Production of Bioalcohol - Study of Various Routes Using Non Edible Renewable Resources

Project Report

By

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(12MCHE04)

Guided By

Dr. R. K. Mewada

DEPARTMENT OF CHEMICAL ENGINEERING

NIRMA UNIVERSITY

AHMEDABAD-382481

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Submitted in partial fulfillment of the requirements

for the Degree of

Master of Technology in Chemical Engineering

(Environmental Process Design)

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MAY 2014

Undertaking for Originality of the Work

I khyati Dipakkumar Bhatt, 12MCHE04, give undertaking that the project report entitled " Production of bioalcohol - Study of various routes using non edible renewable resources" submitted by me, towards the partial fulfillment of the requirements for the degree of Master of Technology in Chemical Engineering (Environmetal process design) of Nirma University, Ahmedabad is the original work carried out by me and I give assurance that no attempt of plagiarism had been made. Due acknowledgement has been made in the text to all other material used.

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This is to certify that

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Certificate

This is to certify that the project report entitled "**Production of bioalcohol - study of various routes using non edible renewable resources**" submitted by **Ms. Khyati Bhatt (12MCHE04)**, towards the partial fulfillment of the requirements for the degree of Master of Technology in Chemical Engineering(Environment Process Design) of Nirma University of Science and Technology, Ahmedabad is the record of work carried out by her under my supervision and guidance. In my opinion, the submitted work has reached a level required for being accepted for examination. The results embodied in this project report, to the best of my knowledge, haven't been submitted to any other university or institution for award of any degree or diploma.

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Abstract

Bioalcohols are attractive alternative to petroleum derived fuel. Combustion characteristics, engine performance , use of widely available low cost non edible biomass without competing with food and feed production leads to selection of biobutanol from bioalcohol family. Rice straw, corn stover and saw dust were hydrolyzed by applying acid hydrolysis pretreatment and fermentation of butanol was carried out by C. acetobutylicum species. Acetone and ethanol are also produced in this process. Hydrolyzates of these substrates resulted in production of butanol yield of 0.167 g/gmol, 0.212 g(or gmol)/gmol, and 0.070 g(or gmol)/gmol respectively. After confirmation of butanol production from non edible sources, effect of change in different parameters was examined. Initial Sugar concentration was changed to half of maximum concentration of rice straw resulted in 0.039 g(or gmol)/gmol yield. Initial concentration of saw dust was changed to maximum concentration resulted in 0.0455 g(gmol)/gmol butanol yield while at lower concentration butanol production was not observed. Seed culture concentrations were also changed but it did not effect to butanol fermentation and give similar results. Temperature apply to shaker bath was changed to 45 $\circ$C and room temperature from 37^{o}C , at 45^{o}C butanol was not observed and at room temperature results was similar to 37 ^{o}C.

Keywords: bioalcohol, biobutanol, rice straw,corn stover, clostridium acetobutylicum NCIM 2337, clostridium pasteurianum

Nomenclature

C. - Clostridium
ABE - Acetone Butanol Ethanol
RCM - Reinforced clostridium media
RCA - Reinforced clostridium agar
NCIM - National Collection of Industrial Microorganisms
MTCC - Microbial Type Culture Collection

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Chapter 1

INTRODUCTION

The search for feasible petroleum substitutes among renewable resources, include bioalcohol, biodiesels, and bioplastics, has become a global priority as atmospheric carbon dioxide levels continue to rise [1]. Biofuel, defined as a liquid, solid or gas fuel produced from biomass, is receiving increased attention in response to the depletion of petroleum fuels and environmental issues like global warming and climatic change, volatility of oil supply, increasing crude oil price, and existing legislations [2]. Further, the generation of biofuel may improve the local employment opportunities and contributes to the reduction of CO_2 emissions [3].

One set of promising alternatives to petroleum derived fuels are bioalcohols, alcohol molecul has additional oxygen therefore having a oxygenate properties leads to reduce combustion heat. In practice it is possible to use any compounds from the alcohol family as a biofuels i.e. bio ethanol , biomethanol, bioethanol biopropanol, biobutanol having a capability to use as bio fuel. Biofuels are generally regard as fuel additive in stead of petrolium substitutes. [4].Bioalcohol fuels are usually of biological rather than petroleum sources. When obtain from biological sources, they are known as bio alcohols (e.g. biobutanol). It is important to note that there is no chemical difference between biologically produced alcohols and those obtained from other sources. Bioalcohol are still in development and research stages. Use of optimized crops with higher yields of energy, elimination of pesticides and fertilizers, and a more rigorous accounting process will help to improve the feasibility of bioalcohol as fuels.

A comparatively known classification for liquid biofuel consist of term “First generation” and “ second generation” fuels. Recently popularized classification for liquid biofuel includes “First generation” and “Second generation” fuels. There are no stringent definitions for these terms,The main difference between them is the biomass used as substrate. Usually sugars, grains, or seeds, one that grow above ground biomass or specific portion of plant and which directly or by simple processing can be used to generation of biofuels. first generation fuels are being used in number of countries from world in a large quantities. Second generation fuels are usually consist of lignocellulosic biomass which are non edible or non edible parts of food crop production. grasses or trees having more energy are examples of second generation .They are not yet being commercially available in any country of world. [5]. Microbes and microalgae used as substrate in third generation biofuels have a potential to convert biomass to biofuel at significant level compare to other first and second generation biofuel and research is going on to overcome the limitation of first and second generation.

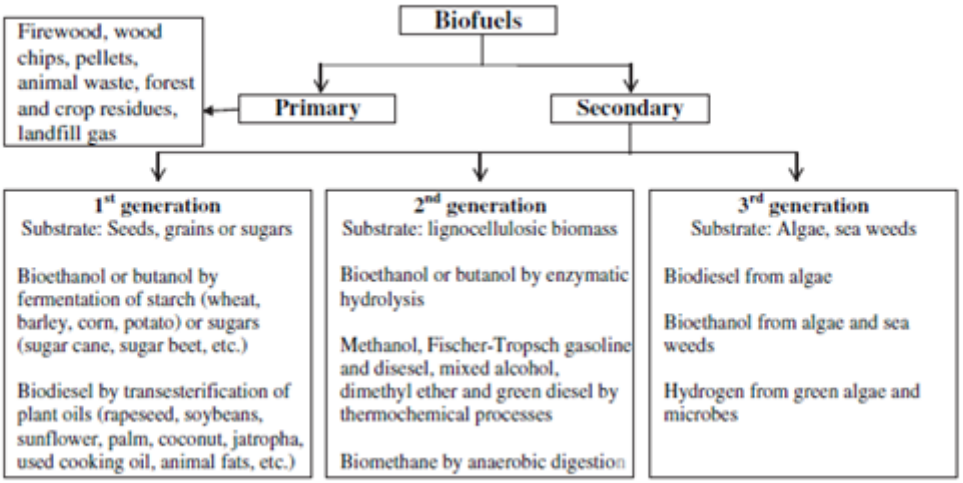

Figure 1.1: Classification of biofuel [6]

Bacteria from the genus clostridium can metabolize a wide range of sugars to produce solvents like butanol. Butanol like, ethanol can be used as a fuel or fuel additives but butanol holds several advantages over ethanol. butanol has 30% greater energy content, lower vapor pressure making butanol easier to store , lower sensitivity to water allowing butanol to be distributed through current gasoline pipelines while ethanol has to be hauled in trunks , lower flammability making butanol safer to handle and butanol can be used in current vehicles without any alteration to current engines [7]. One alternative route for the translate of biomass to alcoholic biofuel basically butanol and ethanol is through the route of acetone butanol ethanol fermentation. It is generally possible by the pretreatment of lignocellulosic biomass through chemical,physical,thermal,mechanical or enzymatic route in translate of fibrous cellulose and hemi cellulose to soluble glucose and other simple sugar. Example of widely used feed stocks are rice straw,corn stover, wood chip, sugar maple,sweet grass,saw dust etc. [8]. From olden times acetone ethanol and butanol fermentation by use of anaerobic microorganism from feed stocks are practiced.The acetone butanol ethanol fermentation is regarded to have huge ability for the industrial production of butanol. A vital compound with a huge marketplace with good properties to be used as alcoholic fuel from renewable and non edible sources.The contemporary butanol producing micro organisms are capable enough to get utilize sugars from cellulosic feed stocks.which formulate the acetone butanol and ethanol fermentation appropriate to be a part of cellulosic bio refineries for the good conversion of feed stocks derived sugars to biofuels. key limitations of these fermentation is high economy of the recovery methods for desired products. This fermentation process is product inhibitory reaction which leads to lethal effect for microorganism used , this product toxicity leads to law yield in batch fermentation. to overcome these limitations research are carried out to enhance the tolerance of microorganism strains to product using genetic and molecular techniques but have partial accomplishment [9].

