Aalto University
School of Chemical
Engineering

Praniesh Ayyanar Ramachandran

ACETONE-BUTANOL-ETHANOL (ABE) FERMENTATION BY CLOSTRIDIUM ACETOBUTYLICUM USING XYLOSE RECOVERED FROM BIRCH WOOD HYDROLYSIS

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Supervisor	Professor Sandip Bankar
Instructor	M.Tech. Vijaya Chandgude

Author Praniesh Ayyanar Ramachandran

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Abstract

The focus of this study is to find the efficiency of xylose and birch wood hydrolysate in Acetone-butanol-ethanol (ABE) fermentation. There is a need to find an effective raw material for the commercial production of butanol which was the reason for this work to be carried out. The literature studies include the background and biochemistry of the process, different types of microorganisms used for this process, pretreatment methods for hydrolysate and detoxification of hydrolysate. The experimental part of this thesis is about finding the effect of glucose and xylose, effect of electron carriers, effect of different concentration of the potent electron carrier and the efficiency of birch wood hydrolysate in ABE fermentation. The results obtained are compared with the results from previous works of different researchers and the discussion are done based on it. The results showed that 60:40 (xylose:glucose) is the best ratio as it yielded 7.99 g/L of overall solvent concentration and also lower residual acid of about 2.68 g/L. Methylene blue was found to give higher solvent concentration of about 9.48 g/L when compared to other electron carriers. The concentration of 0.1 mM methylene blue produced higher solvent concentration and A:B ratio which was about 9.47 g/L and 2.82:1. From this work, it was found that birch wood hydrolysate can be very effective for the ABE fermentation if proper pretreatment and detoxification methods are carried out.

Keywords ABE fermentation, glucose, xylose, electron carrier, birch wood hydrolysate

Preface

First of all, I would like to thank Professor Sandip Bankar and Professor Herbert Sixta for giving me a chance to do my thesis in a very good topic at the right time of my studies. Their guidance and inputs were very helpful for finishing my thesis. Next, my thanks to my advisor Vijaya Chandgude for helping me at different stages of my experimental work.

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Abbreviations

ABE	Acetone-Butanol-Ethanol
AIL	Acid Insoluble Lignin
ASL	Acid soluble lignin
HMF	Hydroxymethylfurfural
MB	Methylene blue
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
CoA	Coenzyme A
CoAT	CoA transferase
TA	Transaldolase
TK	Transketolase
PTA	Phosphotransacetylase
AK	Acetate kinase
PTB	Phosphotransbutyrylase
BK	Butyrate kinase
BADH	Butyraldehyde
BDH	Butanol dehydrogenase

1 Introduction

1.1 Background

Currently, the depletion of fossil fuels created an urge to find an alternative renewable fuel which is environmental friendly. Biomass is found to be an excellent source for producing the biofuel through fermentation which gives solvents such as acetone, butanol and ethanol. These solvents play an important role in energy and fuel industries nowadays.

Ethanol is one of the biofuels that is commonly used as its blending has been increased to 85 % in engines. Whereas, butanol has many advantages over ethanol such as high energy content, high blending percentage with gasoline and no need for engine modification [1]. Butanol has almost same energy as gas so it gives same mileage which is a great advantage. These advantages attracted the researchers to conduct more researches to find whether it could be an alternate for ethanol as biofuel. Butanol can be produced by Acetone-Butanol-Ethanol (ABE) fermentation using microorganism like *Clostridium* species. However, the economic viability of bio-butanol is low when considered to bio-ethanol due its complex and costly production process. The low yield of butanol leads to difficulties in the downstream processing which is a very important factor in industrial production. It has also been observed that bio-butanol production by ABE fermentation gave lower fuel heating value when compared to the bio-ethanol production. These are the main reasons which affects the commercialisation of bio-butanol [2]. Nowadays, many researches have been conducted to find the best raw material for the production of butanol as raw material cost affects the overall production cost. Lignocellulosic biomass are the commonly used raw material for bio-butanol production due to its high availability and low cost. The problems associated with bio-butanol production have to be studied in detail to develop an efficient industrial production process.

The importance in overcoming these factors which affect the bio-butanol commercialisation has led to the initiation of my thesis. It is very crucial to find out the low cost feedstock and the effect of addition of secondary chemicals like electron carriers in butanol production which may be helpful in rectifying the problems mentioned above. The birch wood hydrolysate was used for ABE fermentation in this work. One of the reasons for choosing it over other biomass was due to the high availability of birch wood in Finland which is very essential for the continuous production of butanol. It is also highly available in neighbouring

countries like Russia and Sweden which will be helpful in industrial perspective [3]. Another reason was that it has high composition of xylose which can be used as an alternate to glucose as carbon source for ABE fermentation, it was also the area of focus in this work. Different types of electron carriers were used to check its effect in butanol production as addition of electron carriers were found to be an effective way for increasing the butanol production by different researchers [4].

1.2 Goal, Objectives and Scope

The goal of this thesis was to find an economical and efficient raw material for the commercial production of butanol. This thesis includes three main objectives. The first objective was to study the utilization of xylose as the carbon source for Acetone-Butanol-Ethanol (ABE) fermentation. The second objective was to compare and identify the most effective electron donors for enhancing the butanol production. The third objective was to check the utilization of Birch wood hydrolysate for the commercial production of butanol.

2 Literature review

2.1 Acetone-Butanol-Ethanol (ABE) fermentation

2.1.1 Overview of ABE fermentation

The butanol production through microbial fermentation was reported at first by Pasteur in 1861 whereas the acetone production was first reported only in 1905. During the late 19th century many researchers conducted studies on biobutanol production by anaerobic bacteria. Later the production of synthetic rubber got interest due to depletion of natural rubber. In 1910 Strange & Graham, Ltd with the help of Perkins and Weizmann found that the best way to produce synthetic rubber involved the use of butanol or isoamyl alcohol. Due to this the microbial fermentation of butanol was investigated. As of result of several researches the first industrial plant for butanol production along with acetone from potatoes was began in 1913 Strange & Graham, Ltd by using Fernbach's bacillus. *Clostirdium acetobutylicum* was first isolated by Weizmann which he initially named as BY. It has the ability to produce higher yield of butanol and acetone. During the World War I *Clostirdium acetobutylicum* was used for the commercial production of acetone which is the important solvent for cordite production. After the end of war production from fermentation was declined due to development in automobile industry, cheaper and economical methods. During that time butanol was used mainly for the butylacetate preparation which was used for quick drying lacquers. The need for butanol was increased gradually which led to the spread of ABE fermentation plants in several countries [1].

2.1.2 Biochemistry of ABE fermentation

In ABE fermentation, the solvents (butanol, acetone and ethanol) are produced in a ratio of 6:3:1 on weight basis along with hydrogen and carbon dioxide. The key for increasing the performance of ABE fermentation is to increase the butanol/acetone ratio which is generally 2:1. Many researches have been conducted in the past to get higher butanol/acetone ratio, such as using genetically modified strains, mixed substrates, addition of electron donors to supply more NADH. But in most of the cases the higher butanol/acetone ratios were obtained by losing total solvents productivities [5]. The pH of the culture and the production of solvents are key factors in industrial process due to economic reasons. By knowing the biochemistry of the process the production can be improved by optimizing the necessary parameters. There are several pathways involved in the production of biobutanol such as Embden-

Mererhof-Parnas (EM) pathway, Entner-Doudroff pathway and Pentose phosphate (PP) pathway. The hexose sugars are utilized by the microorganism using EM pathway in which 2 mole of ATP and NADH are produced. The pentose sugars are utilized through PP pathway which produces 5 mole of ATP and NADH. Ferredoxin which occurs in clostridia plays an important role in electron transfer as it both accepts and donates electrons at low potential [1]. The metabolism of xylose by *Clostirdium acetobutylicum* is shown in figure 1.

