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Production of Butanol using *Clostridium Acetobutylicum* in Anaerobic System

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Abstract: In batch culture fermentation, butanol was produced using a pure isolation of the solvent-producing *Clostridium Acetobutylicum*. ABE (acetone-butanol-ethanol) fermentation was conducted using *C. Acetobutylicum* to produce a vast production of butanol. The main objective of this project is to replace the finite sources with renewable resources by undergoing the fermentation process. In order to achieve the main objective, this research aims to formulate the fermentation medium to produce butanol and assess the concentration of butanol and bacteria growth rate. Prior to inoculation, the fermentation media was incubated at 37°C for 24 hours while being sparged with nitrogen gas for 15 minutes. For the results of fermentation products, gas chromatography (GC) was used to analyse the ABE fermentation products. In this study, butanol is produced more frequently than ethanol. However, the production of butanol is lower than acetone due to toxicity, which now restrict butanol synthesis through fermentation. Butanol production peaked at 60 hours, when it was at 4.7 g/L, before falling to 4.3 g/L during the next 10 hours. For the optical density of the fermentation media, the exponential phase started at 15 to 30 hours of incubation and the following consecutive periods are started to lower down due to the toxicity of the ABE production in the reactor.

Keywords: ABE Fermentation, *C. Acetobutylicum*, Butanol, Fermentation Media, Ethanol.

1. Introduction

Natural resources like fossil and nuclear fuels that cannot be replenished as quickly as they are used up are referred to as non-renewable resources or finite sources. Fossil fuels, such as oil, natural gas, and coal, were created from decomposing plants and animals and were found beneath sedimentary and rock strata. These non-renewable materials, however, required a long time to both develop and deplete. Fossil fuels were extracted from fossilised plants and animals that lived between 300 and 360 million years ago. Other non-renewable resources include metal ores and earth minerals in addition to fossil fuels. It

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may also have detrimental consequences on the environment, such as air pollution, global warming, and climate change [1]. Carbon dioxide (CO_2), which has been correlated to both climate change and global warming, can be released through the burning of fossil fuels. The primary causes of climate change are emissions of greenhouse gases like CO_2. In addition, burning fossil fuels sends nitric and sulfuric acids into the atmosphere as acid rain, which is unsafe for the environment. Overall, the distribution, gathering, processing, and burning of these non-renewable resources have an impact on the ecosystem. As a result, attempts have been made to substitute these resources with others that can have a less negative impact on the environment or prevent further environmental harm, such as by employing renewable energy [2].

On the other hand, a renewable resource, often called a flow resource, is a type of natural resource that can be replenished or perpetuated. This resource was known as a perennial resource since it is highly improbable that its recovery rate would ever be higher. In many applications, including power generation, space or hot water heating, vehicle fuels, and rural energy, it is possible for renewable energy to substitute or take the place of fossil fuels. [3]. Such resources as sunshine, wind, waves, biomass, and biofuels can produce renewable energy that is regenerated naturally quickly enough to be used. The term "biomass" refers to biological material derived from alive or recently extinct organisms; the most prevalent examples are plants or products generated from plants [4].

Fuels that are classified as biofuels include solid biomass, liquid fuels, different biogases, and fuels created by the conversion of biomass. Biofuels are defined as fuels that obtain their energy from biological carbon fixation. Bioethanol, a fermented alcohol, is mostly made from the carbohydrates present in plants like maize, sugarcane, and switchgrass that are high in sugar or starch [5-6]. When an alcohol is mixed with lipids like animal fat, soybean oil, or another form of vegetable oil, a chemical process known as transesterification occurs, which results in the creation of a methyl, ethyl, or propyl ester. [7]. In contrast to the vegetable and waste oils used to power converted diesel engines, this biofuel can be easily added to existing diesel engines and distribution networks. A heating oil substitute is also possible with biodiesel mixes. [8].

Biobutanol is a biofuel that is also produced primarily by the fermentation of sugar in organic feedstocks. Butanol has a low vapour pressure. Additionally, it is less corrosive and may be mixed with gasoline or directly being used [9]. Butanol fermentation still has a number of disadvantages, such as low productivity since the butanol produced builds up on microorganisms, high substrate costs, and high butanol recovery costs due to the existence of by-products such as acetone and acetic acid [10]. Simple sugars were fermented in biomass feedstock, which is the most common way of making biobutanol whereby it is less like ethanol than gasoline. In contrast to bioethanol, biobutanol has a higher energy density, lower volatility, higher boiling point, and is more hydroscopic. Compared to other fuel alternatives, biobutanol has a relatively high energy content [11]. The energy density of biobutanol, on the other hand, is 10–20% lower than gasoline. Low volatility means that the substance is more likely to exist as a liquid or solid which means that biobutanol will not easily vaporize in certain temperatures. The volatility of the substances and the boiling point were related to one another [12].