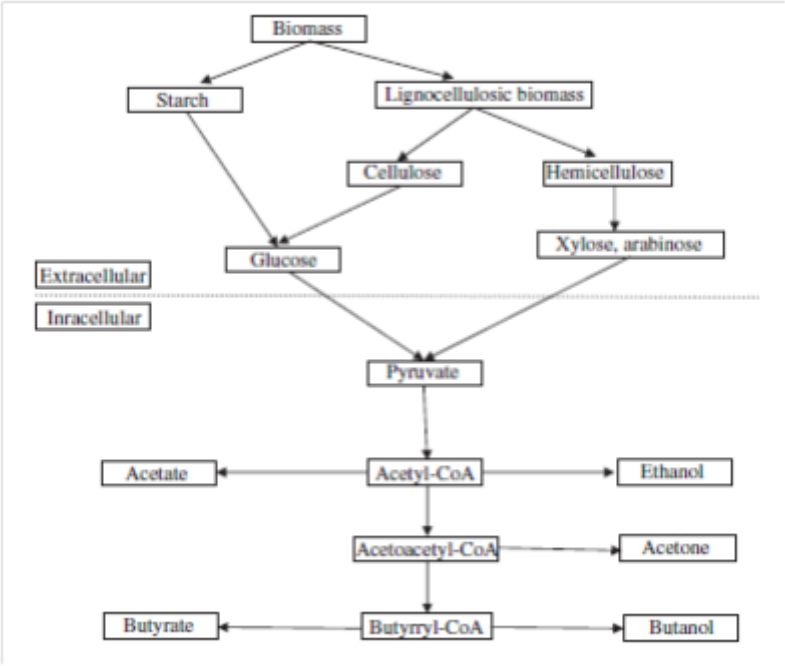

Figure 1.2: Pathway for butanol production [9]

1.1 Scope of the work

The overall goal of this work is to produce butanol from non edible resources. Lignocellulosic biomass obtained as agriculture byproducts and industrial residues can be used for biobutanol production. After pretreatment of biomass, materials are decomposed into simple sugar that can be metabolized by microorganism and converted to alcohol. However selection of bacterial strains and non edible biomass among various sources play imported role in yield of butanol production.

1.2 The objectives of this work

- The main objective of this project is production of bioalcohol from non edible renewable resources. For this butanol was selected from bioalcohol family.
- After production of butanol from non edible sources from one microorganism study of the effect of various microorganisms for butanol production and species with best result will used further for check various parameters.
- To study of different raw materials with one specific microorganism to check the ability of different raw material
- Study of effect of various parameters like concentration of seed culture, effect of concentration of initial sugar, and effect of temperature.

1.3 Organization of thesis

This thesis includes following chapters

- chapter 1st consist of introduction , bio alcohol definition, classification of bioalcohol, reasons for choose of butanol over ethanol, and pathway for butanol production.
- chapter 2 nd is about literature review of butanol, history of acetone , ethanol and butanol fermentation, properties of butanol, description of microorganism and brief explanation of separation and recovery techniques of butanol production.
- chapter 3rd comprises of experimental studies of project work with materials and methods which describes stepwise procedure for butanol production consist various steps like pretreatment, inoculum development and fermentation.
- chapter 4th is the results and discussion of each methods and depicts the results of butanol production, butanol production from various species of clostridia and different substrate, effect of change in various parameter on butanol production.
- chapter 5 th is conclusion it demonstrates that bioalcohol can be generated from route of non edible biomass fermentation.

Chapter 2

LITERATURE SURVE

2.1 Butanol

Butyle alcohol, or butanol is a linear four carbon aliphatic compound from alcohol family having a molecular formula of C_4H_9OH (MW 74.12 gmol-1).Butanol is a drab, combustible, somewhat hydrophobic fluid with an unique banana-like smell and solid alcoholic smell. It is totally miscible with most basic natural solvents, yet just sparingly solvent in water. when inhald in high concentration it cause irritant effect on mucous membranes and a narcotic effect. [10].

2.2 Butanol as Fuel

Butanol has many applications in industries but main important application is as a biofuel is nowadays recieved more attentation. Many reasons leads to received more attention While ethanol has received most of the attention as a fuel additive for many reasons. Butanol could be a better direct option due to its own intrinsic physical and chemical properties and energy content as compared to ethanol. This means butanol consumption is close to that of pure gasoline whereas ethanol-gasoline blends are consumed much faster to obtain the same power input. Additionally, butanol can be mixed with common gasoline at any percentage ratio in a similar way as with existing gasoline-ethanol blends (e.g., 23% in Brazil and 10% in United States and some parts of Europe. Also, butanol usage does not require any modifications in car engines or substitutions, producing similar mileage performance to gasoline. For instance, in 2005, David Ramey, drove a 13-year old Buick across the United States, fueled just by pure butanol with only a 9% consumption increase as compared to standard gasoline (petrol)[13].

Despite this small increase in biofuel consumption the emissions of CO, hydrocarbons and NOx pollutants were drastically reduced. This has a tremendous positive impact on the global environment.Other important advantages over ethanol include: (a) the lower volatility (less explosive). Butanol has a Reid Vapor Pressure (RVP) 7.5 times lower than ethanol ; (b) it does not readily adsorb moisture (lower hygroscopicity), so is less affected by weather changes; (c) less corrosive (Dürre, 2007); (d) is safer than ethanol because of its high flash point and lower vapor pressure; (e) it has a higher octane rating; (f) butanol has approximately 30% more energy/BTU accumulated per gallon (around 110.000 BTU per gallon, as opposed to ethanol, which has 84.000 BTU per gallon); and (g) complete miscibility with gasoline and diesel fuel. This allows butanol to be a much safer fuel that can be dispersed through existing pipelines and filling stations with simple integration into the present fuel delivery and storage infrastructure (pipelines, storage tanks, filling stations, etc.). Ethanol, on the other hand, can only be added shortly prior to use. The vapor pressure of butanol (4 mmHg at 20°C) is 11 times lower than

ethanol (45 mmHg at 20°C) enabling it to be directly added to gasoline without regarding evaporation emissions and consequent related complications. Also, the physical chemical properties of butanol makes possible the blending with gasoline with no phase separation in the presence of water (less readily contaminated with water) than other biofuel/gasoline blends. However, the viscosity of butanol is twice of that of ethanol and 5–7 times that of gasoline. Other physical properties of butanol, such as density and heat capacity, are somewhat comparable to that of ethanol [11, 12].

Table 2.1: Physical and chemical properties of butanol

Properties	Butanol	Gasoline	Ethanol	Methanol
Boiling point (°C)	117–118	27–221	78	64.7
Density at 20°C (g/ml)	0.8098	0.7–0.8	0.7851	0.7866
Solubility in 100 g of water	immiscible	immiscible	miscible	miscible
Energy density (MJ/l	27–29.2	32	19.6	16
Energy content/value (BTU/gal)	110000	115000	84000	76000
Air-fuel ratio	11.2	14.6	9	6.5
Heat of vaporization (MJ/kg)	0.43	0.36	0.92	1.2
Liquid Heat capacity (Cp) at STP (kJ/k-mol.^{o}K)	178	160–300	112.3	81.14
Research octane number	96	91–99	129	136
Motor octane number	78	81–89	102	104
Octane/Water Partition Coefficient (as logPo/w)	0.88	3.52±0.62	-0.31	-0.77
Dipole moment (polarity)	1.66	n.a.	1.7	1.6
Viscosity (10-3 Pa)	2.593	0.24–0.32	1.078	0.5445

2.3 Main Applications of Butanol

Furthermore the normal part as motor biofuel, butanol is really a paramount mass concoction with an expansive extent of mechanical employments. Butanol and inferred mixes are phenomenal diluents in acetones, water driven and brake liquid details. Different requisitions incorporate the production of security glass, cleansers, flotation helps (e.g., Butyle xanthate), deicing liquids, beatufiers (eye cosmetics, nail-mind items, shaving and particular cleanliness items). It is additionally generally utilized as concentrating executor and as a part of nourishment and flavor businesses[13].

2.4 Chemical Synthesis of Butanol

Butanol has been made modernly utilizing three real substance forms: Oxo amalgamation, Reppe combination, and crotonaldehyde hydrogenation In oxo blend (hydroformylation), carbon monoxide and hydrogen are added to an unsaturation utilizing metal impetuses, for example, Co, Rh, or Ru substituted hydrocarbonyl (Falbe, 1970). Aldehyde mixtures are acquired in the first response step, which is trailed by hydrogenation for the handling of butanol. Contingent upon the response conditions, for example, weight, temperature and kind of impetus, distinctive isomeric degrees of butanol could be acquired. In the Reppe combination, propylene, carbon monoxide and water are responded together in the vicinity of an impetus (Bochman et al., 1999) producing a mixture of n-butaraldehyde and isobutaraldehyde where the previous is diminished to n-butanol . The Reppe prepare straightforwardly prepares butanol at low temperature and weight. Then again, this methodology has not been economically fruitful since it

requires unreasonable innovation. Until a couple of decades prior, the basic course for butanol union was from acetaldehyde utilizing crotonaldehyde hydrogenation. The procedure comprises of aldol buildup, parchedness, and hydrogenation (Bochman et al., 1999). In spite of the fact that infrequently used these days, it might again get critical later on. While different courses of action depend totally on petroleum, the crotonaldehyde hydrogenation procedure gives an option course from ethanol which could be handled from biomass. For this situation, ethanol is dehydrogenated to structure acetaldehyde from which the union can continue[14].

