# BUTANOL PRODUCTION FROM BIOMASS

by

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# ABSTRACT

Fossil fuels are a very important source of energy in today's world. They are currently the most economically available source of power for both personal and commercial use. During the combustion of fossil fuels, various emissions and particulates are released into the atmosphere. They contribute to the formation of acid rain that can seriously harm the environment, as well as greenhouse effect which is considered a key factor in global warming. Potential solution of various problems caused by fossil fuels is the use of renewable energy. Production of ethanol as an alternative fuel is already undergoing a massive expansion in US; however another interesting biofuel is getting attention – butanol. During the fermentation process in a well equipped bioreactor, microorganisms consume the biomass converting it to butanol as a main product of the reaction. Amongst various types of biomass that can be used, cellulosic material represents a commonly-available and cost-effective choice especially due to being a renewable energy source that does not harm the environment or jeopardize world food sources. The fermentation process in this study was performed in a small-scale (7L) BIOFLO 110 bioreactor in an atmosphere of 85% $N_2$, 5% $H_2$ and 10% $CO_2$. The bacterium used was Clostridium beijerinckii ATCC 35702, which was grown in a media comprised of a mixture of many chemicals, including corn syrup as a major food source. Samples from the fermenting solution were taken in various stages of the process to allow cell counting which indicates the metabolic action of the bacteria. The objective of this research is to produce butanol and examine butanol potentials from abovementioned bacterium strain.

CONTENTS

# ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Douglas Price and Dr. Gary Walker, whose encouragement, guidance and support from the initial to the final level enabled me to develop a thorough understanding of the subject.

Lastly, I offer my regards to all of those who supported me in any respect during the completion of this project.

# INTRODUCTION

# 1. Fossil Fuels

As a cheap and efficient way of energy production, fossil fuels are among the most used energy sources. The global market economy is completely dependent on burning oil, gas and coal. That dependence is as high as almost 90 percent, and therefore fossil fuels account more than 80 percent of total world energy consumption. Petroleum leads with nearly 40 percent of world consumption, followed by coal with 24 percent and natural gas with 22 percent [1].

Fossil fuels are actually the remains of organic matter, that over hundred of million of years, have undergone substantial physical changes and chemical changes caused by the action of microorganisms [2]. The three main fossil fuels, also referred as carbon based fuels are petroleum, coal and natural gas.

## 1.1 Coal

Of all the fossil fuels, coal is the most abundant in many regions of the world, including developing countries, and therefore its reserves are the most widely distributed. It is very cheap to mine and to transport, but it is also the most damaging to the environment.

At today's rate consumption, coal reserves are estimated to last about 200 years, much longer that oil and gas [3]. Currently, coal produces about half the electric power in the United States.

Coal was formed from the highly aromatic, polymeric component of woody plants called *lignin.* Over a long period of time, those materials were exposed to high pressures

and temperatures, and both water and carbon dioxide were lost. The material polymerized in the process yields carbon-rich material known as coal [3]. The coal has a reputation of being "dirty fuel", because when it is burned, it emits not only carbon-dioxide and water but solid amount of air pollutants, such as sulfur dioxide, fluorides, uranium and other radioactive materials, as well as heavy metals [3].

As mentioned before, in the US coal is mostly used to produce electricity. The heat that combustion of coal produces is used to generate steam, and the steam is used to turn turbines and therefore produce electricity. However, the ratio of $CO_2$ to energy produced is considerably greater than for the other fossil fuels [3].

## 1.2. Petroleum and Natural Gas

Petroleum and natural gas are essentially hydrocarbons, and an important impurity in them is sulfur. Some natural gas deposits contain even more sulfur that methane, which is considered its primary molecular component [3]. The sulfur compounds can be removed from gas and oil, which makes these fuels cleaner than coil.
Before it can be used as a fuel, natural gas actually must undergo extensive processing to remove all materials other that methane. Petroleum and fuels made from it, such as gasoline has big advantage because they are relatively cheap to produce and easy to handle. Transportation system around the globe is mostly based on using cheap petroleum fuel, and natural gas is mainly used to fuel stoves, furnaces, water heaters, clothes dryers and other household appliances. At today's rate of usage, it is estimated that there is about 30 to 40 years' reserves of petroleum and 60 years reserves of natural gas [3].

