A

Project Report

on

WET TORREFACTION OF LIGNOCELLULOSIC BIOMASS FOR THE PRODUCTION OF BUTANOL

Submitted by

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110CH0463

In partial fulfilment of the requirements for the degree in
Bachelor of Technology in Chemical Engineering

Under the guidance of

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May, 2014

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CERTIFICATE

This is to certify that the thesis entitled, "WET TORREFACTION OF LIGNOCELLULOSIC BIOMASS FOR THE PRODUCTION OF BUTANOL" submitted by TAPAS DAS in partial fulfillments for the requirements for the award of Bachelor of Technology Degree in Chemical Engineering at National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by them under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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Date:

ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and indebtedness to Prof. R.K Singh, Department of Chemical Engineering, and NIT Rourkela for introducing the present topic and for his inspiring guidance, constructive criticism and valuable suggestion throughout this project work.

I would also like to thank Mr. Suresh Chaluvadi (PhD scholar) for his timely help and advice. I am also thankful to all staff members of Department of Chemical Engineering, NIT Rourkela.

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ABSTRACT

Lignocellulosic biomass is most abundant renewable resource suitable for continuous supply of biofuel. Ethanol and butanol acquired a dominant place in the partial replacement or blending with gasoline. Butanol could be produced through effective treatment which makes a hope for future energy. Wet torrefaction is pretreatment which enables dissociation of lignocelluolosic fibers and enhances the enzyme saccharification. This work intended to explain the pretreatment effects of acid torrefaction on sugarcane bagasse on the sugars yield. The present study investigates the effect of temperature and HCl concentration in pretreatment of sugarcane bagasse on final glucose yield in production of butanol. The maximum yield of glucose 57.82% was obtained using 200°C and 3% HCl and 30 minutes of residence time. The yield of butanol was 0.924g of butanol produced to glucose consumed.

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CHAPTER-1

INTRODUCTION

1. Introduction:

In our day to day life we need energy in every field such that in cooking purpose, function of vehicles, electricity and many more. From ancient years we are using fossil fuels to fulfill all our requirements but in recent years researches are being done to introduce biofuel instead of fossil fuel as the use of fossil fuel causes greenhouse effect, global warming etc. But use of biofuel is pollution free to a better extent and it's also renewable. Biomass is the biological material of the living organisms which refers to the plants and generally plant derived materials. Biomass is renewable source of energy, so they can be used directly or indirectly if converted to valuable products. Coal, petroleum and natural gas are the conventional energy sources. Economical and energy-efficient processes should be adapted for the production of fuels as petroleum sources are getting depleted, and also there is a high demand for petroleum products. Environmentalists and economists are being bound to have a control over its consumption and to examine renewable and less cost substitute to fossil fuel to meet the energy demand. In regards to this, lots of research work is being carried out around the world on various alternative sources of energy such as wind, geothermal, nuclear, solar, hydrogen, bio fuel or biomass etc. Unlike fossil fuels, butanol is a renewable energy source produced through fermentation of sugars. Butanol is widely used as a partial gasoline replacement in the US. Fuel butanol that is produced from corn has been used in oxygenated fuels since the 1980s. These gasoline fuels contain up to 20% butanol by volume. As a result, the US transportation sector now consumes about 4540 million liters of butanol annually, about 1% of the total consumption of gasoline. Recently, US automobile manufacturers have announced plans to produce significant numbers of flexible-fueled vehicles that can use a butanol blend – B85 (85% butanol and 15% gasoline by volume) – alone or in combination with gasoline. Using butanol-blended fuel for automobiles can significantly reduce petroleum use and exhaust greenhouse gas emission

The vital sources of biomass are agricultural crops and residues (wheat straw, sugarcane bagasse, and corn stover), forestry products, and biological wastes. Biomass is biologically produced and contains carbon, hydrogen & oxygen. It can be converted into energy by various means like biological, thermal, mechanical & physical process.

n-Butanol is a four-carbon primary alcohol, and is currently used as chemical intermediate, solvent, and extractant in cosmetics and pharmaceutical industries. Butanol has been attracting

research attention as an alternative biofuel to bioethanol in the very recent years. Compared to ethanol, butanol is considered as the next generation biofuel due to lots of advantages such as higher energy contentand lower volatility. Butanol can be used directly or blended with gasoline and diesel as fuel additives in the current automobile engine without any modification or substitution and butanol is also compatible with the current transportation pipeline for gasoline.

1.1 Pretreatment & Detoxification of Biomass

1.1. (a) Pretreatment of lignocellulose:

Lignocellulosic biomass mainly contains lignin, hemicellulose and cellulose. Lignin, a highly cross-linked polymer complex comprising of phenolic alcohol monomers, imparts structural support for plant cell wall. Lignin links and forms a rigid physical seal around hemicellulose and cellulose to prevent solvent permeability and microbial attack. Hemicellulose is composed of hetero-polysaccharide backbone (mostly formed by xylose, arabinose, galactose and mannose) with short branches linked also by β-(1-4)-glycosidic bonds. Hemicellulose acts like filler between lignin and cellulose microfibrils. Cellulose is the main structural components in the plant cell wall, and is usually packed into tight micro fibrils due to the hydrogen bond linkage of cellulose long chain. In plant biomass, cellulose is usually in the crystalline form with a small portion in amorphous form, which determines the hard-to-breakdown nature of cellulose by both acid and enzyme hydrolysis. In order to efficiently convert cellulose to fermentable sugars, lignin and hemicellulose must be removed. The goal of the pretreatment is to remove lignin and hemicellulose, reduce the crystallinity of cellulose, and increase the porosity of the lignocellulosic biomass.

1.1.1 Physical/mechanical pretreatments:

Physical pretreatment, also known as mechanical pretreatment, employs machinery chipping, grinding, or milling to reduce the size of biomass and the cellulose crystallinity improving easy acid/enzyme access. Depending on the requirements, biomass can first be sent through a chipping machine to obtain particles at sizes of 10-30 mm; and if fine powder is preferred, the biomass can be further sent for grinding or milling to reduce the size to 0.2-2 mm. In general, the smaller the particle size, the easier for the microorganism or enzyme to digest. Smaller size also helps to disrupt the crystalline structure of cellulose better. However, higher cost is usually associated with finer particle size.