2.5 Economics of the ABE-Fermentation

In 1996, the worldwide annual production of butanol was 2.49×10^6 tons. It has been estimated that around 10–12 billion pounds of butanol is produced annually , which accounts for 7–8.4 billion dollar (USD) market at current price. Butanol has a projected market expansion of 3% annually.In recent years several economic studies have been conducted on the production of butanol from various substrate sources and process layouts . In these studies it was found that recovery of butanol from the fermentation broth by distillation is totally uneconomical when compared with petrochemical derived butanol. Nonetheless, studies employing C. beijerinckii BA101, C. acetobutylicum P260, hydrolyzed DDGS (corn stover, corn fiber, and fiber-rich distillers dried grains and solubles) and wheat straw suggest that commercial production of biobutanol from agricultural wastes is moving closer. For instance, DuPont (US) and British Petroleum/BP (UK) have recently teamed up in a major effort to further develop and commercialize 1-butanol as well as other higher octane biobutanol isomers. Both companies also announced that testing of these advanced biofuels demonstrates the use of biobutanol can increase the blending of biofuels in gasoline beyond the current 10 percent limit for ethanol without compromising performance [16]. It is expected that the first plants would focus on sugar or corn starch; but, it is likely that agricultural waste residues, or their derived hydrolyzates, would become a potential carbon source instead due to their high abundance . If produced directly from a biomass source, there is no net carbon dioxide production [15].

Several recent advances have been performed including the development and optimization of microbial cultures (metabolic/genetic engineering and media formulation), process technologies, and use of waste substrates. However, all these advances will need to be translated into developed technologies and processes that can compete directly with the established petrochemical routes for butanol production. For example, many upstream studies have been focusing on the utilization of low cost by-products from various industrial activities as potential feedstock substrates. Some of these include: industrial waste water from palm oil, corn steep medium , black strap molasses (a secondary product of sugar industries) , corn fiber hydrolyzates , degermed corn , soy molasses , wheat straw hydrolyzates , corn steep water , whole potato media , and hemicelulose Hydrolyzates from the wood and paper industries . It is anticipated that future research might focus on the development of second-generation cultures (as compared to the existing strains of C. beijerinckii BA101, C. acetobutylicum PJC4BK, and C. acetobutylicum P260, which hyper-produce total ABE-solvents on the order of 25–33 gram per liter . Another way where technological advances could be made involves the recovery of fermentation by-products (large waste water streams, cell mass, CO_2, and H_2) for further revenue. For instance, CO_2can be converted into algal biomass and oil when exposed to sunlight. The use of carbon dioxide would benefit the biobutanol industry quite significantly since it is produced at zero cost. Moreover, H_2 gas can be separated and burned to generate electricity. Several studies are available regarding the economical evaluation and feasibility of the ABE-fermentation process .

2.6 Short Description of the Species

Individual vegetative cells of Clostridium acetobutylicum are straight rod-shaped bacillus ranging in size of 0.5–1.5×1.5–6 micrometer. They are Gram-positive in growing cultures but Gram-negative in older cultures, typically strictly anaerobes (oxygen free), heterofermentative, spore-forming and motile by peritrichous flagella. During sporulation, cells swell markedly and store granulose, a polysaccharides based material that serves has carbon and energy source during parthenogenesis. Spores are oval and sub terminal .The optimum growth temperature is 37°C, and biotin and 4-aminobenzoate are usually required as growth factors. ABE-clostridia strains are generally classified into four distinct groups based on their biochemical and genetic characteristics . The best known groups are the oenophiles C. acetobutylicum and C. beijerinckii (formerly known as C. butylicum) and one of the most documented strains in ABE-fermentation research studies [16].

2.7 Characterization of Butanol–producing Strains of Clostridium

A large number of solventogenic clostridia have been reported over the years . C. acetobutylicum harbors a large plasmid which carries the genes for solventogenesis. Loss of the plasmid causes instability leading to degeneration of the bacteria during long fermentation periods which is characterized by acid accumulation without any switch to solventogenesis . In C. beijerinckii, and most probably also in other butanologenic species, the solventogenic genes are localized in the chromosome. Both the chromosome and megaplasmid of C. acetobutylicum have been totally sequenced and the genes involved in acid and solvent production have been identified.

The primary type strain, C. acetobutylicum ATCC 824, was firstly isolated in 1924 from garden soil in Connecticut and is one of the best-studied ABE-solventogenic clostridia along with the C. beijerinckii NCIMB 8052 counterpart. Strain relationships among solventogenic clostridia have been analyzed , and the ATCC 824 strain was shown to be strongly correlated to the historical wild type “Weizmann strain”. The ATCC 824 wild-type strain has been physiologically characterized and used in a variety of molecular biology and metabolic engineering studies both in Europe and United States. DNA sequence analysis of the 16s rRNA gene of several representative strains have shown that the amylolytic C. acetobutylicum ATCC 824 is phylogenetically distant from the saccharolytic strains, including C. beijerinckii NCIMB 8052. A number of reports suggest that C. beijerinckii might have greater potential for the industrial production of solvents than does the previously sequenced C. acetobutylicum since the former has a wider substrate range and pH optimum for growth and solvent formation . The ATCC 824 wild-type strain is well known to metabolize a broad range of monosaccharides, disaccharides, starches, and other substrates, such as inulin, pectin, whey, and xylan, but not crystalline cellulose. A promising route to improve ABE-fermentation is the development of metabolic and genetically-modified clostridia with increased solvent production due to reutilization of carboxylic acids accumulated during the acidogenic phase of carbohydrate uptake, and increased resistance to product inhibition. Metabolic engineering allows the channeling of substrate consumption just to the formation of a specific solvent (e.g., butanol), if desired, resulting in high yields [17].

The C. acetobutylicum ATCC 824 strain has been transformed with a 192-kb megaplasmid designated by pSOL1, which carries a synthetic operon constructed to over-express three homologous acetone-formation genes: ctfA and ctfB encoding a multifunctional coenzyme A (CoA) transferase which transfers the CoA-moiety from acetoacetyl-CoA to acetate or butyrate, and ADC encoding acetoacetate decarboxylase. Subsequently, acetoacetate is decarboxylated to

form acetone, and acetyl-CoA and butyryl-CoA are converted to ethanol and butanol . Therefore, over expression of those genes results in significant increase in ABE solvents formation and decrease in carboxylic acids concentrations. Modification in solvent production in genetically manipulated strains of C. acetobutylicum ATCC 824 due to induced suppression of the solventogenic genes has also been described . Contrary to the super-expression of the solventogenic genes, the prior induction of those genes (suppressed solvent synthesis) resulted in highest solvent production and butanol tolerance reported up till now. Therefore, this strategy appears to be the most promising biotechnological approach for strain enhancement in future commercial applications of ABE-fermentation[16, 17].

The hyper-amylolytic/butanologenic C. beijerinckii BA101 strain generated from C. beijerinckii NCIMB 8052 (formerly just C. acetobutylicum) using chemical mutagenesis . Even though the hyper-butanol producing C. beijerinckii is slightly more tolerant to butanol than the 8052 parent strain, it does not means that it produces more butanol. Recently, pilot plant studies on butanol production by C. beijerinckii NCIMB 8052 parent and mutant BA101 strain in inexpensive glucose/corn steep water medium has been described. The results confirm that C. beijerinckii BA101 grows well and is easy to handle in this simple, cheap medium which is suitable for industrial application (Parekh et al., 1999). Moreover, C. beijerinckii BA101 may be more adaptable to continuous processes than C. acetobutylicum ATCC 824, since it appears to be more stable with respect to strain degeneration. Availability of the genome sequence between these two strains will enable the application of DNA microarrays, gene expression profiling, and comparative genomics in order to better understand the phenotypic differences that exist between C. beijerinckii NCIMB 8052 and C. acetobutylicum ATCC 824 (Ezeji et al., 2004a). The bacterium C. beijerinckii ATCC 55025 was derived from the C. acetobutylicum ATCC 4259 parental strain by treating the cells with aqueous ethyl methane sulfonate (mutagen). The resulting mutant is asporogenic, revealed high butyrate uptake rate, and good tolerance to high initial substrate levels and solvents produced [18].

Table 2.2: Different types of clostridium species used in butanol production

Title Of Reference Paper	Clostridia species	Substrate	Yield	Ref. No
Feasibility of rice straw as alternate substrate biobutanol production	C. acetobutylicum NCIM 2337	Rice straw	13.5g/l	[38]
Butanol Production from Lignocellulosic-based Hexoses and Pentoses by Fermentation of C. Acetobutylicum	C. acetobutylicum DSM 792	Lignocellulose	0.39 g/l	[?]
Butanol Production from Crystalline Cellulose by Cocultured C. thtermocellum and C.saccharoperbutylacetonicum N1-4	C. thermocellum and C.saccharoperbutylacetonicum N1-4	Avicel cellulose(crystalline cellulose)	0.41 g/l	[28]
Fermentation of dried distillers' grains and soluble (DDGS) Hydrolyzates to solvents and value-added products by solventogenic clostridia	C. beijerinckii BA101 C. acetobutylicum 260 C. acetobutylicum 824 C. saccharobutylicum 262 C. butylicum 592	DDGS	20.99, 18.70, 16.72, and 19.9 g/l	[44]
Butanol production by Clostridium beijerinckii. Part I: Use of acid and enzyme hydrolyzed corn fiber	C. beijerinckii BA101	corn fiber	0.39 g/l	[34]
Repetitive domestication to enhance butanol tolerance and production in Clostridium acetobutylicum through artificial simulation of bio-evolution	C. acetobutylicum D64	corn meal	15.3 g/L	[33]
Optimization of butanol production from tropical maize stalk juice by fermentation with Clostridium beijerinckii NCIMB 8052	Clostridium beijerinckii NCIMB 8052	tropical maize	0.27 g/g-sugar	[40]
Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by Clostridium acetobutylicum ATCC824	C.acetobutylicum ATCC824	hemicellulosic hydrolysate	7 g /l	[43]
Optimization of medium compositions favoring butanol and 1,3-propanediol production from glycerol by Clostridium pasteurianum	C. pasteurianum DSM525	Glycerol	0.98 g/L/h	[40]
Enhancing butanol production with Clostridium pasteurianumCH4 using sequential glucose–glycerol addition and simultaneous	C. pasteurianum CH4	crude glycerol, bagasse	0.307 mol/mol	[42]

2.8 Advanced Fermentation–separation Methods

Batch reactors are usually desired in the industry due to its simple mode of operation while reducing the contamination risk. However, the productivity attainable in a batch reactor is generally low due to the lag phase, product inhibition effects as well as the downtime for harvesting, cleaning, sterilizing, and re-filling the reactor. The preparation time and lag phase can be surpassed by using continuous operation and the problem of product inhibition can be resolved through the incorporating an in situ product removal system. One should note that a single-stage continuous operation is not feasible given to the complexity of butanol production by clostridia. To circumvent substrate inhibition and to increase the biomass, fed-batch mode of operation with intermittent or continuous feeding of nutrients has been used for butanol production. Moreover, immobilized cell reactors and cell recycle reactors have also been applied in order to increase the productivity [19].