Biobutanol will become an appealing, cost-effective, and long-term fuel when petroleum oil becomes more costly owing to dwindling oil sources and increased greenhouse gas emissions in the environment [13]. In order to enhance the butanol production, metabolic networks are manipulated through genetic and metabolic engineering. Fermentation routes for manufacturing butanol have made considerable advances, but they are still inefficient. The titer and yields are poor, and separation is costly [14]. Combining electrochemical and microbiological production techniques can produce butanol from sustainable sources, despite the fact that this approach has not been tested commercially. By using Clostridium Acetobutylicum, butanol can be produced during the acetone-butanol-ethanol (ABE) fermentation process [15].

The Firmicutes phylum includes the gram-positive, anaerobic, fermentative, spore-forming bacteria known as Clostridium species. From sustainable carbohydrate sources, such as lignocellulosic biomass, the microorganisms in the ABE fermentation process produce biobutanol. The same raw materials used to make ethanol, including sugar beets, sugar cane, maize grain, wheat, and cassava, are also used to make biobutanol [16]. Furthermore, butanol synthesis from biomass and agricultural by-products may be more efficient than ethanol or methanol [17].

Clostridium species have been demonstrated to be capable of producing biobutanol. *C. acetobutylicum* is well characterized by its biphasic fermentative metabolism. In order to flourish in its vegetative stage, *C. acetobutylicum* requires anaerobic circumstances. It is motile throughout its whole surface via flagella in its vegetative stage. In aerobic settings, it can only live for a few hours before forming endospores, which can remain for years even in aerobic condition [18]. Only when these spores are in anaerobic condition, they can continue to develop vegetative. It shows no activity of catalase which is an enzyme required by aerobic organisms to convert hydrogen peroxide, also known as a harmful consequence of oxygen metabolism, to water and oxygen [19]. Hence, this study has carried out the fermentation process using *C. acetobutylicum* to evaluate its efficiency in producing biobutanol.

2. Materials and Methods

2.1 Pre-culture media (PCM) preparation

In 1000ml of distilled water, 5g of yeast extract, 1 g/L tryptone, 0.01g/L p-aminobenzoic acid and 10g of glucose were combined and stirred. A thorough mixing of the material was performed which are 1.25 g/l NaCl, 0.25 g/l Mg, 0.75 g/l K_2HPO_4, and 0.75 g/l KH_2PO_4. $SO_4.7H_2O$ and Mn at 0.01 g/l. In each beaker, 100ml of distilled water was added to SO_4. H_2O and 0.01g/l of $FeSO_4.7H_2O$. 27ml K_2HPO_4, 27ml KH_2PO_4, 37ml NaCl, 7ml $Mg.SO_4.7H_2O$, 1ml Mn. SO_4. H_2O, 1ml $FeSO_4.7H_2O$ were poured into the glucose and yeast extract mixture and stir well. The mixture was then mixed into 1000 ml and autoclave at 121℃ for 15 mins.

2.2 Fermentation media preparation

900ml of distilled water was mixed with 20g/l of glucose and 5g/l of yeast extract and mixed thoroughly. 2.6ml K_2HPO_4, 0.03ml 4-aminobenzoic acid, 0.8ml $Na.MoO_4.2H_2O$, 0.8ml $CoCl_2.6H_2O$, 5.4ml $CaCl_2.2H_2O$, 0.6ml $CuSO_4$, 1.8ml $Zn.SO_4.7H_2O$, 5.9ml $MnSO_4.H_2O$ and 82ml $MgSO_4.7H_2O$ were poured into the glucose and yeast extract mixture and stir well. The mixture was then poured into a sampling bottle of 1000 ml and autoclave at 121℃ for 15 mins.

2.3 Microorganism and inoculum preparation.

Clostridium acetobutylicum ATCC 824 was made available for purchase from the American Type Culture Collection (ATCC, Japan). The inoculum was created by adding 1 mL of *C. acetobutylicum* ATCC 824 stock culture to 99 mL pre-cultured media (PCM). The PCM was sparged with nitrogen gas for 15 minutes prior to inoculation, and then incubated for 24 hours at 37°C.