1.1.2 Thermal pretreatment:

Steam explosion employs high temperature steam (160-270^{o}C) at high pressure (0.69-4.83MPa) to treat the lignocellulosic biomass for a few seconds to minutes before the biomass is suddenly exposed to atmospheric pressure, during which the biomass undergoes an explosive decompression due to the sudden pressure drop. It was reported that steam explosion can greatly increase the enzymatic hydrolysis efficiency and reducing sugar yield from many different lignocellulosic biomass such as corn stover, wheat straw, and wheat fiber.

1.1.3 Steam explosion:

Steam explosion has been applied to and is recognized as one of the most effective pretreatment methods for lignocellulosic materials, particularly agricultural residues and hardwood. Advantages of steam explosion mainly include reducing the biomass size, effective removal of lignin and hemicellulose without dilution of the resulting sugars and lower energy cost compared to mechanical milling.

1.1.4 Chemical pretreatments:

1.1.4. (a) Acid Pertreatment:

Acid pretreatment can be divided into dilute acid and concentrated acid pretreatment. The goal of acid pretreatment is to partially or completely hydrolyze hemicellulose, break down the lignin structure and disrupt the cellulose crystallinity for further enzymatic digestion to release fermentable sugars. Generally, concentrated acid (H_2SO_4 and HCl) pretreatment is considered to be too corrosive and dangerous to operate. In addition, a large amount of base is required for neutralization, resulting in high salt concentration in the hydrolyzate highly inhibitory to the fermentation. Therefore, dilute acid pretreatment is much more commonly used compared to the concentrated acid pretreatment.

Dilute H_2SO_4 and HCl are commonly used in dilute acid pretreatment of biomass with concentration ranging from 0.5% to 5 %(w/v), or 0.05 to 1N depending on the biomass type or process time. Dilute acid treatment is effect in removing hemicellulose, with almost all the hemicellulose hydrolyzed and recovered as the dissolved sugars such as xylose, glatactose, arabinose etc. in the hydrolysis. The removal of hemicellulose exposes the cellulose to enzyme attack, increasing the enzymatic digestibility and sugar yield in the residue solid left after the acid pretreatment. Various agro-industrial residues, including corn fiber, corn cob, corn stover,

whey straw, whey bran, sugarcane bagasse, and cassava bagasse, have been studied under different acid concentrations and residence times in search for an optimal condition. A variety of degradation products (phenolic compounds, furan derivatives, etc.) usually come with acid pretreatment. Balancing the sugar yield, acid concentration and pretreatment time can control the inhibitors present in the hydrolyzate, alleviating the stress on the following fermentation process.

1.1.4 (b) Alkaline pretreatment:

Alkaline pretreatment with strong bases like sodium hydroxide, potassium hydroxide, calcium hydroxide, and ammonia hydroxide is also widely used. Compared to acid pretreatment, alkaline pretreatment uses relatively mild conditions, such as room or slightly elevated temperature and atmospheric pressure. As a result of this mild condition, the duration of alkaline pretreatment usually takes hours to days instead of few minutes. Elevated temperature can significantly reduce the reaction time; therefore, 80-120^{o}C is often used in alkaline pretreatment. Among all the common strong bases, lime is mostly chosen due to the competitive low price and renewability. Various feedstocks have been treated with alkaline, such as bagasse, wheat straw, Corn Stover, switch grass, wood chips and more. The main goal of the alkaline pretreatment is to remove the lignin from biomass, while hemicellulose is also partially dissolved leaving cellulose accessible to enzymes. It was also reported that in the presence of an oxidizing agent such as oxygen, the removal of lignin is greatly enhanced while cellulose in the biomass is not affected.

1.1 (b) Detoxification of lignocellulosic hydrolyzate:

Various byproducts, also known as inhibitors in the latter bioconversion process, are generated during the pretreatment process. The major byproducts include furan derivatives (furfural and 5-hydroxymethylfurfural (HMF), sugar degradation), phenolic compounds (syringaldehyde, vanillin, syringic acid, vanillic acid, *p*-coumaric acid, ferulic acid, lignin degradation), and weak acid (acetic acid, lignocellulose structure degradation).

Pentose and hexose are released during the hydrolysis of lignocellulosic biomass, and then further degraded into furfural and HMF, respectively. Furfural and HMF are generally recognized as the major inhibitors to the microorganisms. Phenolic, aromatic compounds and aldehydes are degradation products generated from lignin. These compounds, especially the low molecular weight ones, are very toxic to the fermentation microorganisms, even when their concentrations are low. Acetic acid is derived from the acetyl side-groups of hemicellulose, and

is considered as a product of lignocellulosic structure degradation. The inhibitory effect of acetic acid is usually not as severe as furan derivatives or phenolic compounds. At low concentrations, several reports showed that acetic acid actually enhanced the solvent production and prevented the culture degeneration.

When using lignocellulosic hydrolyzate all of the above mentioned substances can cause some degrees of inhibition in the fermentation process. Due to the presence of various inhibitors, the lag phase is prolonged, sugar utilization is reduced, and the product formation (concentration, yield, productivity) is significantly hindered. The inhibitory concentration of each compound cannot be strictly determined due to the diversity of microorganism. Moreover, it was reported that while an individual compound may not cause inhibition, when in the presence with other compounds a significant "synergistic effect" may exhibit. Detoxification is usually needed to re-condition the lignocellulosic hydrolzsates to a suitable substrate for microorganisms to digest. Physical detoxification usually uses vacuum evaporation technique to remove the volatile toxic substances, such as furfural and acetic acid. Usually the furfural can be efficiently removed by this method, and the sugar is concentrated after water evaporates. The down side of this pretreatment is that non-volatile substances accumulate and stay in the concentrated hydrolyzate. In general, chemical detoxification includes using pH adjustment to precipitate and remove toxic substances, and adsorption with activated charcoal or ion-exchange resins. Since some inhibitors are unstable at a certain pH, pH adjustment with $Ca(OH)_2$ (lime) is the most commonly used detoxification method for a variety of lignocellulose hydrolyzates. Generally, lime is added to adjust the pH to 9-10, and then acid (H_2SO_4 or HCl) is added to readjust pH to 5.5-6.5. It was reported that overlime detoxification reduced over 51% of furans, 41% of phenolic compounds, and only 8.7% of sugars.