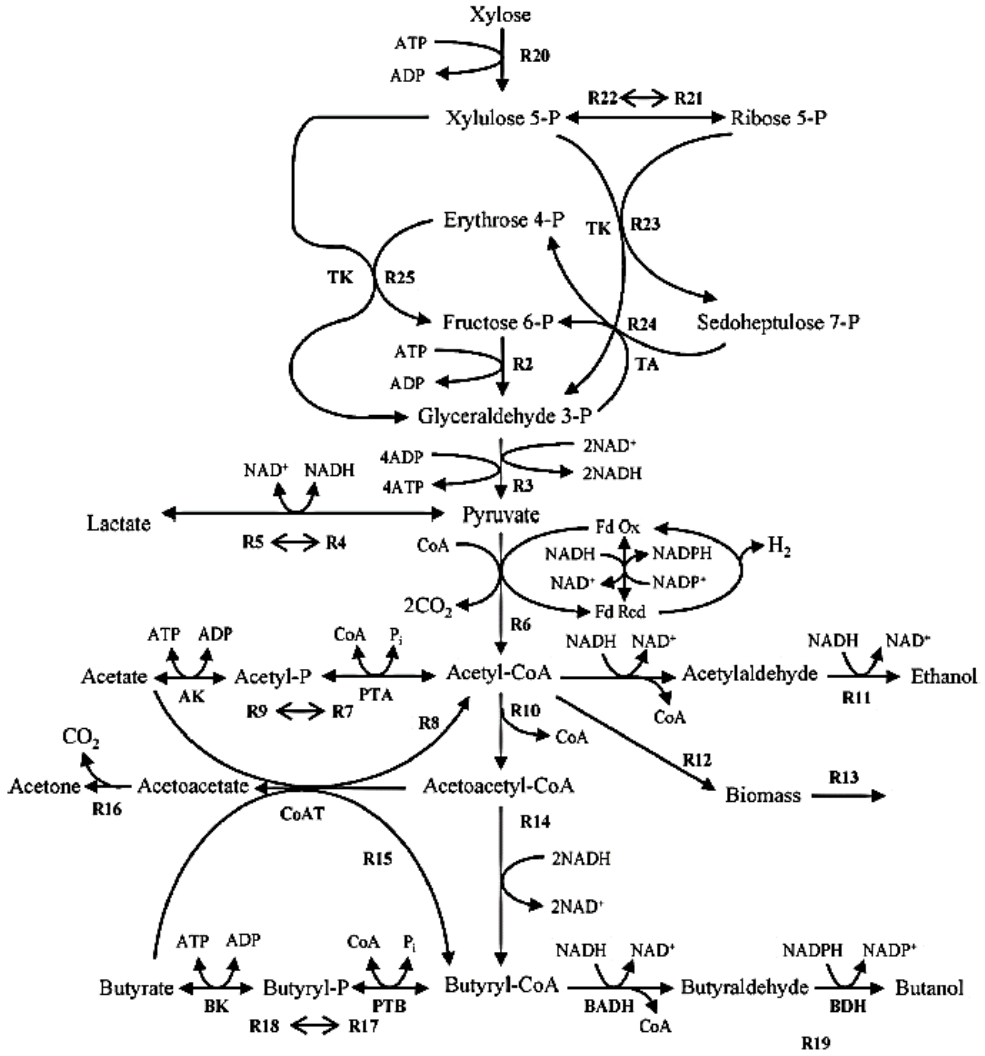

Figure 1. The xylose metabolic pathway in *Clostirdium acetobutylicum* [6].

2.1.3 Microorganisms in ABE fermentation

The organisms involved in ABE fermentation belong to Clostridia family which are anaerobic and Gram positive microbes. Many species of Clostridia have the ability to produce acetone, butanol and ethanol such as *C.acetobutylicum, C.beijerinckii, C.saccharobutylicum, C.pasteurianum, C.sporogens, C.aurantibutyricum*. Among these *C.acetobutylicum, C.beijerinckii* are found to be the high butanol producers [1, 7]. *C.beijerinckii* produces same

amount of solvents as *C.acetobutylicum* except that isopropanol is produced instead of acetone. The main advantage of Clostridium strains over other cultures are that they utilize both hexose and pentose sugars which are obtained from lignocellulose after hydrolysis for ABE production. It was found that potatoes, some root crops, cereal crops and agricultural soil are high sources of these strains. The use of strains in industrial process depends on the raw material used, ratio of solvents required and the need for additional nutrients [1].

2.1.4 Feedstock for ABE fermentation

In the early 20th century the major feedstock for ABE fermentation were food based such as maize, wheat molasses, potatoes which resulted in high production cost. The price of food based feedstock were around 60-80% of the total production cost [8]. It also increased the demand for food supply worldwide, both these reasons resulted in search of low cost, non-food based substrate. As the Clostridia species are able to utilize different types of carbohydrates it leads to wide range of feedstock options like lignocellulosic biomass, glycerol and sugar sources. The lignocellulosic biomass includes agro-industrial waste (corn stover, barley straw, bagasse, wheat straw and switchgrass) and wood chips [5, 9 and 10]. It was found that ABE fermentation utilizing lignocellulosic biomass produce nearly the same amount of solvents as pure sugars [5, 8, and 11]. As the fossil fuel are depleting recently, biobutanol production and use as biofuel have been of great interest because butanol characteristics are similar to gasoline. The low cost and higher availability of lignocellulosic biomass makes it a very good source for production of biofuels, chemicals and other value added products.

2.2 Pretreatment of feedstock

The lignocellulosic biomass consists of cellulose, hemicellulose and lignin which are not accessible for Clostridia as they don't have the enzymes to break them. For utilizing the sugars present in cellulose and hemicellulose the lignocellulosic biomass have to be pretreated and hydrolysed prior to fermentation. Several pretreatment methods are available and each has their own advantages and disadvantages, they are chosen based on the fermentation requirement.

2.2.1 Physical pretreatment

The physical or mechanical pre-treatment includes milling, grinding and chipping which reduce the size of the biomass and increase the surface volume ratio. Based on the requirement, the biomass is sent to chipping machine to get size of 10-30 mm and if the powder is required it is grinded further to 0.2-2 mm. It will be easy for the microorganisms to digest if the biomass size is smaller. Pyrolysis is also used for the pretreatment of lignocellulosic biomass, it uses high temperature (>300^{o}C) which convert the cellulose to gas and char [12].

2.2.2 Chemical pretreatment

In chemical pretreatment the chemicals are used to disrupt the biomass structure. The different kinds of chemical pretreatment are explained below.

2.2.2.1 Ozonolysis

Ozonlysis is one of the chemical pretreatment methods which degenerate the lignin and hemicellulose in many biomasses. Only the lignin and hemicellulose in biomass are degraded whereas the cellulose was not degraded. The advantages of this process is that it removes lignin effectively, don't form any toxic residue for downstream process, the process is carried out in room temperature and pressure making it a safer process but the disadvantage is that it is expensive due to higher requirement of ozone [12].