The Figure 1 shows US energy consumption by source in 2007 (EIA, AER 2007) [4]

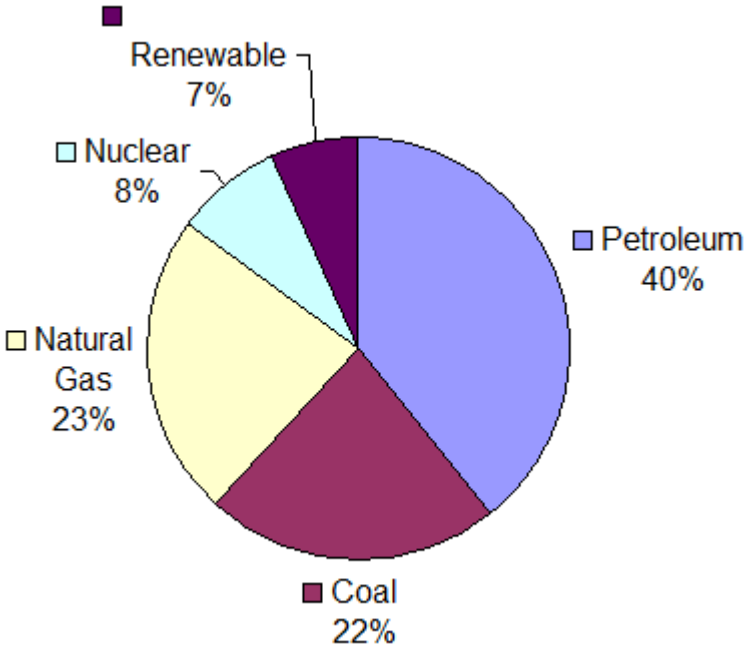


**Figure 1: US energy consumption by source in 2007 (EIA, AER 2007)**

### 1.2.1. Gasoline

Gasoline or petrol is petroleum derived liquid mixture, and it is primarily used as a fuel in internal combustion engines. It is an aromatic, flammable liquid that is manly consisted of hydrocarbons. Gasoline is extremely volatile compound and changes state from liquid to vapor at room temperature. It also vaporizes at increasing rate as temperature increases. For this reason, there are different types of gasoline produced for different regions of the world, as well as for different seasons of the year. More volatile gasoline is used in winter and in the cold locations, and less volatile gasoline is convenient for higher temperatures and in warm locations [5].

Gasoline is a complex mixture of over 500 hydrocarbons that may have between 5 and 12 carbon atoms, and it is usually produced by fractional distillation of crude oil.

Constituents of gasoline include alkanes, alkenes and cycloalkanes in various proportions, which depend on the nature of crude oil used in production process, as well as the grade of gasoline produced.

## 1.3. Environmental issues

Combustion of fossil fuels is considered to be the largest contributing factor to the release of greenhouse gases into the atmosphere. There are many types of harmful outcomes which result from the process of converting fossil fuels to energy. Some of these include air pollution, water pollution, and accumulation of solid waste, the land degradation and human illness.

### 1.3.1. Greenhouse Effect and Global Warming

The burning of fossil fuels is one of the largest sources of emission of carbon dioxide, a greenhouse gas that causes global warming.

The temperature of the Earth is controlled by the balance of the input of energy of the sun and the loss of this energy into the space [6]. Atmospheric gases critical to the temperature balance are known as greenhouse gases. On the average, about one third of the solar radiation that hits the Earth is reflected back to space, and the rest of it is absorbed by the land and oceans [6]. The Earth's surface becomes warm and thus emits long-wave infrared radiation. The greenhouse gases can trap long-wave radiation and warm the atmosphere. This is called a greenhouse effect. Naturally occurring greenhouse gases are water vapor, carbon-dioxide, methane and nitrous oxide [6].

Human activities, including fossil fuels burning are causing greenhouse gas levels in the atmosphere to increase, which can lead to the global warming. Figure 2 presents data on the major global sources of carbon dioxide ($CO_2$) emissions by country, from the beginning of the Industrial Revolution to the present [7].