1.2 Hydrolysis of lignocellulosic materials:

The cellulose molecules are composed of long chains of glucose molecules. In the hydrolysis process, these chains are broken down to "free" the sugar, before it is fermented for alcohol production. There are two major hydrolysis processes: a chemical reaction using acids, or an enzymatic reaction.

1.2.1 Acid hydrolysis:

Acid hydrolysis has been examined as a possible process for treating lignocellulosic materials such as wood chips, the mineral acids act simply and rapidly as reaction catalyzers of polysaccharide fractions. Sugarcane bagasse can be hydrolyzed using dilute acid to obtain a mixture of sugars with xylose as the key component. However, in the hydrolyzate some by-products are generated in the hydrolysis, such as acetic acid, furfural, phenolic compounds, or lignin degradation products. These are potential inhibitors of a microbiological utilization of this hydrolyzate. Processes such as two-stage acid hydrolysis can be employed to produce xylose and glucose. Treatment with dilute hydrochloric acid at moderate temperatures has proven to be an efficient means of producing xylose from hemicellulose. In the second stage more drastic reaction conditions are employed and glucose can be produced from cellulose hydrolysis.

In general, acid treatment is effective in solubilizing the hemicellulosic component of biomass. Proper combinations of pH, temperature, and reaction time can result in high yields of sugars, primarily xylose from hemicellulose. Hydrochloric acid is a catalyst for this reaction and, in this work; it's used to study the hydrolysis of sugarcane bagasse hemicellulose. The effects of temperature, acid concentration and reaction time are also studied, and the effectiveness of the hydrolysis was evaluated in terms of hemicellulose solubilization. Enzymatic hydrolysis of cellulose is carried out by enzymes, which are highly specific. The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50°C) and does not have a corrosion problem. Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as Clostridium thermocellum with high specific activity, they do not produce high enzyme titers. Because the anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi. Cellulases are usually mixtures of several enzymes. The factors that affect the enzymatic hydrolysis of cellulose include substrate, cellulose activity, and reaction condition (temperature, pH, as well as other parameters). To improve the yield and rate of the enzymatic hydrolysis, research has dedicated on optimizing the hydrolysis process and enhancing cellulase activity.

1.3 Fermentation process:

A variety of microorganisms, generally either bacteria, yeast, or fungi, ferment carbohydrates to butanol under oxygen-free conditions. They do so to obtain energy and to grow. Methods for C_6 sugar fermentation were already known (at least) 6000 years ago, when Sumerians, Babylonians and Egyptians began to perfect and describe the process of making beer from grain (starch). After it became possible to free the C6 sugars in lignocellulosic crops (end 19th century), conversion of the C_5 sugars became interesting. They represent a high percentage of the available sugars, the ability to recover and ferment them into ethanol is important for the efficiency and economics of the process. Only in the 1980s research on xylose fermentation began to bear fruit, when a number of wild type yeast were identified that could convert xylose to ethanol. Bacteria have drawn special attention from researchers because of their speed of fermentation. In general, bacteria can ferment in minutes as compared to hours for yeast.

1.4 Product recovery and separation technologies:

No matter whether it is to produce fuel-grade ethanol or butanol, multi-column distillation followed by molecular sieve adsorption has always been the standard operation procedure in the industrial process. Distillation offers a wide range of advantages, such as high alcohol recovery, multi-stage operation, being easy to scale-up and relatively energy-efficient when alcohol concentration in the feed stream is high. There are also many less-attractive facts about recovering alcohol using distillation, such as energy-intensive for low alcohol concentration feed, high-temperature operation which is lethal to microorganisms, and necessity for an additional dehydration step in order to reach the fuel-grade specification. Because the butanol concentration is usually high at the end of the process, distillation is favorable for butanol recovery.

Butanol recovery is the most energy-intensive and costly step in the whole biobutanol production process. In ABE fermentation, the butanol final concentration is usually 1-2% in the fermentation broth. Recovering butanol using distillation is thus extremely energy-intensive and costly. Unlike ethanol, butanol has a low vapor pressure and high boiling point (118^oC), which pose further challenges in distillation and require more energy. Alternative separation technologies that are energy-efficient and suitable to recover low concentration alcohol in the fermentation broth are in demand. Over the years many relatively economic and feasible techniques, including gas stripping, liquid-liquid extraction, adsorption, and pervaporation have

been developed to recover solvents from the fermentation broth. These technologies are more energy-efficient than the traditional distillation approach in terms of lowering the process cost.

There are usually two alcohol recovery approaches from the fermentation broth, "end-of-pipe" and "slip-stream". The end-of-pipe approach refers to the alcohol recovery after the fermentation is completed, and the alcohol-depleted broth is sent to the next step for processing. This approach is usually employed in ethanol recovery from fermentation due to the high end product concentration present in the feed stream. Slip-stream approach refers to alcohol recovery while the fermentation is still on-going in the bioreactor, and the alcohol-depleted stream is returned to or never leaves the bioreactor. This process is also known as the integrated process, meaning that the separation technology is integrated with fermentation and the desired product can be in-situ recovered simultaneously. The slip-stream approach is mostly seen in butanol recovery due to the severe end product inhibition on microorganisms caused by butanol. By employing the slip-stream approach, the butanol inhibition is relived and the butanol-free broth is recycled back into the bioreactor, increasing the volumetric productivity of the reactor. Due to the high temperature employed in the distillation process, distillation can only be used in end-of-pipe approach, while the alternative separation technologies such as gas stripping can be used in slip-stream approach to increase the reactor productivity and overall butanol concentration.