2.2.2.2 Acid pretreatment

Acid pretreatment has two methods, first one is weak acid (dilute acid) hydrolysis and the second one is strong acid (concentrated acid) hydrolysis. In dilute acid hydrolysis, diluted sulphuric or hydrochloric acid are used in concentration range of 0.5 % to 5 % (v/v) based on the processing time and biomass [13]. The dilute acid hydrolysis can be carried out at low temperature for low solids holding and high temperature for high solids loading. Recent processes use higher temperatures to obtain higher conversion of xylan to xylose [12]. The dilute acid is mixed with biomass Concentrated acid hydrolysis are known to degrade the hemicellulose to greater extent giving higher monomeric sugar yield but are highly corrosive

and dangerous to handle. It also requires high amount of base to neutralize the acid which results in high salt formation making it difficult for fermentation [10, 12 and 14]. The concentrated acid has to be recycled in order to make the process economically feasible. Due to these reasons dilute acid hydrolysis are mostly preferred.

2.2.2.3 Alkaline pretreatment

In alkaline treatment bases are used to remove the lignin from biomass and the mechanism of this treatment is based on saponification that crosslinks xylan hemicellulose, lignin and other hemicellulose [12]. The salts are usually formed in calcium or sodium hydroxide treatment, it should be removed before fermentation process. The process conditions are mild that prevents lignin condensation leading to high lignin solubility. As the conditions are mild the sugar degradation to furfural, HMF and organic acids are very low or prevented [15]. The dilute NaOH treatment for hardwood resulted in decrease of lignin content from 24-55% to 20% whereas it doesn't have any effect on softwood having lignin content over 26% [12]. Ammonia treatment at higher temperatures are also used to remove the lignin content and some of hemicellulose but decrystallise the cellulose. The cost and recovery of ammonia plays a key role in this pre-treatment [15].

2.2.2.4 Organosolv process

In organosolv process, the organic solvent or mixture of organic solvents are used with inorganic acid catalysts to remove lignin and hemicellulose. The major solvents used in this process are ethanol, methanol, acetone, ethylene glycol and triethylene glycol. The temperature of the process can be high or low depending on the type of biomass and catalyst used. Usually high xylose yield was obtained in addition to acid. The solvents used in the process can inhibit the growth of microorganisms and so they have to be removed before the fermentation process. The removal and recovery of solvents are needed for reducing the process cost and also for environmental aspect [12].

2.2.3 Biological pretreatment

In biological pre-treatment, the lignin and hemicellulose are degraded using microorganisms like brown, white and soft-rot fungi. Brown-rot fungi have it effect on cellulose whereas white and soft-rot fungi degrade both cellulose and lignin. White-rot fungi such as *Ceriporia lacerate, Stereum hirsutum* and *Polyporus brumalis* are mainly used for biological pre-treatment of lignocellulosic biomass. The advantages of this treatment are it requires low energy and environmental friendly process but the rate of hydrolysis is low and so the residence time is long [12].

2.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis is a heterogeneous process in which the cellulose are hydrolysed using cellulase enzymes. Enzymatic hydrolysis has many advantages over chemical pretreatment like higher sugar yields, lower utility cost, lower energy input, lesser inhibitory products and no corrosion problem [16]. The enzymes are produced by bacteria belonging to *Clostridium, Bacillus, Bacteriodes, Cellulomonas* and *Streptomyces* and fungi like *Sclerotium rolfisii*, species of *Trichoderma, Aspergillus* and *Penicillium*. Enzymatic hydrolysis is affected by several factors like porosity, temperature, chemical composition, agitation speed, polymerization and specific activity [16, 17 and 18]. The disadvantages of this method are low reaction rate, many enzymes are unstable at high temperatures and the separation of enzymes from the biomass after the process is difficult.

2.3 Detoxification of hydrolysate

Several byproducts including furan derivatives (furfural and 5-hydroxymethylfurfural (HMF)), phenolic compounds (vanillic acid, ferulic acid, syringic acid, and vanillin) and weak acid (acetic acid, formic acid) known as inhibitors are formed during the pretreatment process. Furfural and HMF are produced due to degradation of sugars that are released during hydrolysis, they are main inhibitors for microorganism growth [13]. Phenolic compounds are formed by lignin degradation; low molecular compounds are highly toxic to microorganism [19]. Acetic acid is formed from degradation of acetyl groups of hemicellulose, at higher concentration acetic acid inhibits the growth of microorganism [20]. Different detoxification

methods are used to remove these inhibitors in lignocellulosic hydrolysate prior to fermentation

2.3.1 Physical detoxification

Vacuum evaporation is commonly used to remove the volatile toxic compounds like acetic acid, furfural and vanillin. Furfural is effectively removed using this method. The problem in this method is that non-volatile toxic compounds are retained in the hydrolysate [14]. Increase in xylitol production after removal of acetic acid and furfural from lignocellulosic biomass was reported [21]. Membrane separation is another method used to selectively remove the inhibitors from acidic hydrolysates using adsorptive porous membranes. During the process the hydrolysate pass through the membranes and the inhibitors will be adsorbed to the membrane surface.

2.3.2 Chemical detoxification

In chemical detoxification, the inhibitors are removed after precipitating by pH change or adsorption with activated charcoal or ion-exchange resins. Due to highly acidic nature of the acid-pretreated lignocellulosic hydrolysates, they have to be neutralized before fermentation. In general, calcium or sodium hydroxide are used for neutralizing the hydrolysates (pH 6-7). In this process, furfurals and phenolics are removed to some level by precipitation. Many inhibitors are unstable at particular pH and so pH adjustment using $Ca(OH)_2$ is the commonly used method for several lignocellulosic hydrolysates. Over-liming has been found to be an effective detoxification method for the hydrolysates pretreated with dilute sulfuric acid [22]. The addition of calcium hydroxide in acidic hydrolysate produces gypsum that can be removed and used as plaster of paris which have many commercial values. This method removes the furfural and HMF with the additional sugar loss (~10%) by adsorption [23, 24].

The removal of inhibitors by activated charcoal detoxification is cost effective and sugar loss is very low [23, 25]. The effect of this treatment depends on several factors like pH, temperature, time and amount of activated charcoal. By adjusting the initial pH of rice straw hydrolysate from 0.4 to 2.0 and then adding 2.5 % activated charcoal resulted in removal of 72.9 % HMF, 89.3 % of furfural and 34.3 % lignin degradation products with sugar loss of less

than 11.5 % [13]. Ion exchange resins treatment is very effective in inhibitors removal if the pH of hydrolysate is adjusted to 10 but it is not cost effective and also cause considerable sugar loss [26]. Three types of resins (anion, cation and hydrophobic) were studied and it was reported that anion resin was more effective under pH 10 followed by hydrophobic resin and cation resin [27].

3 Materials and Methods

3.1 Culture and media

The sporulated cells of *Clostridium acetobutylicum* were activated by keeping in hot water bath (80^{o}C for 10 min). After the heat shock treatment, 2 ml of activated culture was inoculated in 100 ml of sterile reinforced clostridia medium (RCM) in 125 ml air tight anaerobic glass bottles (purged with nitrogen for 10 min to remove oxygen and then capped with rubber cork and aluminium seal, autoclaved at 121^{o}C for 20 min) and cooled down in ice bath before inoculation. After cooling to room temperature the culture was inoculated at 37^{o}C for 20 h. RCM medium contained (g/L) meat extract, 10; pep- tone, 5.0; yeast extract, 3.0, glucose, 30; starch, 1.0; sodium chloride, 5.0; sodium acetate, 3.0; L-cysteine, 0.50 and pH was adjusted to 6.8 ± 0.2.