Membrane cell recycle reactors are alternative to improve productivity. A hollow-fiber ultra filter was applied to separate and recycle the biomass in a continuous fermentation However, fouling of the membrane with the fermentation broth occurred revealing to be a major obstacle for this system. Researcher proposed a way of overcoming this problem by allowing only the fermentation broth to undergo filtration by using the immobilized cell system[**?**]

2.8.1 Cell Immobilization and Fibrous–Bed Bioreactor

Whole-cell immobilization is presently a widespread technique for laboratory studies in many research fields also with reasonable application in large-scale industrial processes. Generally, cell immobilization can be defined as the physical confinement or localization of cells inside a bioreactor system, with preservation of its catalytic activity and stability, and which can be used repeatedly and continuously. Immobilization allows cells to get confined in a favorable and compatible micro-environment, protecting them from potential harmful reaction media (e.g., organic solvents) and against external shear-stress forces developed inside biocatalytic reactors when freely-suspended cell cultures are utilized . This methodology is not only applicable to microbial cells but also to purified enzymes and animal and plant tissues or even to cell organelles. The industrial use of immobilized cell systems is still limited though, including in ABE-fermentation, and further application will depend upon the optimization of immobilization procedures that can be readily affordable for scaling up.

Fibrous matrices have been developed as support material for cell immobilization because they provide high specific surface area, high void volume, low cost, high mechanical strength, high permeability, and low pressure drop inside reactors. The fibrous bed bioreactor (FBB) with cells immobilized in the fibrous matrix packed in a column reactor has been successfully employed for several organic acid fermentation, such as butyric and lactic acids, with large increased reactor productivity, final product concentration and yield. Other advantages of the FBB include efficient and continuous mode of operation without the need of repeated cell inoculation, elimination of the lag phase, good long-term stability, while enabling simplified downstream processing. The high reactor performance of the FBB can be attributed mainly to the high viable cell density maintained within the bioreactor as a result of the exclusive cell immobilization mechanism on the porous fibrous matrix. Conventional immobilized cell systems normally lose fermentation productivity over long operation periods when the cells are used continually or repeatedly in a continuous or fed-batch fermentation, due to restricted mass transfer and the buildup of dead biomass. Reactor blockage and channeling effects are also frequent to occur, resulting in reactor deterioration with consequent efficiency loss and inoperability. Thus, for stable long-term bioreactor performance, the cells must be renewed continuously to maintain high productivity and avoid culture degeneration. Another advantage

of the FBB is that aged, latent, non-viable and non-productive cells can be immediately removed from the fermentation system and the cell density inside the bioreactor can be adjusted to prevent clogging[21].

Cell immobilization by adsorption onto fibrous matrices usually occurs through three stages: transport of the freely-suspended cells from the bulk liquid onto the fiber surface, cell adhesion at the surface and consequent colonization along the surface. Cell growth in the fibrous matrix can be controlled by the supply of growth nutrients available in the fermentation medium. Upon cell growth the cells get gradually retained at the solid surface until all the available area for immobilization is completely exhausted. The cell-to-fiber adhesion is carried out by simple physical adsorption due to long-range forces such as van der Waals forces and electrostatic (ionic) interactions, and short-range interactions, e.g., dipole interactions and covalent binding established between the bacterial cell wall and the fiber surface. In other cell immobilization systems involving absorption a multiple cell-layer often develops forming a thick biofilm. Although easy to perform, mild on the cells, non-specific character, and potentially free of diffusion limitations, this adsorption method presents some constraints. Since the adhesion process depends on the balance between opposing attraction and repulsion forces in both electrostatic and hydrophobic interactions; changes in the pH, temperature, ionic strength, surfactant concentration, culture age, and the presence of shearing forces may easily lead to cell leakage from the support. Cell detachment can be overcome partially by using fibers with irregular surface or tailor-made surfaces such as modified cotton fabric [**?**, 21].

2.8.2 Butanol Recovery Techniques

High product recovery cost is another problem in the microbial production of butanol. The traditional recovery by using distillation (extraction) suffers from a high operation cost due to the low titer of butanol in the fermentation broth caused by product inhibition. In addition to low product concentration, the boiling point of butanol is higher than that of water (118°C). This makes butanol recovery by distillation very demanding energetically. Philips and Humphrey (1985) evaluated the economics of butanol recovery from fermentation broth using distillation showing that energy savings by a factor of several orders of magnitude can be attained if the final concentration of butanol is increased from 10 to 40 gm per liter. This factor suggests that an enormous amount of energy can be saved if the butanol concentration in the fermentation broth is increased threefold. In order to improve recovery performance and reduce the costs, multiple complementary techniques have been thus investigated including condensation (in situ gas stripping and pervaporation), liquid–liquid extraction, adsorption, and reverse osmosis

Reverse osmosis is most attractive,from an economical perspective. However, it has disadvantages of membrane blockage and fouling. On the other hand, liquid–liquid extraction has high capacity and selectivity, although it can be expensive to perform. There are advantages and disadvantages of using each recovery technique, which need to be carefully analyzed.

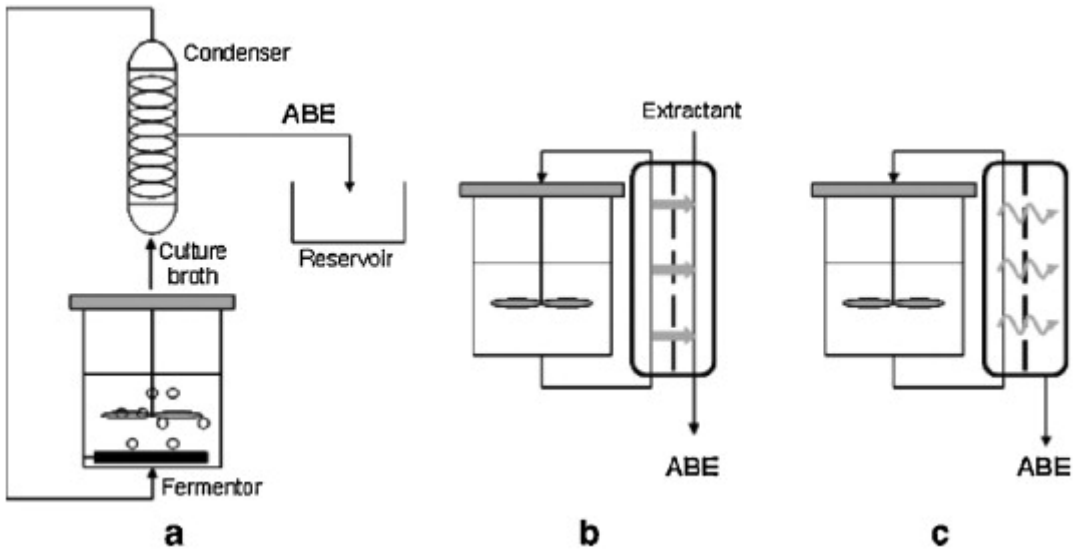

Figure 2.1: Integrated systems for fermentation and in situ solvent recovery: fermentation coupled with (a) gas stripping, (b) liquid–liquid extraction (Perstraction), (c) pervaporation [40]

Gas stripping is a simple and efficient way to recover butanol from the fermentation broth. The fermentation gas is bubbled through the fermentation liquid and then passed through a condenser for solvent recovery. The stripped gas is then recycled back to the fermentor and the process continues until all the sugar in the liquid medium is consumed. Gas stripping enables the use of a concentrated sugar solution in the fermentor and a reduction in butanol inhibition and high sugar utilization[23].