CHAPTER-2

LITERATURE REVIEW

2.1 Literature Review:

Biological butanol production via fermentation was first reported in 1861 by Louis Pasteur, who first discovered and isolated a butyric acid producing strain and later on observed butanol production along with butyric acid. Many scientists including Albert Fitz and Martinus Beijerinck continued the work of butanol-producing microorganisms and isolated several additional strains such as Bacillus butylicus, and Granulobacter saccharobutyricum. In 1926, McCoy first used the name of Clostridium acetobutylicum in their paper and this name was officially recognized and accepted as the butanol producing microorganism. Weizmann, along with a British company Strange & Graham Ltd., later on isolated a strain that showed good acetone and butanol producing ability, and developed and patented a process based on this strain to produce butanol. This process played an important role in World War I, and since 1920 acetone and butanol have become major fermentation products for their outstanding properties as solvents. Many countries, including USA, England, China, Australia, and Canada, made biological butanol plants employing ABE fermentation between 1920 and 1980, but they all finally came to a cessation due to the rise of inexpensive petrochemical synthesis of butanol from crude oils and the high cost of fermentation raw materials.

A reexamine on ABE fermentation over the past few decades has made substantial advances and breakthroughs in the bio production of butanol from numerous alternative feedstocks. Recently, with the depleting fossil fuel reserves and surging crude oil price, biological production of butanol as a superior biofuel has become a beneficial research topic. Compared to ethanol, butanol is a superior fuel source, and the characteristics of butanol and gasoline are similar. A comparison of some properties among butanol, ethanol, methanol and gasoline is concised in Table 2.1. Butanol has a higher energy content and lower volatility than ethanol and methanol. Most importantly, butanol can be directly used as an alternative to gasoline or fuel additive in the current internal combustion engine without any modification. Therefore, butanol could become the next generation liquid biofuel in the near future.

Table-2.1: comparison of some properties among butanol, ethanol, methanol and gasoline

Fuel properties	n-Butanol	Ethanol	Methanol	Gasoline
Energy density (MJ/L)	29.2	19.6	16	32
Heat of vaporization (MJ/kg)	0.43	0.92	1.2	0.36
Research octane number	96	107	106	91-99
Motor octane number	78	89	92	81-89
Air to fuel ratio	11.2	9.0	6.4	14.6
Specific energy (MJ/kg air)	3.2	3.0	3.1	2.9

In 1996 Jones & Woods showed that butanol (and acetone, ethanol and isopropanol) is naturally produced by genus Clostridia bacteria. Clostridia are rod-shaped, spore-forming and gram-positive anaerobic bacteria. In 1997 Cornillot showed that Clostridia are mostly used as the solvent-producing bacteria due to some special genes and various enzymes produced within it. The substrate utilization ability among naturally solvent genic Clostridia is very different from each other and their optimal pH, temperature, and product profiles are also different. Most of the species can ferment pentose and hexose sugars, as well as starch.

In 1965 *C.* acetobutylicum was the main species employed in industrial ABE fermentation until more detailed taxonomy was developed. The strains (*C.* acetobutylicum ATCC 824, P262, P260 and DSM 1731, and *C.* beijerinckii ATCC 55025, NCIMB 8052, and BA101) all showed good butanol production between 10 g/L to 20 g/L. Some of these popular strains have been compared in a study by Gutierrez in 1966 using potato as the substrate, and strong solvent genic abilities were seen.

A very distinctive feature of Clostridia is the biphasic fermentation which was shown by Ezyin in 1967. During the first phase, which is known as acidogensis, acids (acetate and butyrate) and carbon dioxide are produced as the main products during the exponential growth phase, lowering the pH of the medium. Then, through a series of regulations, signals and change in gene expression, the second phase, which is known as the solventogensis in which acids are converted to solvents (acetone, butanol and ethanol).

Knoshaug and Zhang showed that butanol is a severe fermentation inhibitor to Clostridia, changes the phospholipid and fatty acid composition in the cell membrane, alters the membrane structure and compromises the fluidity of the membrane. It also affects the solute transport, membrane permeability, and maintenance of intracellular pH and ATP level. Fermentation is severely inhibited when butanol concentration reaches above 1% and stopped at 2% for most of microorganisms.

Substrate cost is a very important factor impacting on the economics of butanol production via fermentation. Traditionally, corn, molasses and glucose were the major substrates utilized in commercial ABE fermentation in the early 20^{th}century. In a typical batch fermentation, 20-25 g/L ABE can be obtained within 36-72 h followed by distillation as butanol recovery.

Every year, around 2×10^{11} tons of lignocellulosic biomass is produced representing the most abundant renewable sugar source. Lignocellulose consists of mainly cellulose (35-50%), hemicellulose (25-35%) and lignin (10-25%), and a small amount of protein, ash and some extractives. The composition and current use of some common lignocellulosic feedstock are summarized in Table 2.2.

Table-2.2: composition and current use of some common lignocellulosic feedstock

	Composition(% dry basis)			Current use
	cellulose	hemicellulose	lignin	
Cassava bagasse	(Total fiber) 15- 51			Landfill, burnt
Corn fiber	15	23- 64	8	Animal feed, burnt as fuel, compost, soil conditioner
Corn cob	45	35	15	
Corn Stover	38- 40	25- 28	7- 21	
Rice straw	28- 36	23- 28	12- 14	
Wheat straw	35- 40	20- 30	17- 19	
Sorghum stalks	27	25	11	
Fresh bagasse	33.4	30	18.0	Burnt as fuel
Sugarcane bagasse	40- 50	24- 25	25	Burnt as fuel, landfill
Grass	25- 40	25- 50	10- 30	Burnt

Lignocellulose is the largest reservoir of solar energy stored in the form of carbon source on earth, representing a potential group of feedstocks suitable for many bioconversion processes. As shown in Table 2.2, most of the lignocellulosic biomass is considered as waste materials from industrial processing and sold at low prices for animal feed or burnt as a source of energy. It is especially appealing those lignocellulosic feedstocks are renewable and available in abundance. Many processes have been studied and described for the bioconversion of lignocellulosic biomass into various value-added products, such as enzymes, biofuels, and chemicals.

Several lignocellulosic materials such as corn fiber, dried distiller grains and soluble, wheat straw, and switch grass have been reported and successfully applied in ABE fermentation as substrates to produce butanol. In general, cellulose and hemicellulose present in the lignocellulosic feedstocks are not directly accessible to the microorganisms because solventogenic Clostridia do not possess enzymes that can breakdown these materials. In order to utilize the lignocellulosic biomass, the sugars stored in the form of hemicellulose and cellulose must first be released. Therefore, lignocellulose has to be pretreated and hydrolyzed to release all the sugars that can be utilized by the microorganisms in the subsequent fermentation process. Due to the lignin protection and crystalline cellulose micro fibrils, lignocellulosic materials are usually very resistant to enzymatic hydrolysis. In addition, under the extreme conditions employed in pretreatment processes, many toxic compounds that are severe fermentation inhibitors are inevitably generated. Detoxification of lignocellulosic hydrolyzate is preferred in order to obtain better butanol production in the subsequent fermentation process.