The fermentation medium was used with a small modification to that reported in [25] which contained (g/L) ammonium acetate, 2.2; K2HPO4, 0.5; KH2PO4, 0.5; p-aminobenzoic acid, 0.1; thiamine hydrochloride, 0.1; biotin, 0.01; FeSO4, 0.01; MnSO4 H2O, 0.01; MgSO4, 0.2; NaCl, 0.01, both glucose and xylose were used as carbon source (60 g/L) in the ratios of 50:50, 60:40, 80:20, 90:10 to compare with the production in hydrolysate which has both the sugars in it. The pH of the fermentation medium was adjusted to 6.5 with HCl.

3.2 Hydrolysis of birch wood

The birch chips were screened according to SCAN-CM 40:01 (Scandinavian Standard, 2001) and were stored in freezer prior to the beginning of the experiments. The chips were defrosted and the dry matter content (53.8%) in it was determined by SCAN-CM 39 (Scandinavian Standard, 1994). The rotating batch air-bath digester with six autoclaves, each having the volume of about 2.5 L was used for hydrolysis. The dried wood chips were weighed and added to autoclave. The calculated amount of 0.1M sulphuric acid was added to right amount of deionised water. Then the solvents were added to the autoclave to make the liquid to wood ratio of 4:1. The reactor was heated to prehydrolysis temperature (120-200^{o}C) with minimum heating time of 35 min. The wood chips were treated for two hours, then the autoclaves were cooled by keeping in cold water. The free hydrolysate was removed from

wood residue by filtering and the remaining hydrolysate entrapped in the wood chips was separated by centrifugation. The obtained acid hydrolysate was then stored at 5^{o}C. Table 1 and table 2 show the composition of carbohydrates and composition of inhibitors in birch wood hydrolysate before detoxification.

Table 1. Concentration of carbohydrates in birch wood hydrolysate before detoxification

Component	**Monosugars, g/L**	**Total sugars, g/L**
Arabinose	0,8954	0,7771
Rhamnose	1,2155	0,9639
Galactose	1,6075	1,6076
Glucose	4,6490	4,2246
Xylose	43,0172	36,7083
Mannose	3,1536	2,7382

Table 2. Concentration of inhibitors in birch wood hydrolysate before detoxification

Component	**Concentration, g/L**
AIL	4,8544
ASL	10,7719
HMF	0,2002
Furfural	7,4452
Formic acid	4,5873
Acetic acid	7,1036

3.3 Detoxification and neutralization of hydrolysate

Activated charcoal was purchased from Sigma Aldrich. In this detoxification method, 40 g/L of activated charcoal was added to the hydrolysate and was kept in water bath maintained at 50^{o}C. The hydrolysate was manually stirred for 20 min. After this the hydrolysate was vacuum filtered using glass membrane to remove the activated charcoal. The filtered hydrolysate (acidic) was stored at 5^{o}C for further experiments. $Ca(OH)_2$ was used to neutralize the pH of the hydrolysate before carrying out the fermentation process. The neutralization step is important because the acidic hydrolysate cannot be used for ABE fermentation, it will affect the growth of *C.acetobutylicum.* The Concentration of carbohydrates and inhibitors in birch wood hydrolysate after activated charcoal treatment are given in tables 3 and 4. There was a 10.23% reduction in the amount of monosugars in the hydrolysate after detoxification

process. The AIL, ASL, HMF, furfural, formic acid and acetic acid were reduced by 90, 50, 67.3, 70.8, 20.1, 18.8% after the activated charcoal treatment.

Table 3. Concentration of carbohydrates in birch wood hydrolysate after activated charcoal treatment.

Component	Monosugars, g/L	Total sugars, g/L
Arabinose	0,7996	0,7817
Rhamnose	1,0547	0,9443
Galactose	1,4294	1,5750
Glucose	4,1410	4,2486
Xylose	38,7270	36,9132
Mannose	2,8025	2,9072

Table 4. Concentration of inhibitors in birch wood hydrolysate after activated charcoal treatment.

Component	Concentration, g/L
AIL	0,4854
ASL	5,3935
HMF	0,0653
Furfural	2,1733
Formic acid	3,6609
Acetic acid	5,7620

3.4 Electron carriers

Different types of electron carriers were used in the fermentation to improve the butanol production and butanol: acetone ratio. The electron carriers used were methylene blue (MB), phenosafranin, methyl viologen (MV), 2,2 bipyridyl, trimethylamine N-oxide (TMAO), tiron, nicotinic acid, sodium sulfide and glutathione. Most of them were already available in laboratory which were purchased from Sigma Aldrich, except for methyl viologen and tiron which were purchased from VWR, Finland. The electron carriers were chosen based on the literature studies and their ability to alter the electron metabolic flux. The electron carriers were prepared and filter sterilized using 0.2 μm filter membrane before addition to fermentation medium.

3.5 Batch fermentation

All of the fermentation experiments were carried out in 100 ml air tight, anaerobic glass bottles containing 70 ml of the production medium. The fermentation medium was purged with nitrogen to remove oxygen and then the bottles were capped with rubber cork and aluminium seal. They were autoclaved at 121^{o}C for 20 min and then cooled to room temperature. The fermentation medium was inoculated with 5% (v/v) of inoculum containing highly motile cells of *C.acetobutylicum* and incubated at 37^{o}C for 96 h. The anaerobic bottles were manually agitated once per day to maintain culture homogeneity. During the fermentation the samples were collected at regular intervals for analysis of butanol, acetone, ethanol, residual sugars and acids. All the experiments were carried out as triplicates and the results reported are the average of three fermentations.

In model experiments, fermentation medium containing different ratios of xylose: glucose (50:50, 60:40, 80:20, 90:10) were used to check its effect in butanol yield. The fermentation medium with 60 g/L of glucose and xylose were used as controls for all the experiments.

For screening experiments, 0.1 mM of electron carriers were used to study its individual effect on butanol yield. All the electron carriers were prepared as sterile stock solution and added as required to final concentration of 0.1 mM before fermentation in the production medium with xylose: glucose ratio chosen from previous experiments. After the screening experiments, the most effective electron carrier was chosen based on the sample analysis. Then the experiments with different concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1mM) of the selected electron carrier was carried out. The concentration at which the butanol yield and butanol: acetone ratio is higher was chosen for carrying out further experiments. Finally experiments based on time of addition (0, 6, 12 and 24 h) of the electron carrier was studied. The effect of addition time was investigated to see its effect on the microorganism growth, B:A ratio and butanol yield. All these parameters were optimized before fermentation with hydrolysate to obtain better butanol yield.

For fermentation with hydrolysate, the acidic hydrolysate was neutralized using $Ca(OH)_2$ to pH 6.5 prior to fermentation. The neutralized hydrolysate was purged with nitrogen to maintain anaerobic condition and autoclaved at 121^{o}C for 20 min. The fermentation was carried in the same conditions as mentioned above with the addition of optimum

concentration of electron carrier at the right time. The samples were collected at regular intervals and further analysis was carried out.