Liquid–liquid extraction is an efficient technique to remove solvents from the fermentation broth. This approach takes advantage of the differences in the distribution coefficients of the chemicals. Because butanol is more soluble in the extractant (organic phase) than in the fermentation broth (aqueous phase), it is selectively accumulated in the extractant. Common extractants include decanol and oleyl alcohol . Liquid–liquid extraction has critical problems, however, such as the toxicity of the extractant to the cell and emulsification. These problems can be overcome if the fermentation broth and the extractant are separated by a membrane that provides high surface area for butanol exchange between the two immiscible phases; this method is named ‘‘perstraction’’[24]

Pervaporation is a membrane-based process that allows selective the removal of volatile compounds from the fermentative broth. The membrane is placed in contact with the fermentation broth and the volatile liquids or solvents diffuse through the membrane as a vapor which is recovered by condensation. Both liquid and solid pervaporation membranes have been used. A liquid membrane containing oleyl alcohol was used in pervaporation of dilute aqueous butanol solutions . The selectivity of this liquid membrane was better than that of a silicon rubber membrane. When pervaporation using an oleyl alcohol liquid membrane was employed for the pretreatment of butanol purification, the energy requirement was ten times less of that of conventional distillation. To develop a stable membrane having a high degree of selectivity, Qureshi and co-workers (1999) synthesized a siliconsilicalite-1 composite membrane which showed a 2.2-fold enhancement in selectivity[25].

It should be highlighted that the recovery and purification processes are directly affected by the performance of fermentation, which in turn is affected by the strain characteristics. For example, when a strain is metabolically engineered to produce butanol without or much less acetone and ethanol, the purification process will be considerably simplified. When the butanol tolerance of a strain is increased by metabolic engineering, this will also facilitate the recovery

process as higher butanol concentration can be achieved during the fermentation. Thus, the overall process needs to be optimized from strain development to fermentation to downstream processes. This will lead to the reduction in overall production costs.

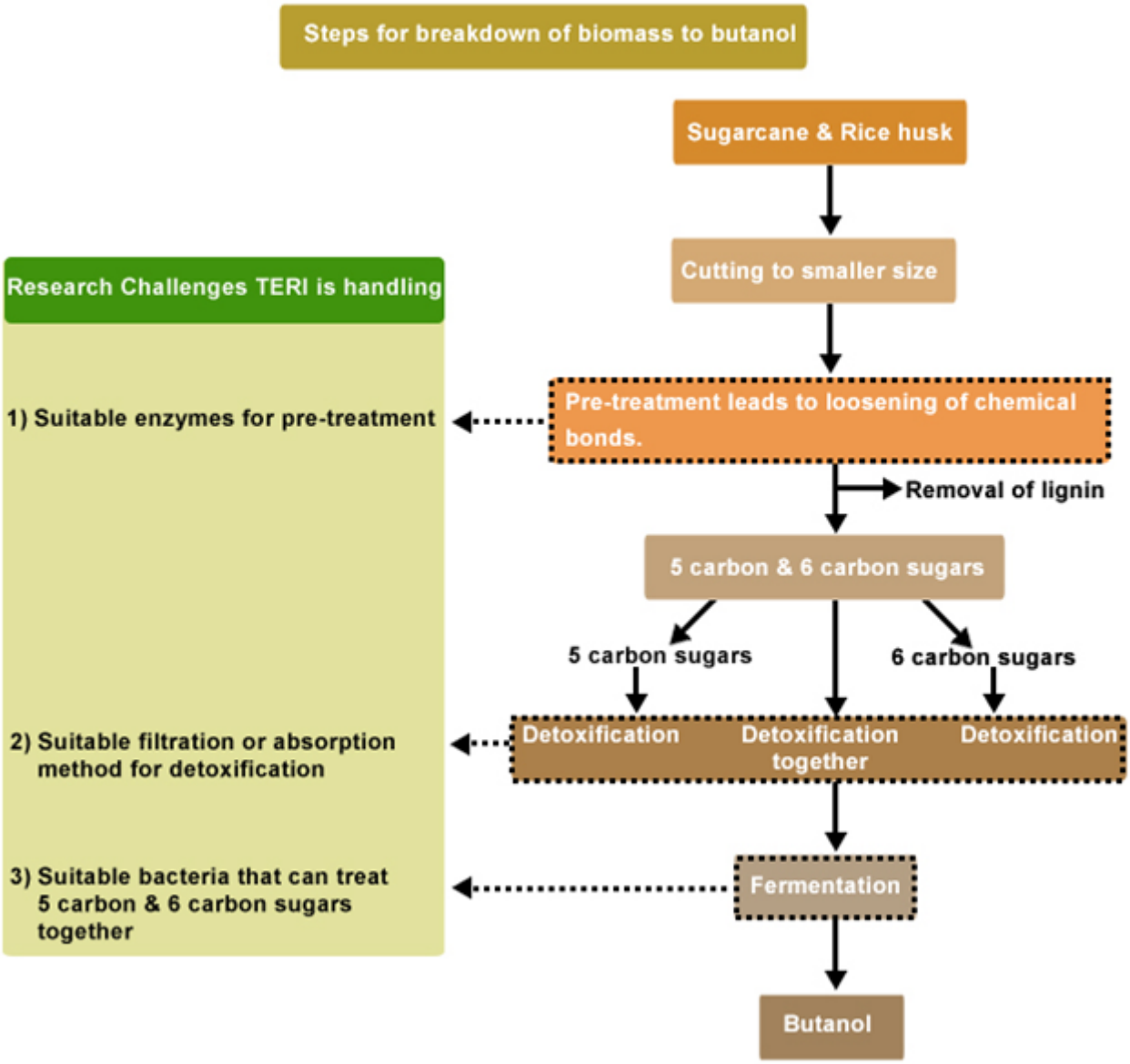

Figure 2.2: Process for butanol production [26]

In this review of literature, properties of butanol are compared with those of the conventional gasoline, diesel fuel, and some widely used biofuel, i.e. methanol, ethanol, biodiesels which shows advantages of higher heating value, lower volatility, less ignition problems, good inter solubility with diesel without any co solvents, more suitable viscosity as a substitute to diesel fuel, a safer fuel to use in high temperatures, easier distribution through existing pipelines, with disadvantages i.e. increasing the fuel-flow due to a lower heating value compared with the gasoline or diesel fuel, incompatible with some fuel system components, a lower octane number than the low-carbon alcohols. Then, the development of n-butanol production is reviewed, and various methods to increasing butanol production are introduced in detailed. Clostridia can secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into monomers. The major limiting factors associated with ABE fermentation include product toxicity. This problem can be eliminated using fed-batch techniques or continuous culture with the application of novel product removal techniques i.e. gas stripping, pervaporation, extraction.

Based on the literature survey carried out, C.acetobutylicum NCIM 2337 species was selected. C.acetobutylicum shows good activity and selectivity for bioalcohol. Rice straw, corn stover and saw dust was selected as substrate biomass because of its easy availability and it requires simple and easy pretreatment. From the results of substrates fermentation, saw dust was further used to evaluate effects of various parameters on biobutanol production.

Chapter 3

EXPERIMENTAL METHODS

3.1 Chemicals

- Reinforced clostridium agar
- Reinforced clostridium broth ,Dinitrosalicylic acid, Sodium sulfite, Sodium hydroxide, Potassium sodium tartrate, Anthrone reagent, glucose, cysteine hydro chloride.

(All chemicals were procured from Himedia and CDH chemicals and used without further purification)

3.2 Culture maintenance and growth

Two different bacterial strains of the Clostridium genus were used in the present study. Dried spores of C. acetobutylicum NCIM 2337 from sand were obtained from NCIM pune and C. pasteurianum were obtained from MTCC Chandigarh.

Spores were germinated anaerobically inside an anaerobic culture jar system (Himedia) in RCA and RCM (Broth) culture media at 37 °C. The inoculums were prepared in RCM containing following components (in 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; cysteine hydrochloride, 0.5. The pH of medium was 6.8 ± 0.2. 100 mL of media was autoclaved at 121 °C, 15 lb pressure and inoculated in a specially fabricated 250 mL screw capped Erlenmeyer flask. [10][11]

3.3 Inoculum development

Revived spores of clostridium culture were incubated anaerobically on Reinforced clostridium agar plates inside an anaerobic jar system (Himedia) at 37 °C until active growth seen. After 48 hours anaerobically successfully incubation period colonies of clostridium seen. From that Single clostridial colony was shifted to Reinforced clostridium media broth, and was reincubated at 37 °C. A result of optimization studies indicates 37 °C as optimum temperature. 5% of actively growing fresh culture (after lag phase of 18–20 h) of the corresponding strain subsequently added to experimental vessel in order to obtain a final seed culture[27]

Figure 3.1: Anaerobic jar

3.4 Substrate hydrolyzate

Rice straw, corn stover and saw dust were used as substrate for butanol production. Saw dust was obtained from local furniture shop which was in powder form. Corn and rice stover was taken from a farmer (Bavla, Ahmadabad), It consists of husk, leaves , stalks, and cob of the maize plants and rice left in field after grains harvest. Collected rice and corn straw is agricultural waste used as packaging material for shifting of some furniture's and equipments. The crude composition of rice has been reported to be cellulose: 32–47%; Hemi cellulose: 19–27%; lignin: 5–24%; ashes: 18.8% and sugar composition as; glucose: 41 43.4%; xylose: 4.8– 20.2%; arabinose: 2.7–4.5%; mannose: 1.8%; galactose: 0.4% Strips of rice and corn straw were debarked and chipped into small pieces of length 4–5 cm. After washed with water these were air dried at 80 °C in hot air oven for 24 hr tp remove moisture content. Corn and rice straw was grind in a mixer grinder to further reduction in size. To convert the hemicellulose of rice and corn stover or polysaccharides of saw dust into sugar monomer. 5% w/w mixture of substrate, i.e. 75 g substrate (rice husk, corn stover or saw dust) added to 1500 ml acidified distilled water (1% H_2SO_4) was taken into 2L beaker. Put beaker under mechanical stirrer for 24 hr at 60 °C set rpm at 200, the resulting liquid solution was than subjected to autoclave at 121 °C for 15 min at 15 lb pressure for further releasing of sugar and was allowed to cool at room temperature. The hydrolysate containing monosaccharide was subjected to filtration, which removed suspended particle. The treated hydrolysate was filtered with use of sterile cotton cloth. The pH of filtered hydrolysate was 1[28, 10].