2.4 ABE fermentation

180 ml of the CRM were poured into 6 different conical flasks. 10% of the PCM that have been inoculated with microbes were poured into the same conical flask and stopper was used to close the conical flask mouth. Nitrogen gas was purged into each conical flask for 15 minutes. The end of the tube was sealed using parafilm and clippers to minimize the rate of oxygen to enter. All of the mixture were incubated at 120 rpm at 37°C for 24 hours to let the microbe grow.

0.3 ml from the diluted solutions were pipetted onto the agar plate. The L shaped glass spreader was dipped into 70% ethanol and flame over Bunsen burner and let it cool for a few second. The sample were spread evenly on the surface by carefully rotating the plate. The steps were repeated until the last

dilution and the plates were sealed with parafilm as a precaution. Then, the plates were incubated for 24 hours at 37˚C.

3. Results and Discussion

From this study, the ABE fermentation was carried out using glucose as the main substrates to culture *C. acetobutylicum.* The optical density, fermentation products and the growth culture were assessed throughout the study.

3.1 Optical Density

The optical density for *C. acetobutylicum* was estimated using UV-Visible Spectrophotometer. A spectrophotometer measures the quantity of light that passes straight through a sample and calculates the turbidity as a result. The quantity of transmitted light decreases as the number of cells rises an increasing in turbidity in a culture indicates the growth and biomass of the bacterial population since turbidity is directly related to cell count. The reading, also referred to as optical density or absorbance, indirectly indicates the number of bacteria [20]. Using absorbance, a logarithmic value, it is possible to graph bacterial growth. The sensitivity of this approach is restricted to bacterial suspensions, which limits its usefulness while being quicker than a traditional plate count [21].

Based on **Figure 1**, the curve shows the absorbance curve for the prepared inoculum of *C. acetobutylicum*. The absorbance peak appears at 600 nm of wavelength. This indicates the amount of light absorbed by the sample which contains *C. acetobutylicum.* The presence and dissolved substances are analysed by the passing light through the sample by the absorbance value [22]. At 0-10 hours, the microbes experienced a lag phase and consequently turned into the exponential phase until it reached 30 hours of incubation. The following hours revealed that the microbes went to stationary phase and gradually moved into the death phase. This can be justified through the appearance of low increment of optical density starting from 30 hours until the end. Hence, it can be concluded that the *C. acetobutylicum* may only be able to ferment actively at 10-30 hours of incubation.

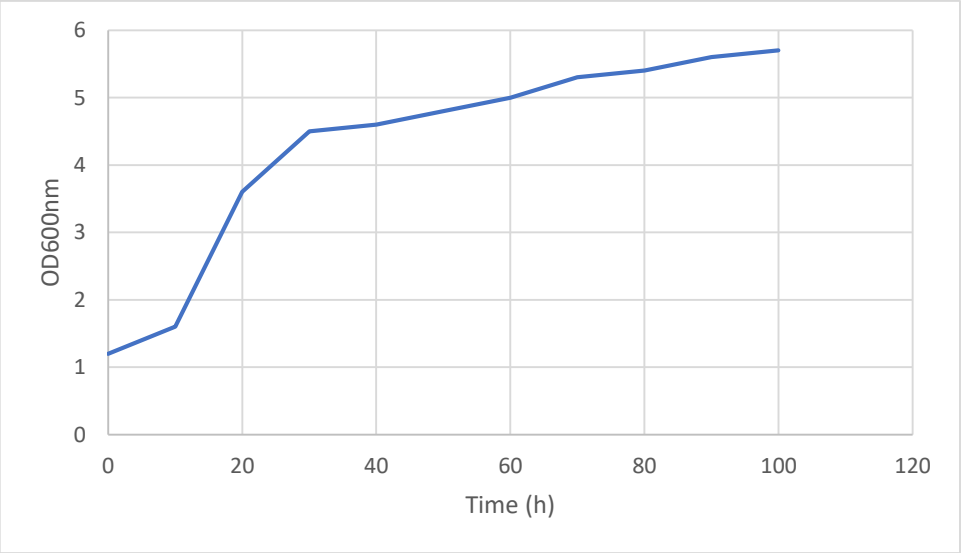

Figure 1: Optical Density of *C. acetobutylicum*

3.2 Fermentation Products

Gas chromatography was used to analyse the ABE fermentation products. **Figure 2** displayed the findings based on the experimental analysis. These values obtained according to a plotted calibration