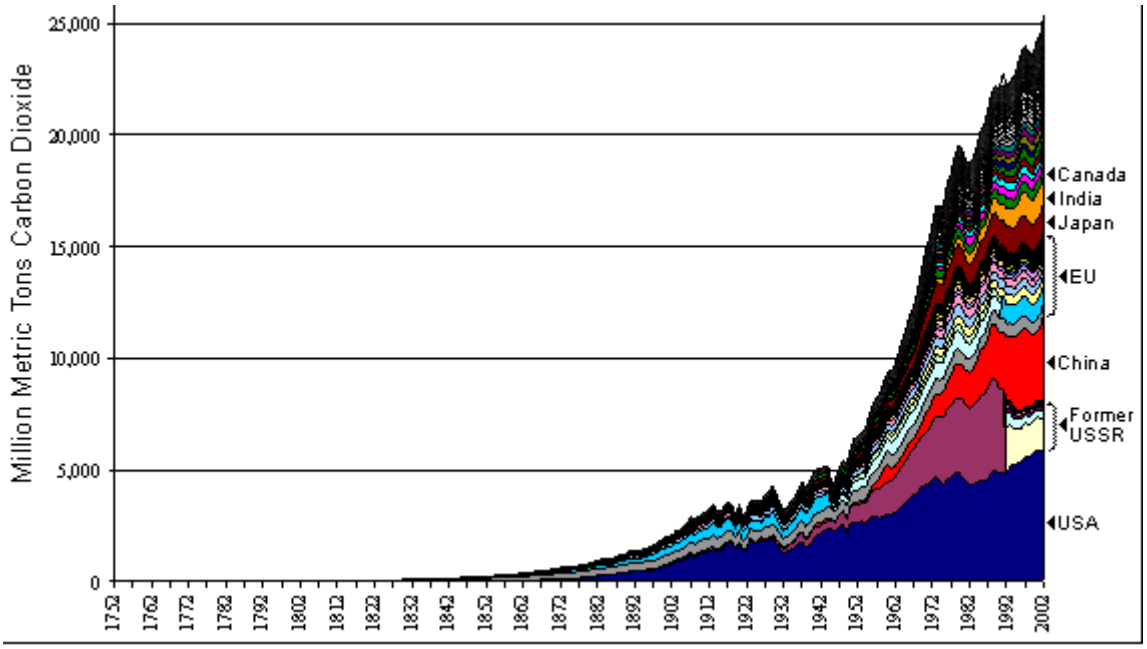


**Figure 2: Global $CO_2$ Emission from Fossil Fuel Burning, Cement Manufacture, and Gas Flaring (1752-2002)**

Atmospheric $CO_2$ levels have increased from about 315 ppm in 1958 to 378 ppm at the end of 2004, which means human activities have increased the concentration of atmospheric $CO_2$ by 100 ppm or 36 percent [8].

# 2. Renewable Energy

Renewable energy represents energy that is generated from natural sources and can be replenished in a short period of time. Renewable types of energy can be subdivided into three areas: *solar energy, tidal power and geothermal energy* [9].

Geothermal energy is derived from heat stored in the earth, or the collection of absorbed heat derived from underground, while tidal power is the only form of energy derived from relative motions of Earth-Moon system in combination with earth's rotation. Solar energy represents the most available energy source on Earth, and only small amount of it is currently used for energy production. Solar radiation along with secondary solar resources such as wind and wave power, hydroelectricity and biomass are the main forms of solar energy.

Overall consumption from renewable sources in the United States totaled 6.8 quads (quadrillion Btu) in 2007, or about 7 percent of all energy used nationally. Consumption from renewable sources was at its highest point in 1997, at about 7.2 quads [10]. The role of the energy consumption in the nation's energy supply in 2007 may be seen in Figure 3. The portion of the renewable energy consumption is still significantly low comparing with the other energy sources.

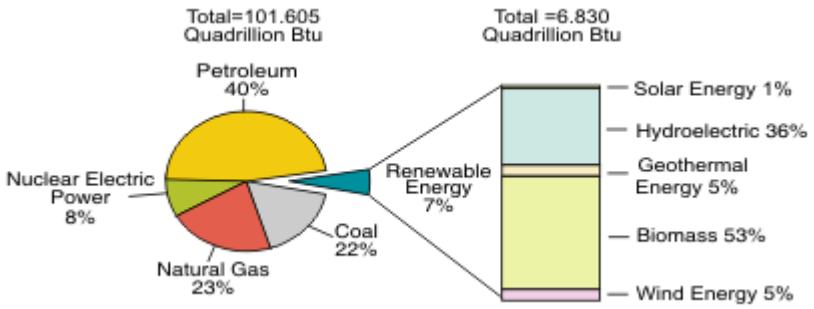


**Figure 3: The role of the energy consumption in the nation's energy supply, 2007** [10]

## 2.1. Energy from Biomass and Ethanol Production

Biomass represents a solar energy stored in organic matter in trees, agricultural crops and other living plant material. During the lifetime plants perform photosynthesis, which is a metabolic pathway that converts carbon dioxide into carbohydrates (sugars, starches and cellulose) using energy from the sun. Carbohydrates are the organic compounds that make up biomass.