3.6 Analytical methods

The fermentation samples were collected and centrifuged at 14,000 rpm for 10 min, and the supernatant was used for further analysis after filtering with 0.45 μm. The solvents (acetone, butanol and ethanol) and acids (acetic and butyric acid) were analysed using gas chromatography. The residual sugars were analysed using HPLC equipped with Agilent HiPlex H 300x7.7 mm ion exclusion column. The eluent used was 5mM H_2SO_4 with a flow rate of 0.6 ml/min. The column temperature was maintained at 65^{o}C.

The sugars in the hydrolysate were measured using Dionex ICS 3000 HPAEC-PAD (Dionex, Sunnyvale, CA, USA) with CarboPac PA 20 column. The acid soluble lignin was measured using UV spectroscopy at 205 nm (Tappi UM 250 1991). The furfural, hydroxymethylfurfural (HMF), acetic and formic acids were analysed by Dionex UltiMate 3000 HPLC (Dionex, Sunnyvale, CA, USA) equipped with an ultraviolet (UV) diode array detector and an Acclaim OA column. The column was maintained at 30^{o}C.

4 Results and Discussion

4.1 Effect of glucose and xylose in ABE fermentation

The experiments were carried out using both glucose and xylose as the microorganism will first utilize glucose and then xylose for the butanol production [19]. It was reported that the conversion rate of sugar to solvent reduces in the order of glucose, arabinose, mannose and xylose [29]. The results of experiments with different ratios of glucose and xylose are shown in figure 2. From the figure it is clear that 60:40 (xylose: glucose) gave higher butanol concentration of around 5.61 g/L with the overall solvent concentration of 7.99 g/L followed by 50:50 which is 7.79 g/L. The total solvent production decreased after 60:40 resulting in an overall ABE concentration of 4.35 g/L in 80:20 and 3.69 g/L in 90:10. It was observed that lower ABE concentration was obtained in xylose control which is 3.01 g/L, this data is in agreement with the results reported Francesca et al., [29] where the maximum ABE concentration was 3.4 g/L. The lower solvent production in xylose control is due to the longer acidogenic phase and the inability of *C.acetobutylicum* to convert the acids to solvent [29]. The residual acid in 60:40 is around 2.68 g/L which is much lower than in 50:50 (3.41 g/L). In all these experiments the glucose was consumed completely at 72 h whereas the xylose consumption was lower than that. There was no much difference in the xylose consumption in experiments with 50:50, 60:40 and 80:20 as they were around 50.75%, 51.60% and 50.21 % whereas it was lower in 90:10 and xylose control with 47.69 % and 45.64 %. From the experiments it is clear that the presence of glucose in the fermentation medium increases both the butanol yield and xylose consumption. The ABE yield was higher in 60:40 (0.19 g/g) followed by 50:50 (0.17 g/g), 80:20 (0.12 g/g) and 90:10 (0.11 g/g). In control with pure glucose the ABE yield was higher than in the model experiments which was 0.24 g/g, whereas in xylose control it was very low to 0.09 g/g. The yield of solvents in different ratios of xylose and glucose is given in figure 2. The ABE yield was the lowest for xylose due to slower growth rate at the start which leads to higher acid concentration and lower solvent conversion. In the mixture of glucose and xylose, the xylose consumption was not as effective as glucose because xylose was initially accumulated as glucose solely served the energy required by the microorganism for metabolic activity. As all the results suggested that 60:40 was better than others it was chosen for the further experiments.

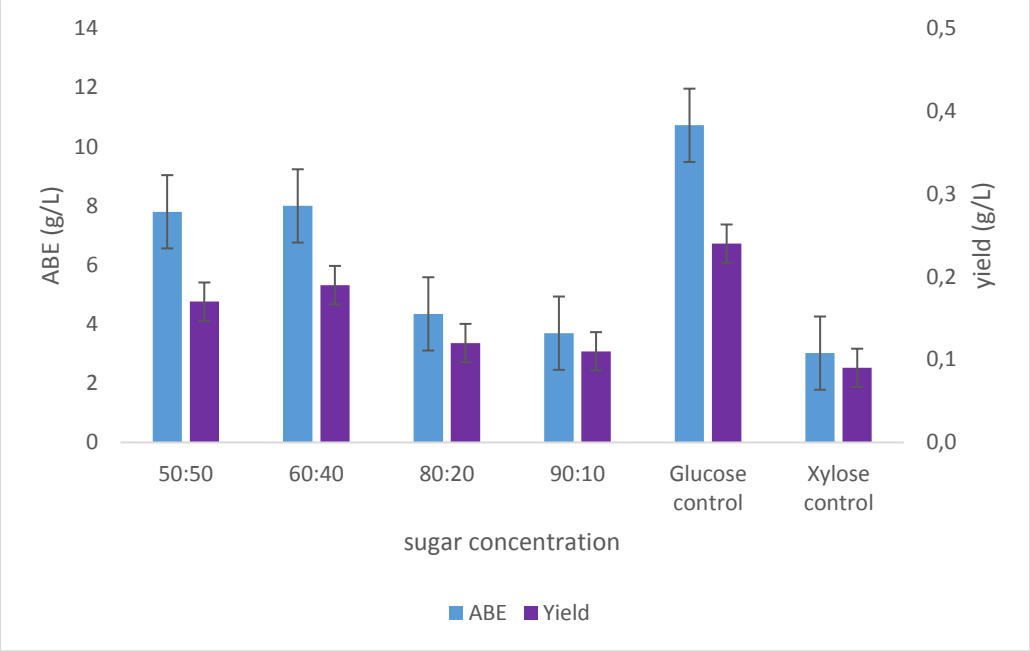

Figure 2. Effect of xylose and glucose concentration in ABE production and yield.

4.2 Effect of electron carriers

The effect of each electron carrier in the butanol production and butanol: acetone ratio by *C.acetobutylicum* was studied. The same concentration (0.1 mM) of different electron carriers was added individually to the fermentation to choose the most effective one. Figure 3 shows the effect of each electron carrier on the solvent and acid production. From the previous studies it was understood that the addition of electron carrier to the fermentation medium increases the butanol yield whereas some of them has a negative effect on it [30]. On comparing the results from this work, it was found that methylene blue (MB) gave higher solvent concentration and ABE yield. The total solvent concentration was 9.48 g/L and the ABE yield was 0.24 g/g which is higher than in the control (8.01 g/L) that was not supplemented with electron carrier. Li et al., reported that the addition of methylene blue directs the carbon flow towards acetone production which will result in lower B:A ratio but it resulted in higher overall solvent production [31]. It was also evident from the results as the B:A ratio (2.85:1) is lower with methylene blue addition than that in the control.

When nicotinic acid was added to the fermentation medium the B:A ratio (3.02:1) was higher than in methylene blue addition whereas the ABE concentration (9.48 g/L) was lower than that. It was reported that when nicotinic acid was added it increases the level of NADH and NADPH which is important in butanol production. The carbon flux was diverted towards

butanol production which leads to higher B:A ratio [31]. The addition of TMAO resulted in ABE concentration of 9.28 g/L with the B:A (2.92:1) ratio similar to that of control. The residual acid (4.61 g/L) was much higher than that of the control (2.90 g/L) which shows the inhibitory effect of acid in butanol production.

The addition of Phenosafranin, 2,2 bipyridyl and sodium sulfide also increases the solvent concentration and butanol yield when compared to the control but with lower B:A ratio. The result was in accordance with that reported in [30]. The use of sodium sulfide affected the fermentative pathway as it increased the carbon flux towards the ABE formation and reduces the carbon flux towards acid formation [32]. A similar result was obtained from the experiments as the residual acid (2.85) was lower than in control.