Qureshi in 1970 showed that besides fed-batch and continuous fermentation technologies for butanol production, simultaneous saccharification and fermentation (SSF) are also another feasible technology for ABE fermentation. Usually, separate hydrolysis and fermentation (SHF) process was employed when using lignocellulosic biomass as substrate for fermentation. The advantage of SHF is that the hydrolysis process and fermentation process can be operated under their optimal conditions (usually pH 5.0 and 50^{o}C for enzymatic hydrolysis, and fermentation temperature 30-37^{o}C). However, as the end product of the hydrolysis, sugars inhibit the enzyme activity and lower the enzyme efficiency. Simultaneous saccharification fermentation can solve this problem by integrating the two processes together, with the enzymes, pretreated lignocellulose and microorganism all present in the same reactor. A compromised condition, usually pH 5.0 and 37^{o}C, is used in SSF. Enzyme converts the cellulose and hemicellulose into

sugars, and enzyme inhibition by sugars is relieved due to the simultaneous utilization of the released sugars by the microorganism. SSF is commonly employed in butanol fermentation from lignocellulosic biomass, lowering the process energy requirement and improving the enzyme efficiency and butanol production. Using wheat straw as the substrate, 13.12 g/L ABE were produced from SHF by *C.* beijerinckii P260, whereas similar ABE production of 11.93 g/L was obtained from SSF, indicating that SSF is also a feasible option for ABE fermentation using lignocellulosic biomass.

Lignocellulosic biomass mainly comprises lignin, hemicellulose and cellulose. Lignin, a highly cross-linked polymer complex containing of phenolic alcohol monomers, instructs structural support for plant cell wall. Lignin links and forms a rigid physical seal around hemicellulose and cellulose to inhibit solvent permeability and microbial attack. Hemicellulose is composed of hetero-polysaccharide backbone (mostly formed by xylose, arabinose, galactose and mannose) with short branches linked also by β-(1-4)-glycosides bonds. Hemicellulose acts like filler between lignin and cellulose microfibrils. Cellulose is the key structural components in the plant cell wall, and is usually packed into tight microfibrils due to the hydrogen bond linkage of cellulose long chain. In plant biomass, cellulose is usually in the crystalline form with a small portion in amorphous form, which determines the hard-to-breakdown nature of cellulose by both acid and enzyme hydrolysis. In order to efficiently convert cellulose to fermentable sugars, lignin and hemicellulose must be removed.

CHAPTER-3

OBJECTIVE

3.1 Objective:

The aim of the present work is the production of butanol from cellulosic materials through wet torrefaction.

In this project we are performing three steps to get butanol.

1. Pretreatment of lignocellulosic material using torrefaction
2. Saccharification of pretreated biomass.
3. Fermentation.

In pretreatment process wet torrefaction of biomass is done. Process of "Wet torrefaction" of biomass composes of establishing a desired reaction condition within a reaction chamber where the desired reaction condition is of at least 160^0c & a pressure sufficient to maintain water in a condensed state. The saccharification process undergoes enzymatic hydrolysis followed by biochemical conversion of sugar intermediates. The yield of butanol is to be analyzed after fermentation.

CHAPTER-4

MATERIALS & INSTRUMENTS

4.1 Materials Used:

4.1.1 Chemicals Required:

(1) Dilute sulfuric acid = 0.5%, 1%, 2%, 3%

(2) Concentrated and diluted NaOH for pH adjustments.

(3) DNS = 10 gm

(4) Phenol = 2 gm

(5) Sodium sulphite = 0.5 gm

(6) Sodium hydroxide = 10 gm

(7) Na-K tartrate (40%)

4.2 Instruments Used:

4.2.1 Mesh (Scale):

Mesh analysis is used to determine the particle size of granular material.

4.2.2 Beaker:

A beaker is a simple container for stirring, mixing and heating liquids commonly used in many laboratories. Beakers are generally cylindrical in shape, with a flat bottom. Beakers are available in a wide range of sizes, from one milliliter up to several liters.

4.2.3 Furnace:

The furnace transfers heat to the living space of the building through an intermediary distribution system. If the distribution is through hot water (or other fluid) or through steam, then the furnace is more commonly called a boiler. One advantage of a boiler is that the furnace can provide hot water for bathing and washing dishes, rather than requiring a separate water heater. One disadvantage to this type of application is when the boiler breaks down, neither heating nor domestic hot water are available.

4.2.4 Vertical Autoclave:

This equipment is primarily used for sterilization purpose. It is an enclosed space where steam bath is given to any equipment placed inside it. Water filled in it is heated by electric coils present at bottom. It has a vent at top, from where steam can be released to maintain the required pressure. For our case, sterilization is required after maintaining the pH and also for each filtration. Pressure around 2.02 kg/cm^2 is used. Once the pressure reaches 2.02 kg/cm^2, it is

maintained for half an hour. The equipments are allowed to cool down before removing from the autoclave.

4.2.5 Laminar flow chamber:

Firstly, the whole chamber is wiped on the inside by spirit solution or ethanol. UV light is turned on with the shutter completely closed and left for 15 minutes. After switching off the UV, we again wait for 15 minutes. Next the fan is turned on and shutter opened. Any transfer taking place in these conditions should be done close to a Bunsen burner flame. All flasks or jars should be properly capped inside the chamber itself.

4.2.6 Vacuum filtration:

For this filtration, a filter having sintered base is fitted into a Buchner-type funnel which in turn is air tightly attached to a conical flask having a side outlet to provide vacuum. For every filtration, Whitman filter papers are attached onto the filter. Also after every filtration, the conical flask and filter media are cleaned and sterilized.

4.2.7 UV-Spectrophotometer:

To analyze the samples, UV-Spectrophotometer is used. For our analysis, we use the particular wavelength measurement. The equipment consists of two cuvettes, one of which acts as a reference while in the other cuvette the sample is kept. Once kept inside, a UV-light is used to know the absorbance of a particular sample at a particular wavelength.