Using biomass to produce energy can possibly solve the problems that world faces because of excessive use of fossil fuels, and can significantly reduce greenhouse gas emissions, pollution and waste management problems.

Biomass can be used as a fuel in various forms. One of the most important biomass fuels is wood, but wood is often too valuable to burn and it is usually used for other applications [11]. On the other hand, the residues from the forest and wastes from wood processing and manufacturing operations could be used as an alternative energy source to produce fuel. In most industrial countries, this waste is available at low or even bargain prices.

Many agricultural products can be used in biofuel production. They are specially grown for this purpose and they include corn and switchgrass in the United States, wheat and sugar beet in Europe and sugar cane in Brazil. The current principal fuel used as a petrol substitute for motor vehicles is *ethanol* and it is produced by sugar fermentation process. Ethanol or ethyl alcohol ($C_2H_5OH$) is a clear colorless liquid. It is biodegradable, low in toxicity and causes little environmental pollution if spilt. Ethanol burns to produce carbon dioxide and water. It is a high octane fuel and has replaced lead as an octane enhancer in petrol. By blending ethanol with gasoline we can also oxygenate the fuel mixture so it burns more completely and reduces polluting emissions [12].

Of the global fuel ethanol production of about 40 billion liters in 2006, around 90 percent was produced in Brazil and the United States. Brazil is the most competitive producer, and has the longest history of ethanol production (Figure 4). They use about the half of their sugarcane to produce ethanol, while the consumption is mandated by the government [13].

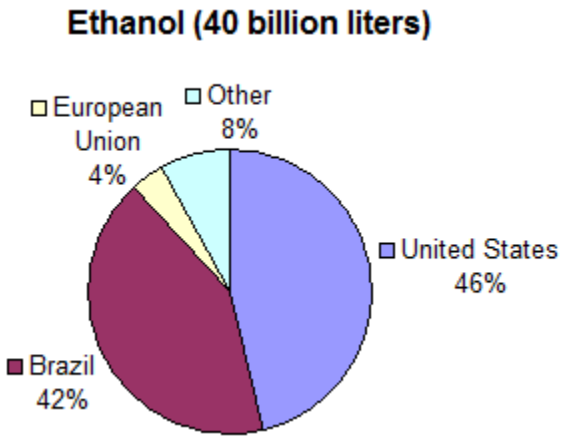


**Figure 4: World Ethanol Production in 2006 [13]**

In the United States ethanol is mostly produced using corn as a biomass, and it currently constitutes only a small fraction of fuel supply. Production capacity significantly increases and the production in 2007 reached 6,500 million gallons [14].

The biggest issue connected with the ethanol production is rising agricultural crop prices from demand for biofuels, which makes the potential conflict between food and fuel. The grain required to fill the tank of a sport utility vehicle with ethanol (240 kg of corn for 100 liters of ethanol) could feed one person for a year, so it could be seen that competition between food and fuel is real [13].

### 2.1.1. Cellulosic Material

In order to overcome problems that ethanol production from agricultural crops can cause, cellulosic material can be used instead and the major attention is on various wastes, such as agricultural waste and waste from different industries. Cellulosic material can be supplied from a variety of resources at a very low price and can be classified in four groups based on the type of resource:

- Wood
- Municipal Solid Waste
- Waste Paper
- Crop Residue Resources

Cellulosic material is composed of lignin, cellulose and hemicellulose and it is sometimes referred to as lignocellulosic material. Cellulose molecules consist of long chains of glucose (6-carbon sugars) as do starch molecules, but have a different structural configuration. These structural features plus the encapsulation by lignin makes cellulosic

materials more difficult to hydrolyze than starchy materials. Hemicelluloses are also comprised of long chains of sugar molecules, but contain pentoses in addition to glucose [15].