From the experiments with methyl viologen, it was found that the electron flux was directed towards butanol production and thus acetone was produced in low concentration resulting in higher B:A ratio. The same trend was observed in the experiments performed by Najeeb et al., where 6.09:1 of B:A ratio was reported [30]. It was reported that the addition of methyl viologen inhibits the activity of hydrogenase enzyme [33]. Because its addition results in competition between reduced ferrodoxin and methyl viologen to occupy the active sites of hydrogenase enzyme. The addition of methyl viologen increases the NADH and decreases the hydrogenase activity which results in solventogenesis and increased production of alcohol [33].

The addition of glutathione and tiron resulted in negative effect on the overall production of solvents. The glutathione reduced the acetone production (1.47 g/L) which is 15% lower than the control (2.06 g/L) but it increased the butanol concentration (5.16 g/L) when compared to the control. Though it resulted in lower overall ABE concentration (7.21 g/L) it gave the higher B:A ration of 3.5:1 because of increased butanol and decreased acetone production when compared to all other electron carriers.

From all the experiments it is clear that all the electron carriers increase NADH level in the fermentation medium that directs electron flow towards the solvent production. MB gave the higher ABE concentration and hence in the next experiments different concentrations of methylene blue were used to find its effect on ABE production.

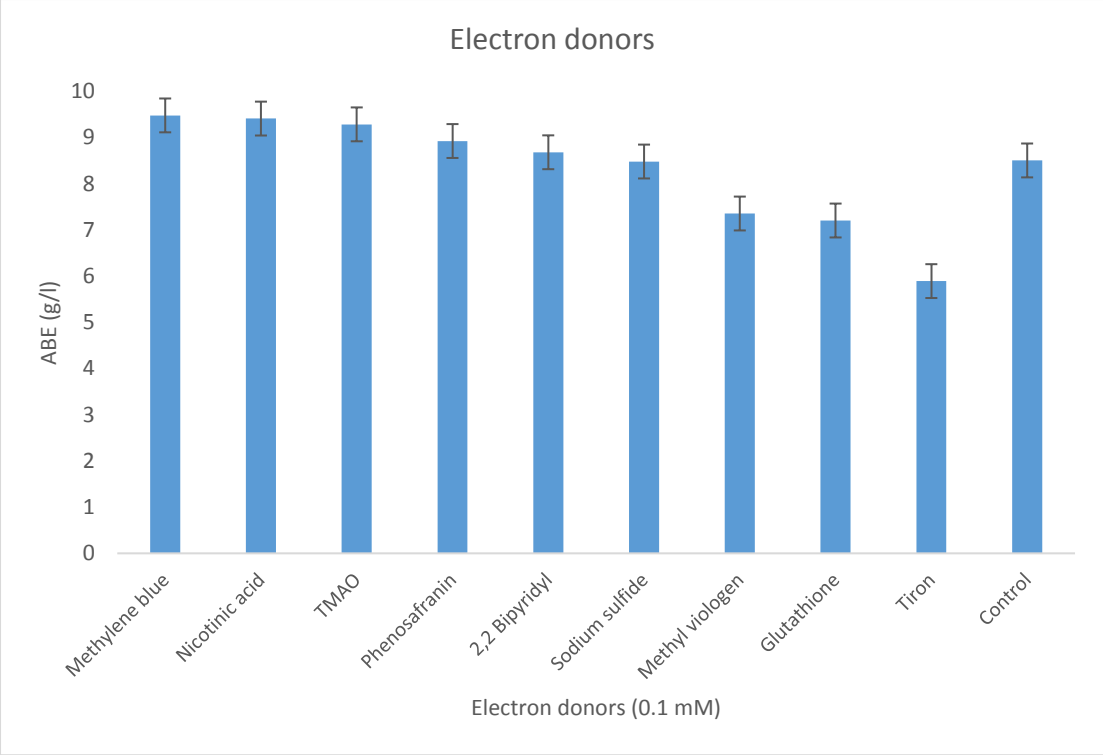

Figure 3. Effect of electron carriers in ABE fermentation.

4.3 Effect of different concentration of methylene blue

Methylene blue with different concentration from 0.05 to 1 mM (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM) were added in the fermentation medium and its effect in solvent production was studied. The solvent production and B:A ratio increased from 0.05 to 1 mM which then started decreasing gradually. It explains that the concentration of methylene blue above 0.1 mM can have negative effect on the butanol production. The experimental results are reported in figure 4.

When 0.05 mM MB was added to the fermentation medium, the total solvent production was 9.07 g/L and the B:A ratio was 2.75:1. The highest ABE concentration was obtained on using 0.1 mM MB, it produced 9.47 g/L of ABE and the B:A ratio was 2.82:1. As mentioned above [28], the use of MB gave lower A:B ratio and it can be seen that there was no much difference in A:B ratio on using 0.05 and 0.1 mM MB.

The increase in the concentration of MB from 0.1 to 0.2 mM lead to 7% decrease in the solvent production. Further increase in the concentration to 1 mM cause 12% decrease in the total solvent formation. It is due to the fact that increase in concentration of MB affects the growth

of *C.acetobutylicum* which in turn reduces the solvent production. This shows the effect of the concentration of MB in ABE production. The amount of acids formation increased on increasing the concentration of MB. The acid production was high on using MB above 0.1 mM, the assimilation of acids into solvents were poor which led to low ABE concentration.

Though, the use of 1mM methylene blue resulted in lower ABE production, it still produced higher amount of solvents on comparing with control (fermentation medium without the addition of methylene blue). It is evident from this that even the addition of higher concentration of MB leads to increase in the solvent production. On comparing the results from the experiments, it is clear that the concentration of 0.1 mM MB is the most effective. It was used for the rest of the experiments to check the effect of addition time of MB in ABE fermentation.

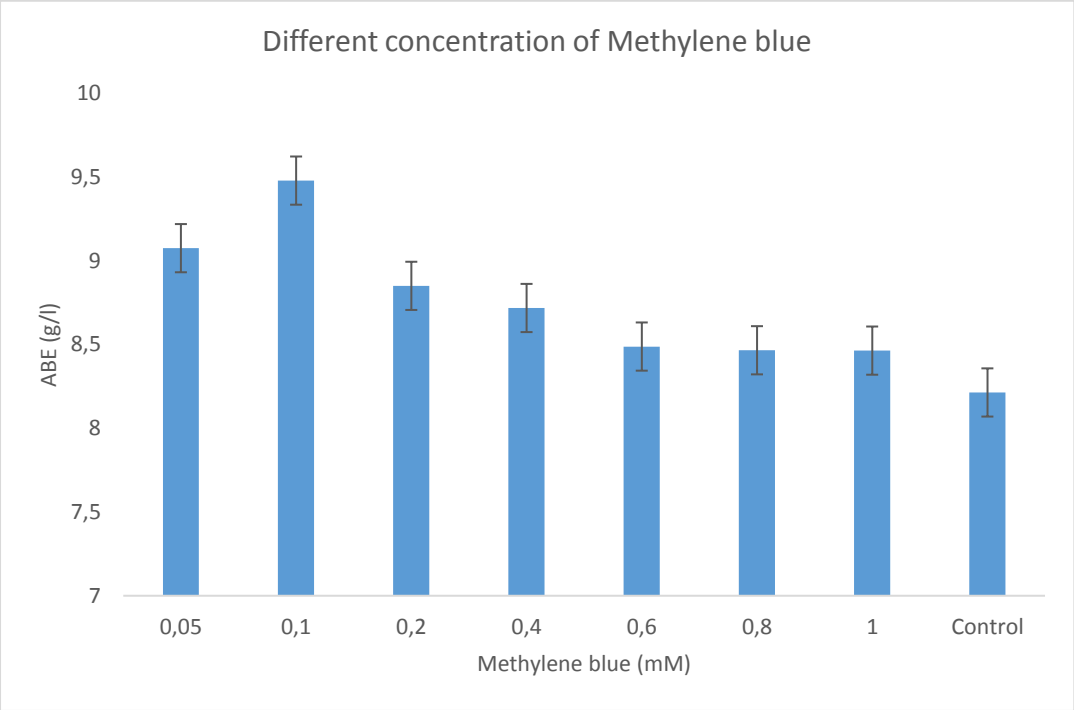

Figure 4. Effect of different concentration of methylene blue in ABE production.