4.2.8 Shaking incubator:

This instrument is used for fermentation. It is an enclosed space where given conditions are maintained at a particular rpm. Flasks are kept on a shaking platform moving at a particular rpm.

4.2.9 PH-meter:

This instrument is used for measuring the pH of a given solution.

CHAPTER-5

EXPERIMENTAL METHOD

5.1 Raw materials:

Sugarcane bagasse (SB) was obtained from sakthisugars, cuttak. The material is well dried, milled and sieved to 1mm size and stored in plastic bags in dark place until use. Dilute Hydrochloric acids of 3%v/v, 2%v/v, 1%v/v and 0.5% v/v are used. Biomass loading of 10% which means 2g of biomass and 18ml of dilute solvent.

5.2 Wet Torrefaction procedure:

Wet Torrefacation experiment was carried out in the batch reactor of volume 380ml at various temperatures of 180^0 C, 200^0 C, 220^0 C and high pressure. Two residence times of 15min and 30 minutes were chosen. The temperature of the reactor was controlled by PID controller. The biomass loading was 10% w/v i.e. 2g of biomass in 18ml of dilute solvent. Three parameters were chosen based on severity in pretreatment. The pressure developed in the reactor was water vapour pressure. Care was taken to minimize loss of vapours from the reactor. The hydrolyzate was collected from the reactor after completion and cooling of reactor**.** The weight of hydrolyzate was measured before and after the torrefaction. The hydrolyzate was adjusted to neutral pH using sodium hydroxide. Reducing sugars are measured using Miller assay also called, DNS assay.

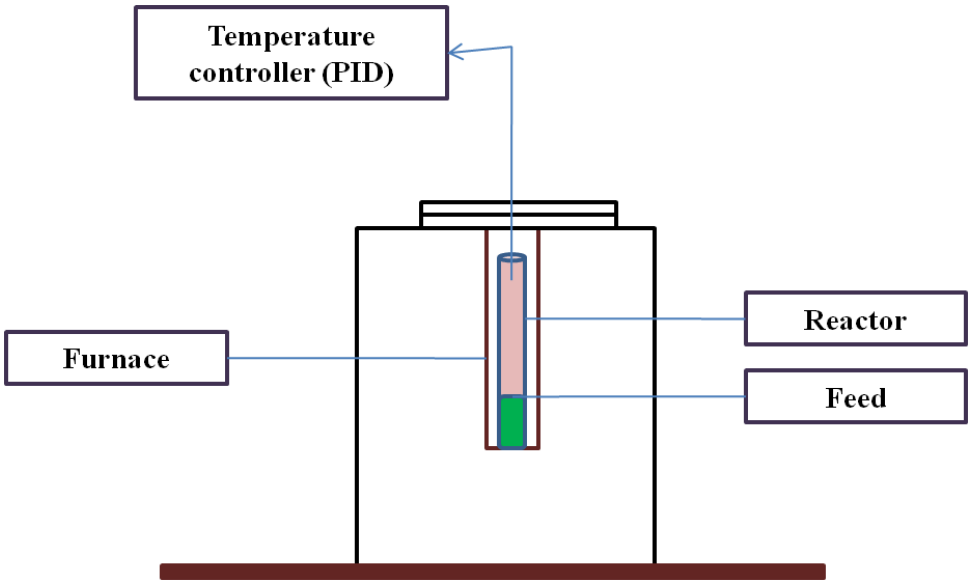

Fig 5.1: Experimental set up

5.3 pH adjustment:

1. O.5M dilute sulfuric acid hydrolyzed bagasse is taken and pH is adjusted with the help of litmus paper.
2. The aim is to neutralize the mixture and it is done easily by observing the color change of the litmus paper.
3. As the samples are acidic, so NaOH solution of 0.2M is added to make that neutral.

After maintaining the pH, the samples are kept in a Vertical autoclave for ½ hr at 120^{o}C(around 2.02 kg/cm2 pressure) and allowed to cool.

5.4 Sodium Citrate Buffer

Citric acid monohydrate	210 g
DI water	750 ml
NaOH – add until pH equals 4.3	50 to 60 g

Dilute to 1 L and check pH. If necessary add NaOH until the pH is 4.5. When the 1 M stock citrate buffer stock is diluted with water to 50 mM the pH should be 4.8. After diluting the citrate buffer check and adjust the pH if necessary to pH 4.8.

5.5 Enzymatic hydrolysis:

The hydrolysis with enzymes of acid torrified 1g biomass (dry weight basis) SB was carriedout in 150 ml stoppered conical flasks with enzyme celluclast 1.5L acquired from sigmaladrich. The enzyme loading was 60 FPU/g of biomass and the total reaction volume was made up to 50 ml with 50mM Sodium citrate buffer (pH 4.8). The incubation was carried at 50^{o}C for 72h in a shake flask incubator of 100rpm. After incubation, samples were centrifuged to remove the unhydrolysed residue. The hydrolyzate was used for reducing sugar analysis by Miller method.

5.6 Inoculum preparation:

Lyophilized Clostridium acetobutylicum MTCC 481 has been procured from microbial type culture collection, IMTECH, Chandigarh, India. It was maintained as spore suspension in sterile water. This culture has been rejuvenated in RCA (Reinforced Clostridial Agar) and RCM (Broth) culture media at 37 ^{o}C. The inoculums were prepared in RCM containing (g/L): glucose, 5.0; yeast extract, 3.0; starch, 1.0; beef extract, 10.0; peptone, 10.0; sodium chloride, 5.0; sodium acetate, 3.0; Agar,0.5 and cysteine hydrochloride, 0.5; pH 6.5 ± 0.1. 100 mL medium were autoclaved at 121^{0} C and inoculated in 250 mL screw capped Erlenmeyer flasks, and then

incubated for 72 h at 37 ± 0.5 ^{o}C at 120 rpm. In addition, Cooked Meat Medium (CMM) is also used for the growth and maintenance of clostridia. These were incubated anaerobically inside an anaerobic culture bag system till active growth was seen (72 h). Actively growing cultures (after lag phase, 18–20 h) of the Clostridia were added subsequently to experimental flasks.