The structural composition of various types of cellulosic biomass materials is given in Table 1 [15]:

**Table 1: Composition of various type of cellulosic biomass material (% dry weight)**

| Material | Cellulose | Hemicelluloses | Lignin | Ash | Extractives |
|---|---|---|---|---|---|
| Algae (green) | 20–40 | 20–50 | — | — | — |
| Cotton, flax, etc. | 80–95 | 5–20 | — | — | — |
| Grasses | 25–40 | 25–50 | 10–30 | — | — |
| Hardwoods | 45±2 | 30±5 | 20±4 | 0.6±0.2 | 5±3 |
| Hardwood barks | 22–40 | 20–38 | 30–55 | 0.8±0.2 | 6±2 |
| Softwoods | 42±2 | 27±2 | 28±3 | 0.5±0.1 | 3±2 |
| Softwood barks | 18–38 | 15–33 | 30–60 | 0.8±0.2 | 4±2 |
| Cornstalks | 39–47 | 26–31 | 3–5 | 12–16 | 1–3 |
| Wheat straw | 37–41 | 27–32 | 13–15 | 11–14 | 7±2 |
| Newspapers | 40–55 | 25–40 | 18–30 | — | — |
| Chemical pulps | 60–80 | 20–30 | 2–10 | — | — |

Alcohol production is accomplished by fermentation action of microorganisms. In this application, microorganisms use sugar and convert it into alcohol through complex metabolic pathway. To produce ethanol from lignocellosic materials, it is essential to hydrolyze it before fermentation, since microorganisms do not have enzymes to digest cellulose. In other words, treatment of cellulosic material is necessary in order to break it down to simple sugars. The first step is pretreatment and it is needed to liberate the cellulose from the lignin seal and its crystalline structure. Most pretreatments are done through physical or chemical applications, and the available techniques include acid hydrolysis, steam explosion, ammonia fiber expansion, alkaline wet oxidation and ozone

pretreatment [16]. The second step is hydrolysis where the long chains of sugar molecules are broken down to free the sugar, so the microorganism are able to use it. Two major processes are used: a chemical hydrolysis using acids, or enzymatic reaction using specific cellulase enzymes that brakes cellulose into glucose molecules.

### 2.1.2. Algae Fuel

As can be seen from the Table 1, algae could possibly be used as very effective cellulosic material for biofuels production. Algae only consist of cellulose and hemicellulose, while crystalline structure of lignin is absent. This means that the preparation of algae material for fermentation does not need pretreatment in order to remove lignin.

Algae are a large and diverse group of simple, typically autotrophic organisms that use photosynthesis to transform carbon dioxide and sunlight into energy. They grow so efficiently that they can double their weight several times a day. Algae can grow in salt water, freshwater or even contaminated water, at sea or in ponds, and on land not suitable for food production [17].

## 2.2. Butanol vs. Ethanol

Butanol is an alcohol that also can be produced by fermentation from corn, grass, leaves, agricultural waste and other biomass. Butanol is usually produced by the traditional ABE fermentation - the anaerobic conversion of carbohydrates by strains of *Clostridium* into acetone, butanol and ethanol [18]. It has many superior properties as an alternative fuel when compared to ethanol. These include:

- Butanol is a four carbon alcohol. It has doubled the amount of carbon of ethanol, which equates to a 25 percent increase in harvestable energy-Btu's (110,000 Btu's per gallon for butanol vs. 84,000 Btu per gallon for ethanol). Gasoline contains about 115,000 Btu's per gallon [19].
- Butanol is six times less "evaporative" than ethanol and 13.5 times less evaporative than gasoline.
- Butanol is far less corrosive than ethanol and can be shipped and distributed through existing pipelines and filling stations (no car modification is required).
- Butanol can be used as a replacement for gasoline gallon for gallon e.g. 100%, or any other percentage. Ethanol can only be used as an additive to gasoline up to about 85% and then only after significant modifications to the engine. Worldwide 10% ethanol blends predominate [19].

# 3. Butanol Production

## 3.1. Fermentation Processes

Fermentation has been known and practiced since prehistoric times, long before the scientific principles were understood. The term 'fermentation' is derived from the Latin word *fervere* which means *to boil.* The boiling appearance is due to the production of carbon dioxide bubbles caused by the anaerobic catabolism of the sugars present in the extract. The production of alcohol by the action of yeast on malt or fruit extract was the very first industrial process for the production of a microbial metabolite and has been carried out on a large scale for many years [20]. Thus, industrial microbiologists have extended the term fermentation to describe any process that involves action of microorganisms on sugars to produce certain product.