curve for each material. Acetic acid is produced at a higher rate than butyric acid due to the fact that the acidic fermentation phase for acetic acid occur earlier than the butyric acid. Butanol is produced at a lower rate than acetone. This is due to a variety of issues, such as low product yield, insufficient selectivity, and its toxicity, which now restrict butanol synthesis through fermentation. Butanol production peaked at hour 60, when it was at 4.5 g/L, before falling to 4.3 g/L during the next 10 hours. Meanwhile, the production of acetone was 5.1 g/L on the 60th hour and drop into 4.8 g/L on the 70th, whereas the production of ethanol was 4.3g/L on the 60th hour and drop to 4.0 g/L on the 70th. This is because there were two unique phases in the ABE fermentation. An acidogenic phase where a surge in bacterial population and growth tended to promote the production of butyric acid, acetic acid, and fermentation gases (CO_2 and H_2). Glycolysis, which resulted in the formation of pyruvate from the sugars, also involved two distinct fermentation gases (CO_2 and H_2). The bacterial metabolism changed at the end of the exponential growth phase, leading to the formation of acetone, butanol, and ethanol from the acids. Because butanol was present in fermented alcohol, bacterial culture development and metabolism were inhibited during the solventogenic phase, which resulted in low product concentrations.

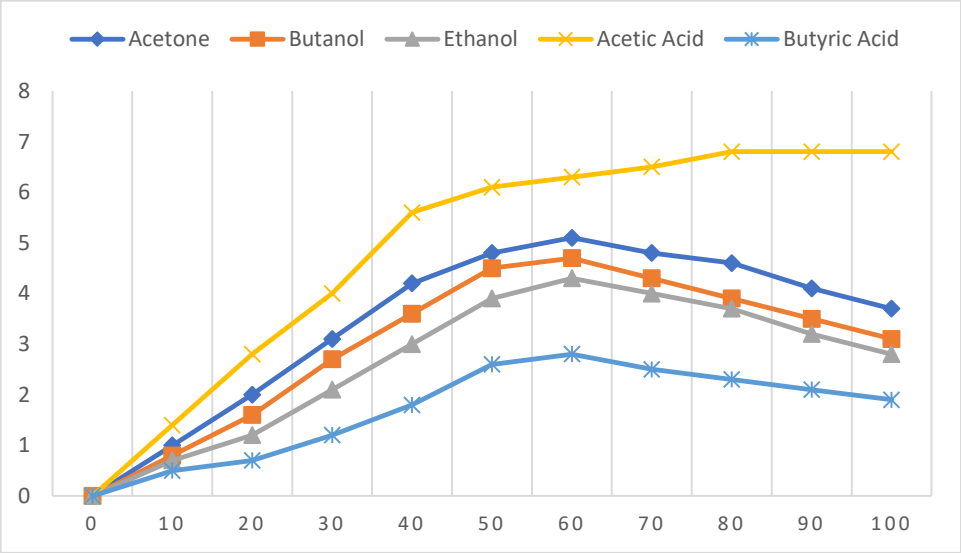

Figure 2: Fermentation Products

3.2 Microbe growth

Spread plate method was used to achieve single colony bacteria for the fermentation process. Using the spread plate approach, which is used for viable plate counts, the total number of colonies forming units on a single plate is counted. The number of cells in the tube from which the sample was plated may also be calculated using this method. Based on **Figure 3**, single colony can be seen clearly. Each colony represents the progeny of a single bacterial cell. Spread plate technique is used as it can equally distribute and count all of the microorganisms in a mixed culture. The method makes it simpler to calculate the number of bacteria present in a solution. The plate was sealed with parafilm as a precaution to prevent contaminations and from oxygen entering the plate.

Figure 3: Colonies of *C. acetobutylicum*

4. Conclusion

The aim of this study is to replace the finite sources with renewable resources by undergoing the fermentation process. Based on the results of this investigation, the productions of butanol were carried out by utilising *C. acetobutylicum* as the medium for fermentation. For the optical density, it shows the exponential phase occur from 10-30 hours of incubation. This indicates that *C. acetobutylicum* growing actively during these hours. As for butanol, it happened to appear at maximum amount of 4.5 g/L at 60 hours of incubation whereas the following hours portrayed a timid decrement along the incubation period. In order to identify the existence of *C. acetobutylicum*, the growing of microbes on the agar was conducted and the incubated agar expressed the colonies of *C. acetobutylicum*.

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