4.4 Effect of time of addition of methylene blue

From the previous experiments it was concluded that the effective concentration of methylene blue was 0.1 mM as it gave higher butanol yield and overall solvent concentration. Thus, the effect of addition time of methylene blue on B:A ratio and ABE production was studied. Methylene blue was added at various times to the fermentation medium (0, 6, 12

and 24 h) and its effect on ABE production was analysed.

The initial (at 0 h) addition of methylene blue increased the growth of the *C.acetobutylicum* slightly as the OD of the medium was higher when compared to the control. The effect of time of addition of methylene blue on the growth of *C.acetobutylicum* and B:A ratio are shown in figure 5. The maximum OD_{600} (2.524) was obtained when the methylene blue was added at 0 h and it decreased gradually as the time of addition increased. The lowest OD_{600} of 1.916 was obtained when methylene blue was added at 24 h.

The highest butanol concentration (6.61 g/L) was obtained when 0.1mM methylene blue was added at 0 h to the fermentation medium. The butanol concentration was found to decrease with the increase in addition time of methylene blue. The addition of methylene at 24 h resulted in the lowest butanol concentration of 5.63 g/L.

As expected, the B:A ratio was higher at the initial addition of methylene blue. The B:A ratio (2.92:1) was maximum as the butanol production was higher at 0 h. The B:A ratio followed the same trend as the butanol concentration, it reduced following the increase in addition time. The lowest B:A ratio (2.61:1) was reported when the time of addition is 24 h.

There was no significant effect of addition time of methylene blue in the acetone production. The results showed that the acetone production was not affected to an extent due to time of addition. The maximum and minimum acetone concentration obtained were 2.26 and 2.16 g/L at 0 and 24 h. As mentioned above the presence of methylene blue in the production medium directed the carbon flux towards the acetone production irrespective of the addition time. Figure 5 shows the effect of addition time of methylene blue on ABE concentration.

The overall solvent concentration at 0, 6, 12 and 24 h were 9.66, 9.15, 8.84 and 8.49 g/L. The experiments with control (without addition of methylene blue) gave the butanol and ABE concentration of 5.61 g/L and 8.36 g/L. This revealed that the addition of methylene blue increased the sugar consumption in spite of the addition time. The glucose was completely consumed after 72 h in all the experiments and the residual xylose after 96 h was 19.14 g/L (when methylene blue was added at 24 h) which was lower than in the control (21.20 g/L).

From all the experimental results, it is evident that the optimal time for addition of methylene

blue is 0 h as it gave higher butanol concentration, B:A ratio and ABE concentration.

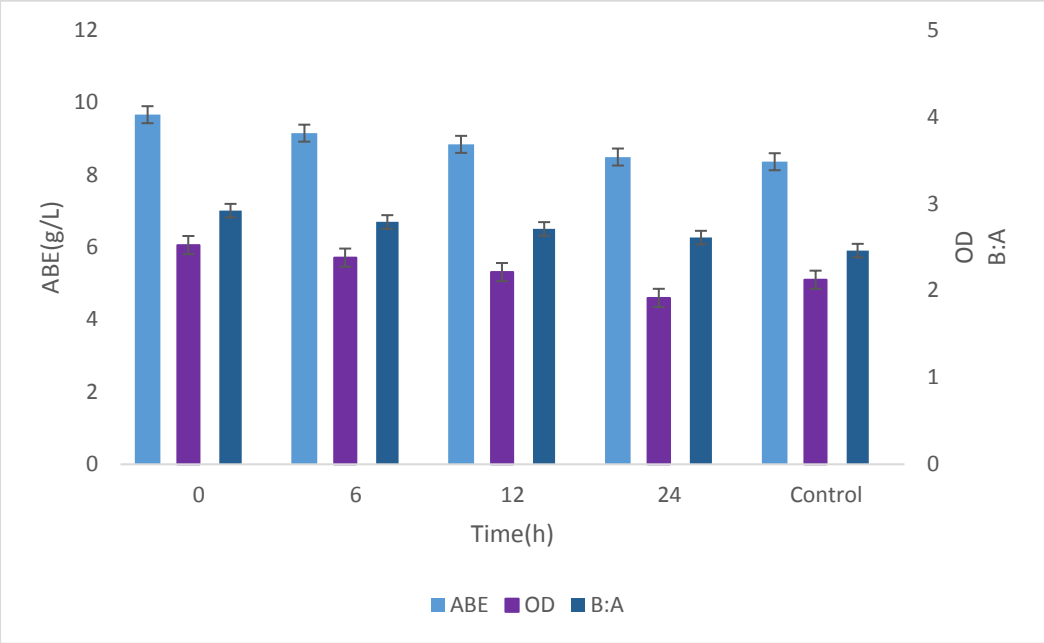

Figure 5. Effect of time of addition of methylene blue in ABE concentration, growth of *C.acetobutylicum* and B:A.

4.5 ABE fermentation using birch wood hydrolysate

After carrying out the experiments with pure sugars, the birch wood hydrolysate after activated charcoal treatment was used for ABE fermentation. The experiments with hydrolysate was not as simple as the pure sugars due to the presence of lignin and acids. The growth of *C.acetobutylicum* in the acid treated birch wood hydrolysate is lower when compared to the pure sugars. This is due to the presence of the inhibitors like furfural, HMF and which inhibits the growth of the organism. The activated charcoal treatment removed the inhibitors to certain level but not completely. The complete removal of inhibitors will lead to reduction of the sugar content which will result in low solvent production. The choice of detoxification method is important; it should have higher removal of inhibitors without affecting the concentration of sugar in the hydrolysate. By diluting the hydrolysate, the concentration of inhibitors in it can be reduced but it will also reduce the concentration of sugars.

The parameters such as electron carrier (methylene blue), concentration of electron carrier (0.1 mM) and time of addition (0 h) that were optimized using pure sugars were used for the

ABE fermentation using detoxified birch wood hydrolysate. Both the detoxified birch wood hydrolysate and medium with pure sugars (60:40 of xylose: glucose) without the addition of methylene blue were used as controls in these experiments. On comparing the xylose utilization, the medium with pure sugars was found to have higher xylose consumption (51.25 %) followed by hydrolysate with 0.1 mM methylene blue (48.12 %) and hydrolysate without electron carrier (46.87 %). This shows that the presence of inhibitors in the hydrolysate prevents the xylose consumption by *C.acetobutylicum* which is in agreement with the results reported in [34] where the xylose utilization was lower in the hydrolysate when compared to xylose control.