An established fermentation process may be divided into six basic component parts [20]:

1. The formulation of media to be used during the development of inoculum and in the production bioreactor.
2. The sterilization of media, bioreactor and additional equipment.
3. The production of an active, pure culture in sufficient quantity to inoculate the production vessel.
4. The growth of the organism in the bioreactor under optimum conditions for product formation.
5. The extraction of the product and its purification.
6. The disposal of effluents produced in the process.

Figure 5 presents major processes involved in fermentation processes [20].

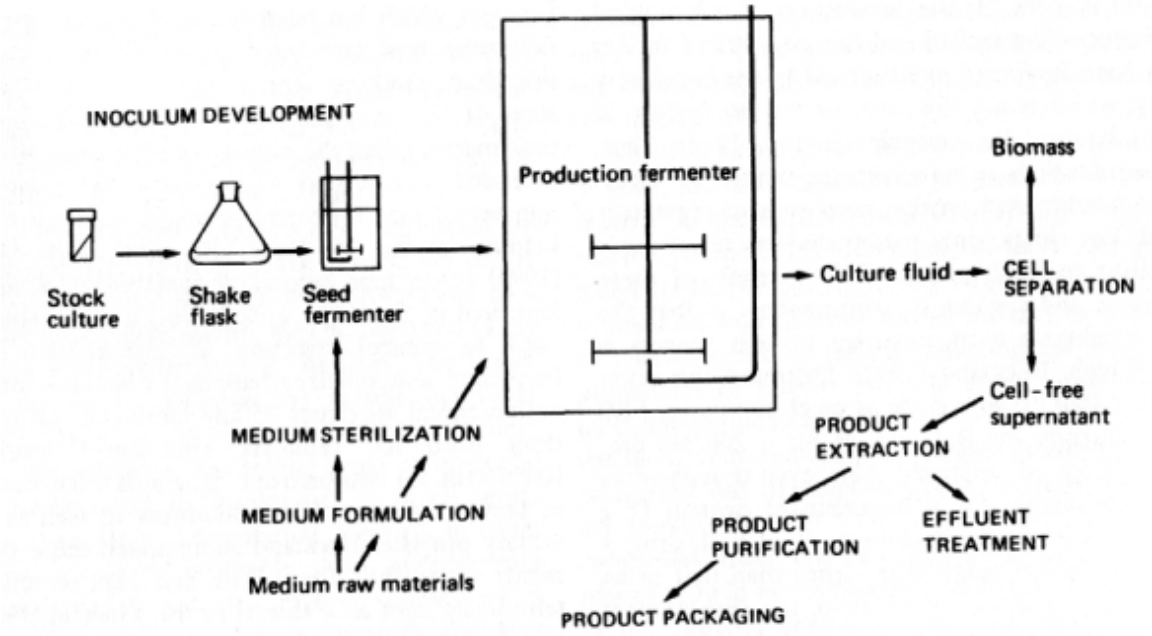


**Figure 5: Major fermentation processes**

### 3.1.1. ABE Fermentation

ABE fermentation is a process that utilizes bacterial fermentation to produces acetone, butanol and ethanol from biomass. It is well known process and it was primary used to produce acetone during World War II. The process is strict anaerobic, which means that it must not have any oxygen present. Anaerobic environment is usually maintained by bubbling nitrogen gas. The process produces acetone, butanol and ethanol in a ratio of 3-6-1. It usually uses a strain of bacteria from *Clostridium* family. *Clostridium acetobutylicum* is the most well known strain, although *Clostridium beijerinckii* is used for this process, with very good results.

## 3.2. Microbial Growth Kinetics

Microbial growth can be defined as an orderly increase in cellular components, resulting in cell enlargement and eventually leading to cell division [21].

Fermentations may be carried out as a batch, continuous and fed-batch processes. The mode of operation is dictated by the type of product being produced. This chapter will consider the kinetics and application of batch processes.

### 3.2.1. Batch Culture

Batch culture is a closed culture system which contains an initial, limited amount of nutrient. Cell suspension in a system increases in biomass by cell division and cell growth until a nutrient availability becomes limiting [22]. During this period, the inoculated cell culture will pass through a number of phases, as illustrated in Figure 6. After inoculation there is a period during which it seems that no growth take place. This is a phase referred to as the *lag phase* and may be considered as a time of adaptation. In a commercial process the length of the lag phase should be reduced as much as possible and this may be achieved by using a suitable inoculum [20]. The next phase is *exponential* or *log phase* when the growth rate of the cells gradually increases. The cells grow at the constant, maximum rate. Mathematically, this exponential growth can be described as follows:

- rate of change of biomass is **$dx/dt = \mu x$**

where x is concentration of microbial biomass, t is time in hours and μ is specific growth rate per hour.