Figure 3.2: Pretreatment of biomass

3.4.1 Anthrone test for total sugar measurement of rice straw and corn hydrolysate

Principle: Carbohydrates are dehydrated with concentrated $H_2SO_4$to form "Furfural", which condenses with anthrone to form a green color complex which can be measured by using UV spectrophotometer at 620nm. Anthrone react with dextrins, monosaccharides, disaccharides, polysaccharides, starch, gums and glycosides. But they yields of color where is to form carbohydrate to carbohydrate.

Procedures

- Prepare mixture of 4 ml Anthrone reagent and 3 ml of sample in a lightly capped test tube. Same as prepare a mixture of 4 ml Anthrone reagent and of 3 ml working standard in a test tube.
- After giving high temperature around 85-90 °C for 15 minutes it develop the red- brown color.
- Cool to room temperature in a cold water bath, measure the absorbance with a spectrophotometer at 620 nm[29].

3.5 Fermentation

5 different 250 mL of screw capped Erlymentry flasks containing 50 mL of substrate hydrolysate were used as anaerobic fermentor. Acidified substrate hydrolysate was neutralized at 6.5 pH using 5 M NaOH. These solution were subjected to autoclave at 121°C for 15 minutes, after cooling in room temperature flasks were inoculated with 6 ml of actively growing culture.

0.5 g/l cysteine hydrochloride was added to these flasks in order to provide the anaerobic condition. Sample were taken at regular time interval throughout the fermentation course for the analysis of biomass, substrate and products concentrations[30]

Figure 3.3: Fermentation process using shaker

3.6 Analysis

for the analysis of pretreatment 5 ml of sample was taken at regular time interval. first sample was taken after 6 hr of pretreatment, than 12 hr,18 hr 'after 22 hr sample was collected at time interval of 1 hr anthrone test was carried out for find out total sugar concentration. these results give concentration of sugar released from biomass. growth study of micro organism species were done by measuring optical density at 575 nm with use of UV spectroscopy. concentration of products i.e. acetone , butanol and ethanol were quantified by gas chromatography.

Chapter 4

RESULTS AND DISCUSSION

The presentation of this thesis is divided into two phases. The first phase was the production of butanol from non edible sources and with use of two different clostridium species. rice straw , corn stover and saw dust chosen as non edible sources, especially in regard to its product conversion efficiencies. The second phase was designed to improve the butanol fermentation based on the defined parameters of the fermentation. The results from these two phases are presented in the following section accordingly.

4.1 Pretreatment

One of the key steps in the lignocellulosic biomass to fermentable sugars conversion is pretreatment. The major goal of pretreatment is to disrupt the lignocellulosic structure into momosaccharide so that enzymatic hydrolysis of the polysaccharides to monomeric sugars can be achieved with greater rapidity and yield, prior to ABE fermentation[31]

Due to the large variations of the chemical compositions of agro-residues, especially polysaccharides and lignin, it is important to choose an appropriate method of biomass fractionation for their effective biotechnological utilization. In this context, the complex cellular structure of the agro-residues must be fractionated into C_6 or C_5 sugars with minimum side (or by)products. Thus the procedure applied will be a hydrolysis and/or a pretreatment, depending on the purpose of the released sugars. Due to the combination of several sugars and for presenting majority part of amorphous structure, the hemicellulose is more soluble in water and easily degraded than cellulose. In the lignocellulosic materials the cellulose and lignin are intimately linked, because hemicellulose acts as glue between those fractions. Next to cellulose, lignin is the most abundant and important polymeric organic substance in the plant world. Lignin increases the mechanical strength properties[32]

The composition of various substrate used in this project are mainly cellulose, hemicellulose and lignin. It is reported that rice straw will consist approximately dry weight bases of 28.7-35.6 % cellulose, 11.96-29.3 % hemi cellulose, 15.4-20 % lignin, corn stover 35.1-39.5% cellulose, 20.7-24.6% hemicellulose,11-19.1% lignin and saw dust will consist of 45-51% cellulose,25-28% hemicellulose,10-21% lignin

Hydrolysis of this lignin and hemicellulose is required to be removed and form simple monomer sugar, elevated reaction rates and significantly advanced cellulose hydrolysis can be achieved by dilute acid hydrolysis.

The achievement of high levels of sugar after pretreatment is a crucial factor for the commercial competitiveness of the use of lignocellulosic materials. Pretreatment in the form of dilute acid hydrolysis is one of the most important cost contributing factors in overall bio conversion processing of agro-residues. The concentration of released sugars during pretreatment is

directly dependent upon the type of lignocellulosic material, composition of substrates, temperature, time, acid concentration, solid-to-liquid ratio and the reactors employed in the process. Biomass with high lignin content released a smaller amount of fermentable sugars due to its structural compactness[33]

The lignocellulosic biomass chemical composition differs with the source of plant species. Cellulose is the main constitute(s) of straws. Hemicellulose is the second major compound of these biomass sources. Cellulose is the most common polysaccharides in nature and consists of repeating units of cellobiose.

Among the factors that influence the efficiency of acid hydrolysis, temperature, reaction time and acid concentration are the most widely investigated factors. The acid concentration is considered one of the most important factors regarding the release of sugars. High concentrations of acid may decompose the hemi cellulosic structure, producing inhibitors and also causing damage to the equipment used. Therefore, an appropriate acid concentration is essential for acid hydrolysis of Lignocellulose. 1% of hydrochloric acid was used for pretreatment.

Temperature is also a crucial factor that effects directly the degradation of sugars into inhibitors, which eventually effect microbial metabolism. Temperature is directly connected to the energy waste of the process. In general, it is observed that mild temperature led to a significant recovery of sugars while higher temperatures caused more sugar degradation, aiding the formation of inhibitors. 60 $\circ$C was selected for 24 hrs pre treatment.

Hydrolysis consist of two stages, first one is impart shear stress by mechanical stirring at higher temperature and second is by autoclaving of acidified substrate solution which applies high temperature and pressure. Hydrolysis affects the porosity of fibres which helps in loosening of lignin and hemicellulose network .thereby converting the solid straw into smooth slurry that assists hydrolysis with release of most of the sugar[11]

Table 4.1: Release of sugar during pretreatment

Time(hr)	Total sugar (g/l)		
	Rice straw	Corn stover	Saw dust
6	15.09	47.8	8.59
12	20.69	51.32	12.36
18	31.14	55.26	16.51
22	37.78	57.98	27.84
23	38.01	58	27.96
24	38.89	58.11	28

Total sugar concentration during the first 6 hrs of rice straw pretreatment was 15.09 g/l , corn stover pretreatment was 47.8 g/l and saw dust was only 8.59 g/l. reason for this differences are variation in composition of substrate. Corn stover consists of highest amount of cellulose which results in more amount of sugar release.

amount of sugar released in saw dust is lower than two substrates because saw dust used in this experiment contains less % of cellulose. Other reason is during pretreatment of saw dust many compounds such as Furfural, HM, acetic acid, leyunic acid, formic acid and phenolic compounds could be generated at an inhibitory level. Resin adsorption and activated charcoal adsorption are Methods to remove these inhibitors from saw dust hydrolysate can be employed for more sugar to be released[33]

Three different substrate used in the present study have resulted in release of varied amount of glucose from the processed substrate.

4.2 Growth study of C.acetobutylicum NCIM 2337 and C. Pasteurianum

c.acetobutylicum NCIM 2337 and C. pasteurianum clostridium species were used in this project in order to evaluate the ability of different clostridium species to butanol production. all experiments were conducted with c.acetobutylicum NCIM 2337 while C. pasteurianum was used to compare the ability of clostridium species for conversion of sugar to butanol, therefore rice straw and corn stover were experimented with both species.

Figure 4.1 shows a colony growth from spore of c.acetobutylicum NCIM 2337. For enumeration, reinforced clostridial agar was used and pour plate technique was employed. after incubation of 48 hrs anaerobically inside anaerobic jar, colony growth seen. Reinforced Clostridial Agar contains casein enzymatic hydrolysate as sources of carbon, nitrogen, vitamins and minerals. Yeast extract supplies B-complex vitamins which stimulate bacterial growth. Dextrose is the carbohydrate source. Sodium chloride maintains the osmotic balance. In low concentrations, soluble starch detoxifies metabolic byproducts. Cysteine hydrochloride is the reducing agent. Sodium acetate acts as a buffer[25]

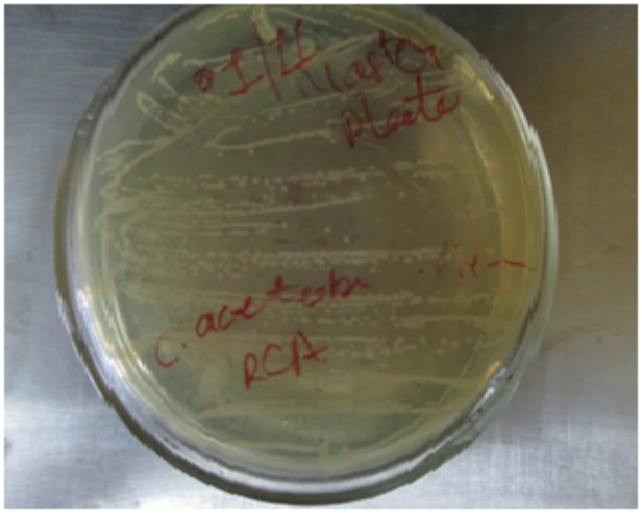

Figure 4.1: Colony growth of C.acetobutylicum

colony was shifted to reinforced clostridial broth to prepare seed culture. In fermentation procedure Substrate hydrolyzate was mixed with seed culture . Clostridium acetobutylicum NCIM 2337 and clostridium pasteurianum was grown during fermentation, utilizing substrate hydrolyzate as its feed stock. Fig 4.1describes batch fermentation of rice and corn stover hydrolyzate.