5.7 Fermentation:

Batch fermentation experiments were carried out in 250 mL of screw-capped Erlenmeyer flasks under anaerobic conditions. Anaerobic condition in the flask was generated by addition of 0.5% cysteine hydrochloride to the SB hydrolyzate. The fibrous remains collected after sieving the filtrate through cotton cloth was dried at 70 ^{o}C in a hot air oven, and then was weighed. The reduced weight of bagasse after pretreatment was noted and was considered for the final yield calculation. An initial sample (0 h) was taken immediately after pretreatment for sugar analysis. Regular samples (1 mL per day) were taken to study Clostridium acetobutylicum's growth curve, so as to detect the growth stage at which respective products were produced. Experiments were run for nearly 120 h (5 days). At the end of fermentation final sample were taken for determination of butanol production and sugar utilization.

5.8 Analysis:

The samples were taken for every 24 hours for Butanol and sugar analysis with the help of UV-spectrophotometer. In case of Butanol analysis, the filtrate is directly placed in UV-spectrophotometer and its absorbance is noted down at 197 nm wavelength. But in case of sugar analysis, DNS method is followed.

5.9 Estimation of reducing sugar:

After enzymatic digestibility/hydrolysis sample was centrifuged in an eppendorf at 10,000 rpm for 10 min at 4 ^{o}C to determine sugar concentration; acid hydrolyzate can also be used to determine the reducing sugar present in it, but caution need to be taken to raise the pH from 2 to 8 by 5N NaOH. 100 µL of the supernatant or the hydrolyzate was then pipetted into the test tube followed by 300 µL of DNS reagent (DNS reagent preparation is mentioned in below). Blank was also prepared simultaneously by adding 100 µL of DW and 300 µL of DNS reagent. They were then kept in hot water bath at 90 - 100 °C for 10 min. Test tube along with blank were then taken out followed by cooling under running water. 10 ml DW was added to test tubes and then

absorbance of the sample and blank were taken at 540nm. Enzymatic hydrolyzate was diluted 5 to 10 times as required to bring the absorbance value in the range of standard curve.

CHAPTER-6

RESULT, DISCUSSION & CONCLUSION

6.1 Composition of raw material:

The composition obtained for the SCB was (average values of three replicates, error lower than 1% in all compounds, weight per cent on dry basis): Glucan38.9%; Xylan20.6%; Arabinan5.56%; lignin 23.9%; others (by difference)11.0%. These values are in the range found for this kind of materials.

6.2 Sugar Analysis:

For Wet torrefaction of 15min duration:

Table-6.1: Glucose yield at different time

Sl No.	Glucose 24hr	Glucose 48hr	Glucose 72hr
05s180	0.88	1.63	1.9
05s200	1.21	2.29	5.63
05s220	3.73	5.23	12.5
1s180	4.5	11.6	24.5
1s200	5.96	15.6	32
1s220	9.26	18.5	33.5
2s180	6.1	12.6	26.3
2s200	8.6	16.5	32.1
2s220	7.8	14.5	28.8
3s180	7.8	14.5	26.1
3s200	10.6	23.5	32.1
3s220	14.6	15.4	37.98

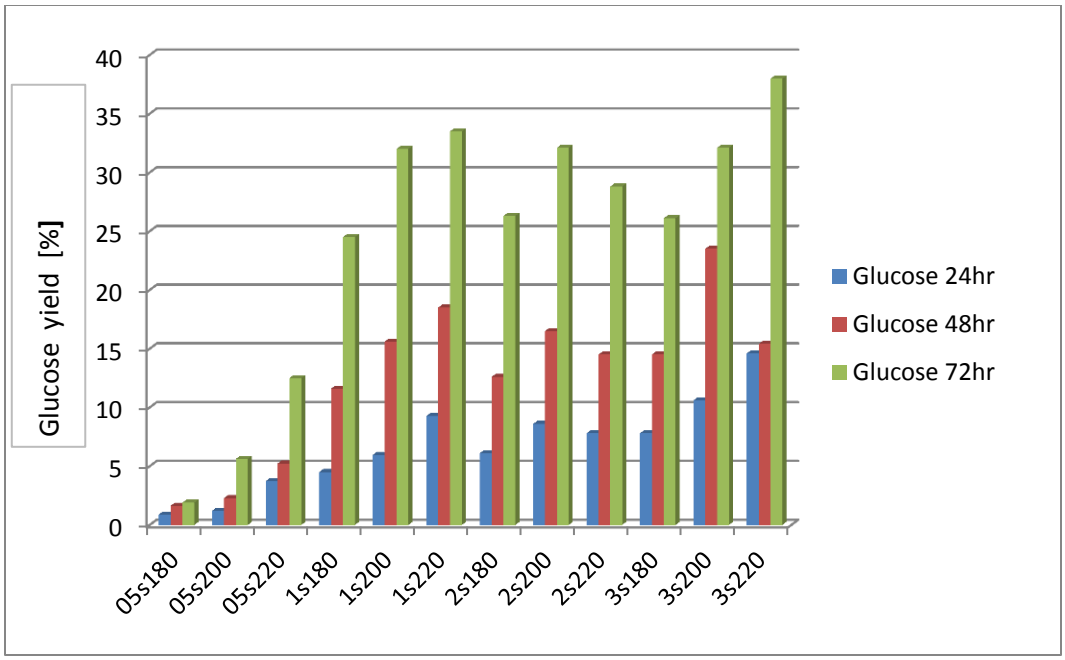

Figure-6.1. Glucose concentration after enzymatic saccharification of pretreated sugarcane bagasse with (0.5%; 1%; 2%; 3%) HCl of 15min pretreatment

For 30min duration:

Table-6.2: Glucose yield at different time

Sl No.	Glucose 24hr	Glucose 48hr	Glucose 72hr
05s180	1.1	2.65	4.52
05s200	2.46	5.1	10.5
05s220	5.4	12.1	19.5
1s180	5.1	13.4	26.3
1s200	6.25	16.2	34.5
1s220	11.1	23.46	35.6
2s180	8.6	17.5	36.5
2s200	9.6	23.5	41.2
2s220	11.1	23.5	46
3s180	12.1	25.6	45.5
3s200	15	28.1	57.8
3s220	14.6	23.2	50.2

