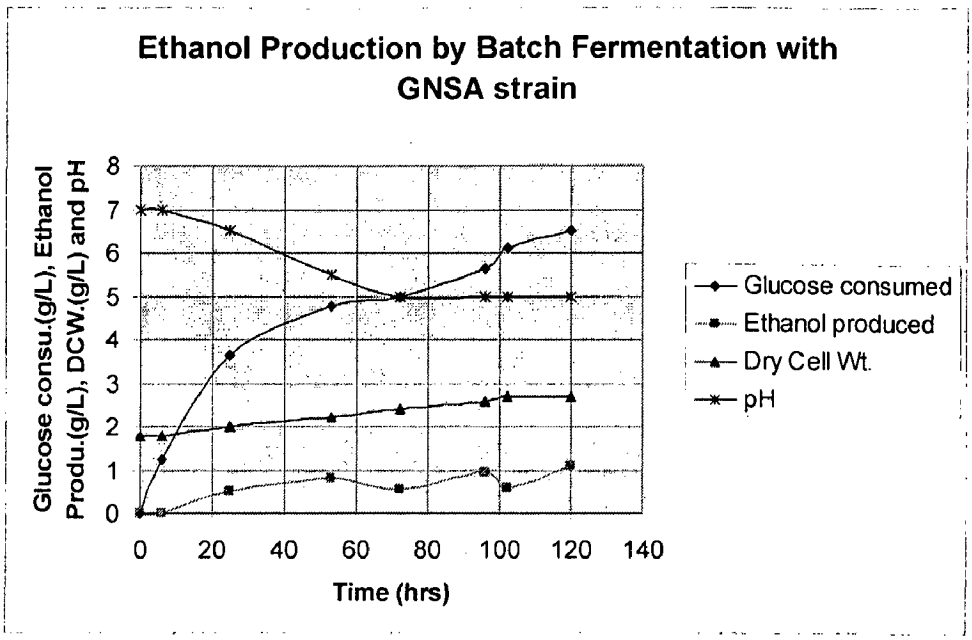

Fig-7: Glucose consumption, Ethanol production and Dry Cell Wt. and pH at different time intervals in Batch Fermentation with GNSA strain

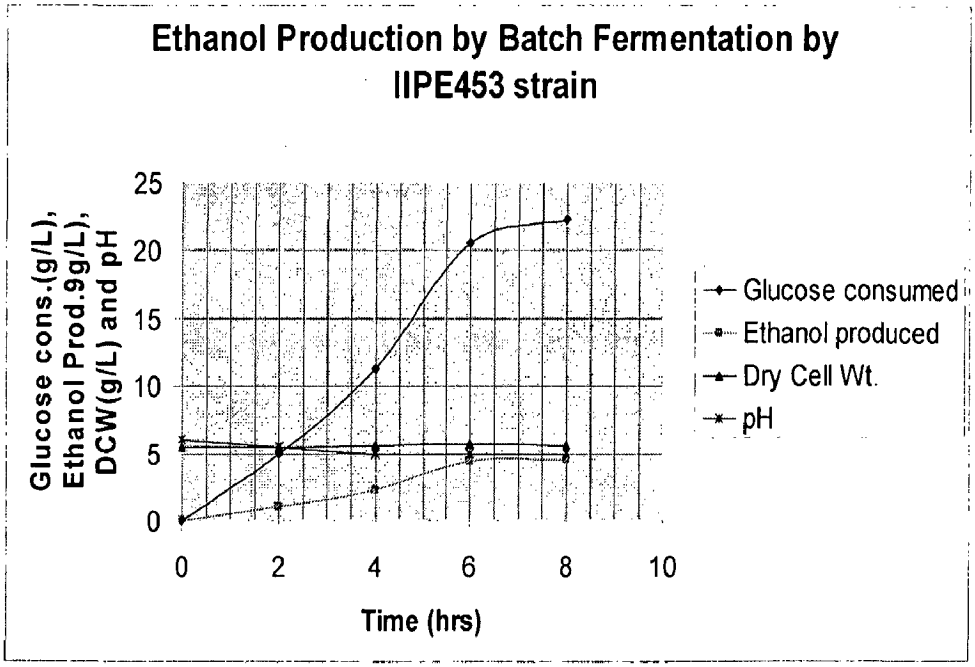

Fig-8: Glucose consumption, Ethanol production and Dry Cell Wt. and pH at different time intervals in Batch Fermentation with IIPE453 strain.

From the fig-7, it was observed that till to 53 hrs ethanol produced gradually, but after 53 hrs the production of ethanol was cyclic (i.e., production of ethanol was gradually decreased and then increased). Batch fermentation with GNSA strain to produce ethanol from bagasse hydrolysate was taken more time to complete consumption of glucose rather than IIPE453 strain. Using IIPE453 strain, it was taken only 8 hrs to

90% consumption of glucose. From the fig-8, it was observed that the production of ethanol was increased gradually.

From the above data, it was observed that strain IIPE453 was better than strain GNSA to produce ethanol from bagasse hydrolysate by batch fermentation.

4. Conclusions and recommendations

Experimental study of Biocatalytic conversion of biomass to biofuels (i.e., ethanol) was carried out in two steps. In 1st step, it was acid hydrolysis was conducted with 2% H_2SO_4 solution and in 2nd step, it was fermentation was conducted in batch mode. From the experiments it was concluded that,

1. In 1st acid hydrolysis nearly double amount of glucose was obtained rather than 2nd acid hydrolysis for the same residence time.
2. In acid hydrolysis's, residence time of 120 min was better than others (i.e., residence time of 60 min and 90 min) due to high glucose concentration was obtained.
3. Maximum glucose concentration of 28.125 g/L was obtained in 1st hydrolysis with the residence time of 120 min.
4. Three times extractions of hydrolysate carried out with ethyl acetate was better detoxification process to remove furfural with less removal of glucose, because it removes 9.34% glucose and 91.02% of furfural. The remaining processes were removed more than 9.34% glucose.
5. Fermentation with IIPE453 strain was better than GNSA strain, because IIPE453 was given highest product yield of 21.06% and productivity of 0.74 g/L-hr at 6 hrs. IIPE453 was taken only 8 hrs to 90% consumption of glucose.

Following are the recommendations for the future study,

1. Strain DNA characterization is required to know the name of the strain.
2. Acid hydrolysis can be conducted with other acid concentrations (i.e., other than 2%).

3. Detoxification processes can be conducted with ion exchange resins to remove the furfural.
4. Fermentation can be conducted with other strains to check the performance of those strains to produce ethanol from biomass.
5. Continuous fermentation can be conducted.
6. Recovery of ethanol from fermentation broth is required to get pure ethanol with high concentration by extractive distillation.
7. Kinetic study of ethanol fermentation can be conducted.

5. References

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