The efficiency of the detoxified birch wood hydrolysate in ABE fermentation was analysed by comparing with the controls. *C.acetobutylicum* gave higher solvent concentration (5.46 g/L) on using the hydrolysate with 0.1 mM methylene blue added at 0 h when compared to the hydrolysate control (5.01 g/L). From the results, it is evident that the methylene blue in hydrolysate increases the solvent concentration by increasing the growth of organism and diverting the electron flux towards solvent production. The highest solvent concentration (8.1 g/L) was obtained in pure sugars control. The presence of the acids and other inhibitors reduced the conversion of acids to solvents in detoxified hydrolysate. The same trend was seen in the experiments carried out by [34] where the ABE concentration was higher in xylose control than in the hydrolysates. The results obtained from experiments using birch wood hydrolysate are shown in figure 6.

The acid treated birch wood hydrolysate has furfural, formic and acetic acid in the concentration of 2.17, 3.66 and 5.76 g/L after the activated charcoal treatment. The presence of formic and acetic acid affected the re-assimilation of acid and conversion of acids to solvent. It has been reported that the acetic acid has positive effect on the butanol production whereas formic acid has negative effect on the butanol production [35]. This explains the reduction in solvent production using detoxified birch wood hydrolysate in my experiments. It was mentioned that by maintaining the pH at 5.1 during the batch fermentation, the solvent production has been increased by avoiding the effect of formic acid [34]. This can be a solution for preventing the inhibitory effect of formic acid which will increase the conversion of acids to solvent. The neutralization of acid treated hydrolysate with $Ca(OH)_2$ formed some salts which affected the solvent production by *C.acetobutylicum*. This is due to the presence of the

acids in hydrolysate and it can be avoided by reducing the acid concentration through effective detoxification method.

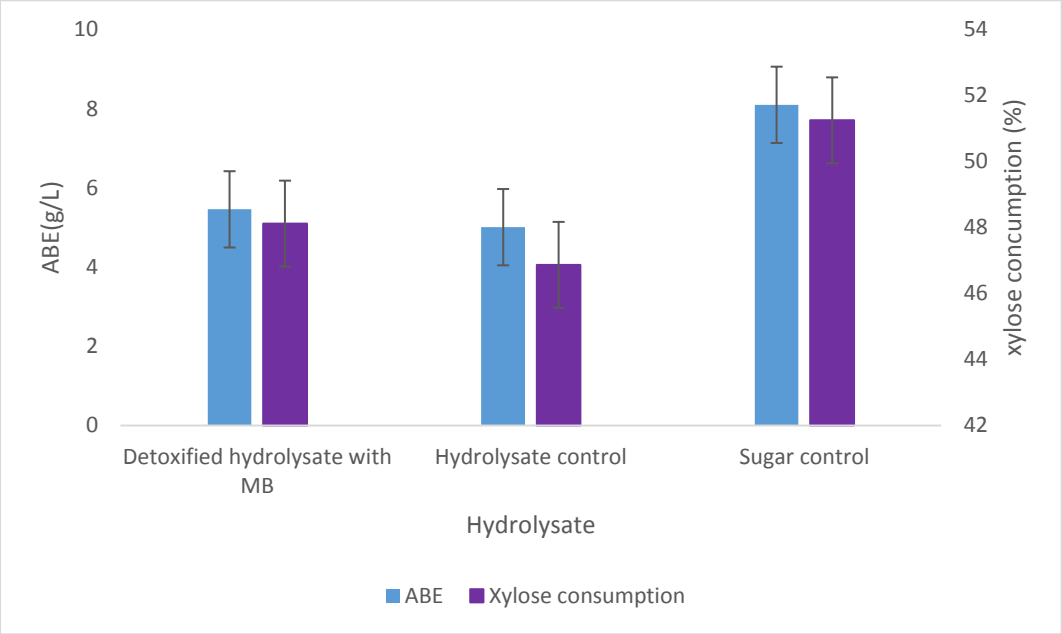

Figure 6. ABE fermentation and Xylose consumption of birch wood hydrolysate by *C.acetobutylicum*.

5 Conclusions

The main aim of this thesis was to study the use of birch wood hydrolysate for ABE fermentation and to find whether it can be an alternative raw material for the commercial production of biobutanol. This has been achieved by carrying out the experiments with the information obtained from literature review. The results gave enough information to show that the work has been justified.

Most of the studies carried out on ABE fermentation used other lignocellulosic biomass such as agricultural wastes and very few on wood hydrolysate. The wood hydrolysate has several inhibitory compounds like HMF, furfural when compared to other lignocellulosic feedstocks which complicates the ABE fermentation. The birch wood hydrolysate used in this process mainly has xylose which was used as the main carbon source. The production of biobutanol from xylose may not be as efficient as glucose but it still offers great opportunity as an alternate carbon source. More research need to be carried out using xylose to understand the problems associated with it and the methods to overcome it. The use of wood hydrolysate for commercial biobutanol production has to be investigated more as it has high sugar contents in it and also its availability is abundant.

This study shows that the use of electron carrier increases the butanol concentration and it will be helpful in industrial scale whereas the cost and reusability of the electron carrier are the important factors to be taken into account. Additional experiments with different electron carriers that are not used in this work will give more information and give options to choose the most effective and economical one that will be suitable for the commercial process.

The acid pretreatmet carried out in this project converted the cellulose, hemicellulose and lignin in birch wood to monomeric sugars which was easier for *C.acetobutylicum* to produce the solvents. But it also produced higher concentration of acetic and formic acid which inhibited the ABE fermentation. The choice of pre-treatment method is very important and several methods have to be tested to obtain the hydrolysate as efficient as possible. Only one detoxification method was used in this project and it was chosen based on the literature study. The activated charcoal treatment did not reduce the sugar content in the hydrolysate which is one of the most important aspects for ABE fermentation. However, it did not cause

significant removal of the acids like formic acid which is a major inhibitory compound that affected the solvent production. Different detoxification methods have to be analysed using the birch wood hydrolysate to select the best method that has higher reduction of inhibitory compounds and lower reduction of sugars.

Through this project, it was found that *C.acetobutylicum* can be used as an effective microorganism for the ABE fermentation using birch wood hydrolysate. Several researchers are working with engineered strains to check its potential in biobutanol production. Experiments with such engineered strains for ABE fermentation using birch wood hydrolysate will wider the chances of selecting the best strain that will give higher butanol concentration.

Overall, this project suggests that birch wood hydrolysate can be a better lignocellulosic feedstock for biobutanol production if few improvements are done. Further research on pre-treatment and detoxification of the birch wood hydrolysate, and the use of different electron carriers and strains for ABE fermentation using this hydrolysate will help to improve the butanol production.

6 Future perspectives

During this thesis there was a big problem with the inhibitors in birch wood hydrolysate as it hindered the butanol production and analysis. The future research work should be focused on using different hydrolysis methods to produce low acidic hydrolysate and detoxification methods for removing the inhibitors from birch wood hydrolysate. So that the inhibitors will be reduced to an extent that it will not affect the ABE fermentation. More number of electron carriers can be used to find if any of them is more effective than methylene blue. It will be useful to study the use of engineered strains for ABE fermentation using birch wood hydrolysate as those strains have better resistance to butanol concentration in the medium and also to the inhibitors.

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