When integrated this equation gives:

$$\mathbf{x}_t = \mathbf{x}_0 e^{\mu t}$$

where $x_0$ is the original biomass concentration, $x_t$ is the biomass concentration after the time interval t and e is the base of the natural logarithm.

On taking natural logarithms, equation becomes:

$$\mathbf{\ln x_t = \ln x_0 + \mu x.}$$

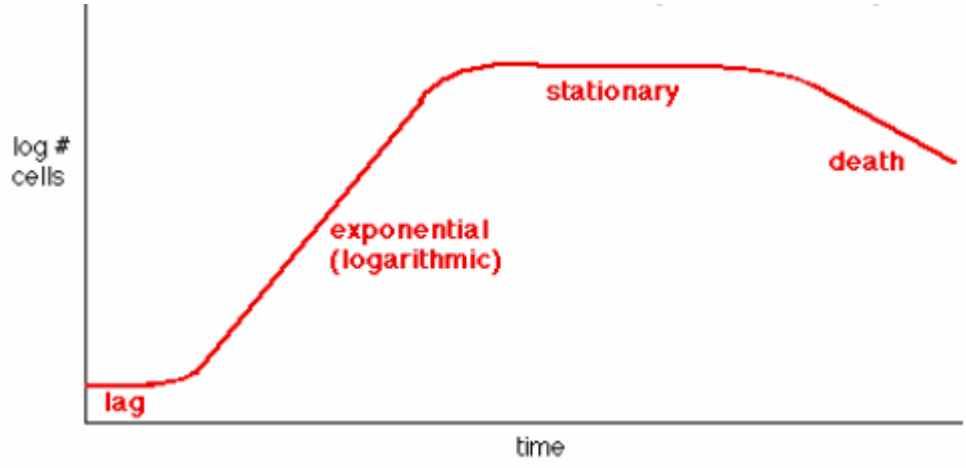


**Figure 6: Phases of Microbial Growth in Batch Culture** [23]

A plot of the natural logarithm of biomass concentration against the time (Figure 6) should yield a straight line, the slope of which would equal μ. During the exponential phase nutrients are in excess and the organism is growing at its maximum specific growth rate $\mu_{max}$ [20].

During *stationary phase*, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust resources that are available to them. This phase is a constant value as the rate of bacterial

growth is equal to the rate of bacterial death. At *death phase*, bacteria run out of nutrients and die. After this phase, the fermentation process is considered to be completed. Thus, productivity in batch culture will be greatest at $\mu_{max}$ and improved product output will be achieved by increasing both μ and biomass concentration [20].

## 3.3 Media for Fermentation

Design of a media for fermentation is a very important issue today. It depends on the microorganism used for the process, as well as the product that is desired. Agricultural grains represent a very good source of food for microorganisms, since it is very easy to liberate simple sugars from the grains. But, use of agricultural product significantly affect the price of the food, so rationally thinking it is not a good idea using them as a food source for microorganisms. Cellulosic material is mentioned as a very cheap and efficient way to utilize waste from different industries. But there is a negative side of this approach. Microorganisms can not digest cellulose and it must be broken down in order to be utilized. Several techniques are used to brake down cellulose and liberate glucose, but the cost of this is still considered as a problem. In order to overcome the problems, microbiologists use different genetic modification techniques to develop new bacterium strains able to directly digest cellulose and produce butanol.

In order to perform fermentation, all microorganisms require water, sources of energy, carbon, nitrogen, mineral elements and vitamins. Medium prepared for this application should meet as many as possible of the following criteria [20]:

- It will produce the maximum yield of product or biomass per gram of substrate used

- It will produce the maximum concentration of product
- It will permit the maximum rate of product formation
- There will be the minimum yield of undesirable products
- It will cause minimal problems during media making and sterilization
- It will cause minimal problems in other aspects of the production process (agitation, purification and waste treatment)

The very important requirement in media formulation considers the price of the substrates used. The use of cane molasses, starch, glucose, sucrose and lactose as a carbon sources, and ammonium salts, urea, nitrates, corn steep liquor as nitrogen sources, have tended to meet most of the abovementioned criteria because they are very cheap [20].