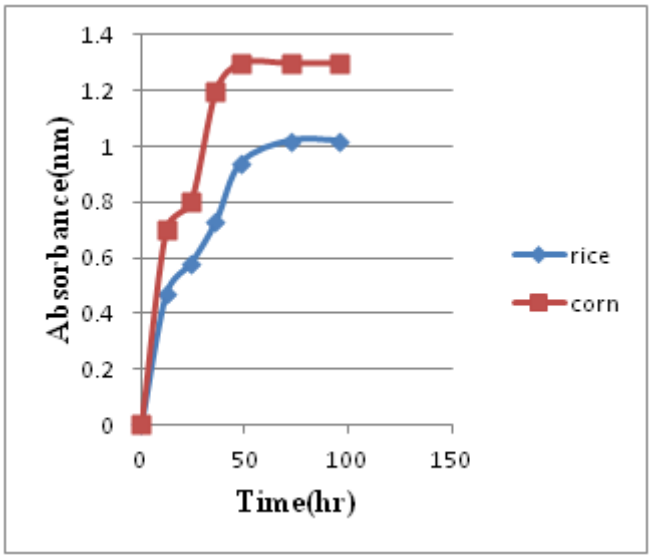

Figure 4.2: Growth curve of C.acetobutylicum NCIM 2337 in rice straw and corn stover hydrolyzate

The hydrolyzates of rice straw and corn stover obtained with acid hydrolysis were allowed to undergo anaerobic fermentation for 5 days fig 4.2 shows clostridial growth cycle with different feedstocks, samples were taken out at regular intervals to study the ABE production at various growth stages of C. acetobutylicum.

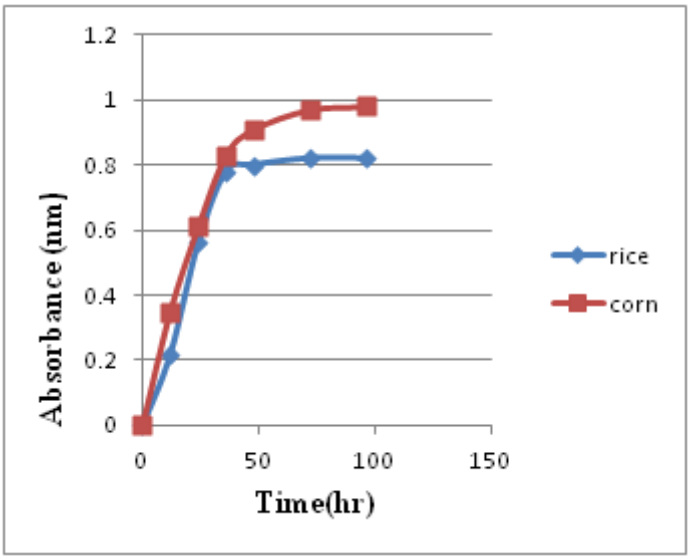

Figure 4.3: Growth curve of C. pasteurianum in rice straw and corn stover hydrolyzate

From figure 4.2 and 4.3 it is clear that growth of clostridial species in corn stover is more compare to rice straw. In both hydrolyzates as the time increases concentration of microorganism is start to increases, after reach to exponential phase growth of microorganism tends to enter into stationary phase in fermentation broth. There is not much deference in absorbance of two species. Initial 18 hr of lag phase seen in both species curve. Exponential phase was last for 24-48 hr followed by stationary phase of 48-96 hr. The lag phase of C.acetobutylicum, which is believed to be the phase at which clostridia adapts its nutritional environment, lasts for nearly 11–18 h. Exponential growth phase, during which bacteria show rapid growth in terms of count and size, continues for 17–18 h. This phase is acidogenic phase, during which bacteria produces acid and other precursors required for the production of solvents. After the end of 30

h of its life cycle, clostridia were observed to enter in a long stationary phase, during which the microorganism starts producing solvents (acetone, butanol and ethanol). This stage is known as solventogenesis. Solventogenesis stage starts with the sole production of acetone. Growth curve of clostridium species in the respective hydrolyzates demonstrate that the solventogenic clostridium species selected for this study are good butanol producers. Which justifies carrying out use of them .[21, 34]

4.3 Utilization of sugar

figure shows the pattern of consumption of sugars available in the rice straw hydrolyzate by use of species C. acetobutylicum NCIM 2337.

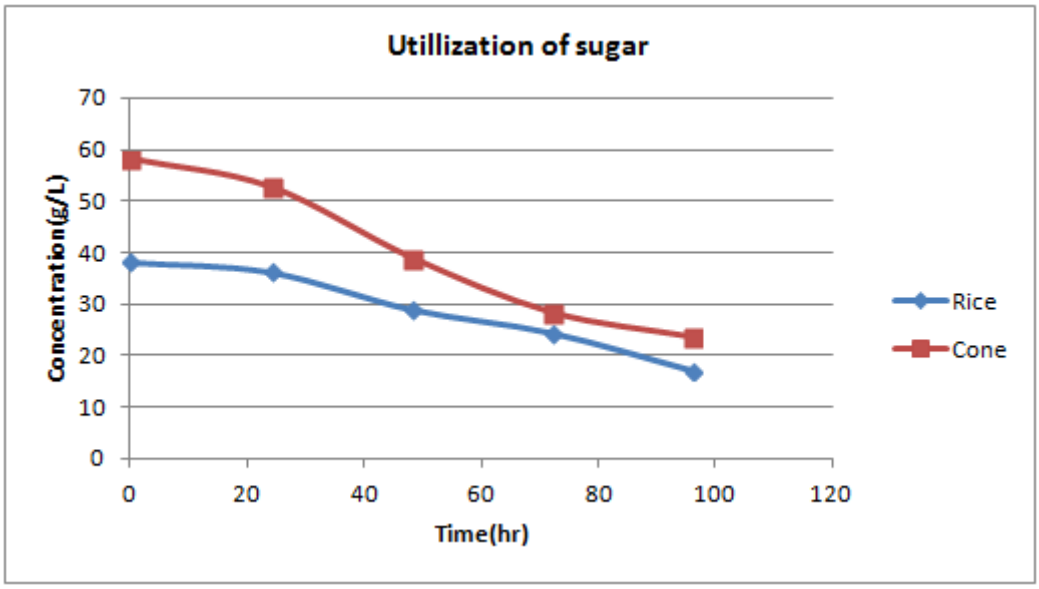

Figure 4.4: Utilization of sugar by C. acetobutylicum from rice straw hydrolyzate

It is clear from the figure 4.4 that as time increases sugar concentration decreases and conversion of biomass to fermentable product increases. corn stover gives maximum 59% of conversion compare to rice straw which give 55 % conversion.utilization of sugar is depend on the potential of microorganism used in the fermentation process.

It indicates that sugar utilization of substrate to convert butanol is based on initial concentration of sugar to the fermentation broth. Sugar concentration affect to the growth rate of clostridia species responsible for conversion. For utilization of sugar minimum initial sugar concentration must be required. To prove that experiments were conducted in which rice straw and saw dust hydrolyzate were fermented with different initial sugar concentration in first experiment rice straw experimented with 38.14 g/l, 19 g/l initial sugar concentration resulted in 54% and 47% of sugar conversion respectively. Same as saw dust also experimented with 28g/l, 56 g/l, 14 g/l and 7 g/l resulted in 68%, 71%, 43% and 14% respectively. rice straw and corn stover feed stocks contains inhibitors like fur fural , HMF etc, these inhibitor does not much affect to butanol conversion as compare to saw dust . saw dust contains phenolic compound which leads to cell lysis of microorganism and leads to poor yield of desired product and forms byproduct.It has been reported that higher carbohydrate utilization, higher stationary cell population and higher levels of butanol in the final fermentation broth if inhibitors present in less amount[34]

wene seed culture concentration varied from 3ml, 9ml, 12ml in saw dust hydrolyzate compare to 6 ml there is not major change in sugar utilization. which proves that consumption of sugar

depend on microorganism used and composition of inhibitors present in biomass.

To overcome this problems inhibitors removal process has to be applied after pretreatment. example of detoxification process uses resin or charcoal adsorption. Other option is develop improved microorganism strain by genetic technology to enhance capacity against tolerance compound.

4.4 Production of solvents during fermentation

In this study hydrolyzates of acid pretreated rice straw, corn stover and saw dust was used for butanol fermentation. Hexoses and Pentoses sugar of hydrolyzate supported growth and butanol fermentation by solventogenic clostridia species C.acetobutylicum NCIM 2337 and C. pasteurianum. Though the clostridia utilize other sugars such as galactose, mannose,cellobiose,arabinose and xylose, glucose is the preferred carbon source.