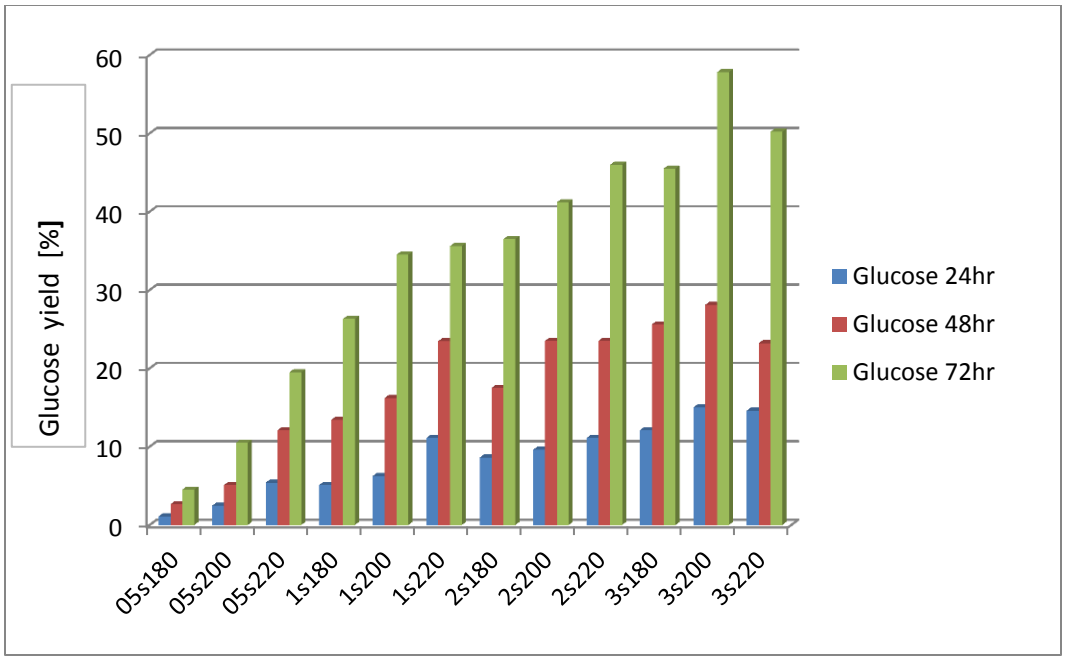

Figure-6.2. Glucose concentration after enzymatic saccharification of pretreated sugarcane bagasse with (0.5%; 1%; 2%; 3%) HCl of 30min pretreatment

6.3 Analysis of butanol:

Butanol concentration with succeeding days:

Table-6.3: Butanol concentration vs glucose concentration after each day.

Sl No.	glucose (g/l)	Butanol(g/l)
0	15	0.00
1	14.325	0.40
2	13.35	1.10
3	11.025	3.60
4	8.6	5.40
5	0.4	13.50

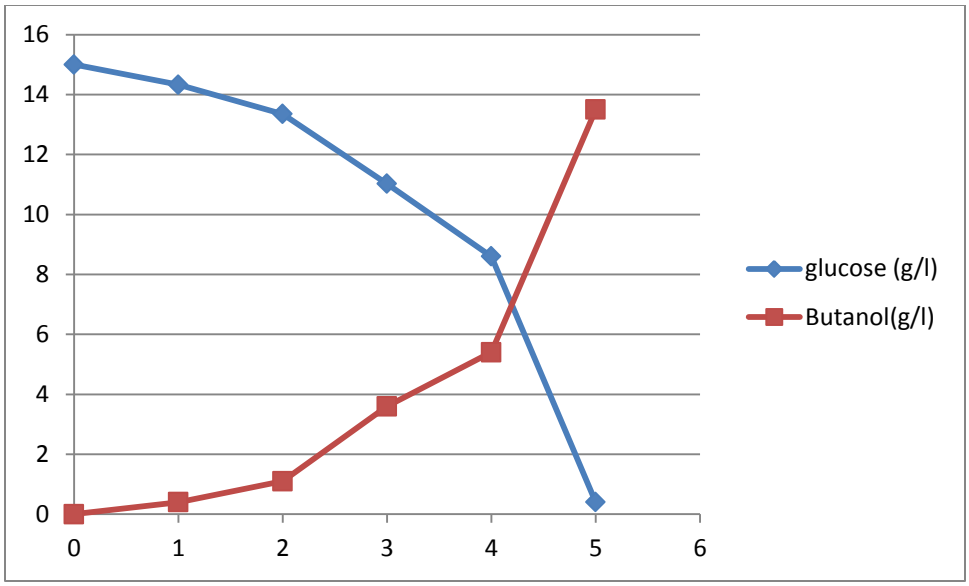

Figure-6.3 Butanol concentration vs glucose concentration

6.4 Discussion:

From sugar analysis at 200°C we are getting maximum yield of glucose concentration. For a 15min process we are getting fewer yields compared to 30min process. For a time period of 72hr the glucose yield is more than from a period of 24hr & 48hr.At 3% HCl concentration we are getting more yields compared to 0.5% HCl concentration & 2% HCl concentration. For a 30min process the sugar concentration is more than a 15min process. A time period of 72hr gives more glucose than periods of 24hr & 48hr. From the theoretical knowledge we know that we can get a maximum yield of 85%. From the result we are getting a yield of 0.924g/g with maximum glucose utilized.

6.5 Conclusion:

At a temperature of 200°C butanol concentration is more than at 180°C & 220°C and acid concentration of 3% in pretreatment maximum amount of glucose yield was obtained compared to 0.5%, 1%, 2%. The butanol concentration in g/lt increased proportionally with increase in days. The maximum yield of glucose is 57.82% after enzymatic saccharification & yield of butanol is 0.924g/g

6.6 Future work:

The sugar yield can be further optimized in future by changing the pre-treatment parameters. The glucose yield could be increased using antimicrobials in enzymatic saccharification. Additional supplementation of glucosidase could increase the yield of the glucose. The inhibitors analysis and effect to be studied in fermentation of 5-carbon sugar to biofuels on different organisms.

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