Prior to carrying out hexose based fermentation; a control batch fermentation was carried out using 50 g/l glucose as the carbon source in order to evaluate the ability of different clostridium species and substrate to produce acetone, ethanol and butanol production. Fermentation were run for 96 hr. butanol yield, and total (acetone, butanol, ethanol) yield were achieved during fermentation. These result demonstrate that the solventogenic clostridium species and substrate selected for this study are good butanol producer, which justifies carrying out further investigation with them.

Throughout the fermentation, law level of acids were present in the fermentation media.(data not shown), which indicates an efficient fermentation.

The lag phase or exponential phase of C. acetobutylicum during which bacteria show rapid growth in terms of count and size, continues for 17-18 h. this phase is acidogenic phase, during which bacteria produces acids and other precursors required for butanol production. After the end of this phase clostridia were observed to enter in a long stationary phase, during which the microorganism starts producing butanol. This stage is known as solventogenesis. Fermentation in hydrolyzate resulted in large amount of acetone and ethanol during the initial phase, which was found to be drastically reduced towards the end of stationary phase on the other hand, For the first 48 hr no butanol production was observed which steadily increased to reach a maximum concentration. at the end of stationary phase large amount of butanol were observed in all experiments[35]

4.4.1 Effect of different clostridium species

In order to examine the ability of two different clostridia species to get utilize sugar for butanol production, Rice straw and corn stover were selected. Rice straw and corn stover were fermented with both C.acetobutylicum NCIM 2337 and C. pasteurianum species. The fermentation with rice and corn hydrolyzate resulted with more yield of butanol by C.acetobutylicum. fermentation with C.acetobutylicum using rice straw as substrate resulted in 0.106 g/g . Total yield by C.acetobutylicum observed higher in rice straw while lower in corn stover hydrolyzate. One reason for higher total yield in corn stover by C. pasteurianum is higher production of ethanol and acetone compare to C. acetobutylicum[37]

Table 4.2: Comparison between clostridium species

Substrate	Micro organism	Sugar conversion(%)	Selectivity	Butanol yield	Total yield
Rice straw	C.acetobutylicum	54	0.311	0.167	0.182
Corn stover	C.acetobutylicum	59	0.36 0.	0.212	0.214
Rice straw	C.pasteurianum	49	0.278	0.136	0.149
corn stover	C.pasteurianum	55	0.397	0.218	0.228

4.4.2 Effect of different substrate on butanol production

The present methodology of stress assisted acid hydrolysis of lignocellulosic biomass has resulted in maximum total sugar concentration of 0.130 gmol, selectivity of butanol 0.36 butanol yield and total yield resulted 0.187 g/g , 0.190 g/g respectively from the 5 % w/v corn stover hydrolyzate while maximum 68% sugar was get utilized through fermentation of saw dust but due to presence of inhibitor could not get desired product. rice straw hydrolyzate also give a good result sugar conversion 54% , butanol selectivity 0.311, yield of butanol was 0.106 g/g and total yield 0.121 g/g.

Table 4.3: Effect of different biomass on butanol production

Substrate	Sugar conversion(%)	Selectivity	Butanol yield	Total yield
Rice straw	54	0.311	0.167	0.182
Corn stover	59	0.36	0.212	0.214
Saw dust	68	0.103	0.070	0.086

Despite higher % of sugar utilization by saw dust higher yield observed in corn stover and rice straw hydrolyzate because each type of biomass is unique in its composition and the respective hydrolysate requires a specific detoxification method. The concurrent sugar uptake and metabolism of these sugar is a desirable feature since it depend on the ability of solventogenic clostridia species to ferment substrate hydrolyzate[38].

Among the lignocellulosic biomass studied till date wheat straw contains least inhibitors while hydrolyzate obtained with acid hydrolysis of saw dust has a high lignin content, which could resulted in a high level of phenolic compounds. Phenolic compounds are more toxic than other inhibitors during fermentation. Even at reduced concentration levels might have induced cell autolysis and sporulation contributing to the early fermentation cessation[39]

4.4.3 Effect of initial sugar concentration

to evaluate the effect of initial sugar on butanol fermentation saw dust and rice straw selected among three substrates, and C. acetobutylicum used for seed culture.results are shown in table 4.5 and 4.6

Table 4.4: Effect of initial sugar concentration of saw dust

Initial sugar(gmol)	Sugar conversion(%)	Selectivity	Butanol yield	Total yield
0.049	68	0.103	0.070	0.086
0.088	71	0.075	0.053	0.078
0.044	43	0.0249	0.010	0.036
0.033	14	0	0	0

Table 4.5: Effect of initial sugar concentration of rice straw

Initial sugar(gmol)	Sugar conversion(%)	Selectivity	Butanol yield	Total yield
0.095	54	0.311	0.167	0.182
0.055	47	0.082	0.0385	0.063

From the table 4.5 and 4.6 it is clear that in the acetone, butanol, ethanol fermentation , the carbon source concentration in the medium must not fall below the threshold limit, which required to keep the fermentation solventogenic. Otherwise, the fermentation will tend towards acid production. It can be observed from the table 4.5 that butanol production was possible from 0.049gmol , 0.088gmol, and 0.044 gmol initial sugar concentration but for 0.033 gmol initial sugar concentration butanol production was not observed. It means that 0.044 gmol sugar concentration is threshold limit for butanol production while using saw dust as substrate.

while change in sugar concentration of rice straw there is also decrease in selectivity, butanol yield and total yield.

It has been reported higher carbohydrate utilization, higher stationary cell population and higher levels of butanol in the final fermentation broth, if law level of inhibitor compounds are present[40]

4.4.4 Effect of seed culture concentration

These experiments were also carried out by C. acetobutylicum microorganism using saw dust as substrate.

Table 4.6: Effect of seed culture concentration

Seed culture	Initial sugar(gmol)	Sugar conversion(%)	Selectivity	Butanol yield	Total yield
3	0.069	58	0.128	0.074	0.11
9	0.040	73	0.133	0.097	0.12
12	0.050	67	0.171	0.114	0.13

Seed culture concentration was changed to evaluate the effect on butanol production i.e. 3 ml, 9ml, 12ml but there is not much difference in butanol production compare to regular 6 ml seed culture. experiment which carried out to compare capability of different substrates for convert butanol 6 ml seed culture was used for saw dust , by use of 3 ml , 9 ml, 12 ml resulted in butanol yield of 0.066 g/g, 0.088 g/g, 0.122 g/g.

4.4.5 Effect of temperature variation

Temperature is important parameter to survive or maintain growth of microorganism.

Table 4.7: Effect of temperature

Temperature	Sugar conversion(%)	Selectivity	Butanol yield	Total yield
45	0	0	0	0
room temperature	69	0.172	0.114	0.139

$37 \circ$C temperature is reported as optimum temperature for microorganism growth, therefore $37 \circ$C temperature was maintained constant for all studies. Furthermore one experiment was conducted at 45 $\circ$C and other one was conducted at room temperature to check effects

of temperature on fermentation. As can be seen from table... results obtained for room temperature are same as 37 ∘ C, but at 45 ∘ C microorganism could not survive and no solvent production observed[41]

Chapter 5

CONCLUSION

This study demonstrated that the feasibility of producing butanol from non edible lignocellulosic biomass hydrolyzate in fermentation. In India, rice straw, corn stover and saw dust forms most abundant agro residue and renewable source. These feed stock has thus tremendous potential for alcoholic biofuel production. Among the three substrates corn stover has liberated highest amount of sugar (0.322 gmol/l) by acid pretreatment. Species Clostridium acetobutylicum NCIM 2337 and clostridium pasteurianum were capable of utilizing all the three kinds of substrate hydrolyzate but C. acetobutylicum was able to convert more amount of sugar than c.pasteurianum. Entire fermentation studies depicts that performance of corn stover hydrolyzate was best among all substrates as it contains higher amount of sugar concentration and least inhibitors which results in more amount of sugar utilization. Saw dust has capability to utilize higher % of sugar but due to presence of phenolic inhibitors very law yield of butanol produced.

In this project work non edible renewable resources were used to fermentation of bio based fuel. Reason for selection of non edible sources is restrict the feed vs food debate. Substrates which are used in this studies are agro residue and easily available in India. These substrates contains higher amount of sugar but these sugar is complex in structure which restrict microorganism to utilize them directly. To overcome this problem pretreatment must be required.which convert these complex sugar to simple form. Acid hydrolysis was selected for pretreatment. As per review from literature clostridia species are most used microorganism for anaerobic fermentation. As per availability in India, C.acetobutylicum NCIM 2337 and C. pasteurianum species were selected. C. acetobutylicum NCIM 2337 was used for all experiments to evaluate effect of different parameter.

from this study it is clear that threshold initial sugar must be require for microorganism for their growth and conversion process of sugar to alcoholic fuel . For rice straw and saw dust threshold sugar concentration was 0.055 gmol/l and 0.044 gmol/l respectively. seed culture concentrations were also changed but there was not change observed. Two experiments were carried out to check effect of temperature on butanol production. 45^{o}C and room temperature was selected, C. acetobutylicum bacterial species was unable to grow at 45^{o}C, while there is not much change observed at room temperature compare to 37^{o}C.

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