Water is the major component of any fermentation process. It is used in a fermentation media, as well as in other services such as heating, cooling, cleaning and rinsing. The reuse and efficient use of water is always of high priority.

Energy for microbial growth comes from either the medium components or from light. Most industrial microorganisms are chemo-organotrophs and the common energy sources for them are carbohydrates, lipids and proteins that are provided in a media [20].

The choice of carbon sources is always determined by the microorganisms used in the process, and the main product they produce. It is common practice nowadays to use carbohydrates as the carbon source. The concentration of sugars in the media should be carefully controlled, since it is recognized that the rate at which the carbon source is metabolized can often influence the formation of products.

Most industrially used microorganisms can utilize both organic and inorganic sources of nitrogen. Inorganic nitrogen may be supplied as ammonia gas, ammonium

salts or nitrates [20]. Ammonia is usually used for pH control in the reactor and as the major nitrogen source. Organic nitrogen may be supplied as amino acid, protein or urea [20].

All microorganisms require certain mineral elements for growth and metabolism [20]. In most media, magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential components.

## 3.4. Design of a Bioreactor

After obtaining microorganisms and preparing the adequate media for them, they should be combined in a well equipped bioreactor. The main function of a bioreactor is to provide a controlled environment for microbial growth, to obtain a desired product. In designing and constructing it should be considered that the vessel should be capable to operate aseptically for some period of time. It is extremely important issue, since no contamination is desirable during fermentation. Depending on metabolic activities of microbes, adequate gas bubbling and agitation should be provided. If the process is aerobic, aeration is very important step, while for the anaerobic processes usually nitrogen gas should be introduced through a sparger situated near the bottom of the vessel in order to remove any oxygen present. The agitator is required to achieve a number of mixing objective. The speed of agitation should be carefully controlled and should not cause damage to the organism. Agitators may be classified as disc turbines, vaned discs, open turbines of variable pitch and propellers [20]. Agitation and gas bubbling will produce foam that is controlled by chemical antifoam agents. Operating temperature is one of the most crucial things in any fermentation process, and a system of temperature control should be provided. Heating to a desirable temperature may be achieved using water

jacketed vessels or heat-blanketed vessels. During the fermentation some heat is normally generated and internal cooling coils are usually the part of the reactor design to allow cooling. Changes of pH during the process indicate that metabolic activity of microbes took place. Therefore, a system of pH control should be available. Dealing with anaerobic fermentation, it is important to control oxygen amount in the vessel. Dissolved oxygen probe along with pH probe are immersed into the vessel and provide adequate measurements throughout fermentation process. In order to examine each phase of the process, sampling procedures must be performed. Therefore, sampling facilities are a regular part of any bioreactor. Some of the principal features of bioreactor design are illustrated in Figure 7 [24].

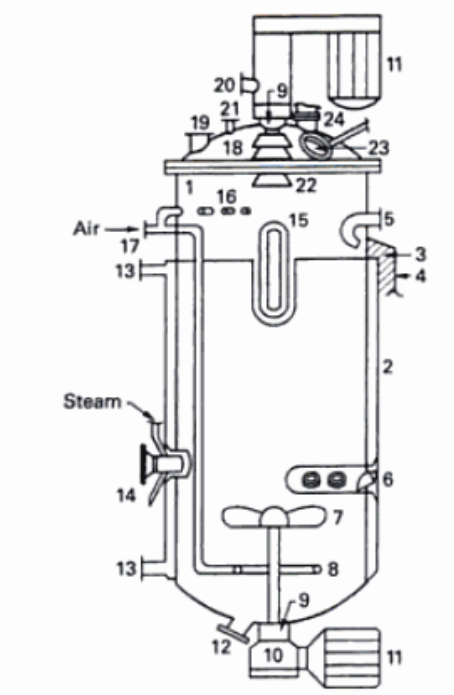


**Figure 7: A typical bioreactor: (1) reactor vessel; (2) jacket; (3) insulation; (4) shroud; (5) inoculum connection; (6) ports for pH, temperature and dissolved oxygen sensors; (7) agitator; (8) gas sparger; (9) mechanical seals; (10) reducing gearbox; (11) motor; (12) harvest nozzle; (13) jacket connections; (14) sample valve with steam connection; (15) sight glass; (16) connections for acid, alkali and antifoam chemicals; (17) air inlet; (18) removable top; (19) medium or feed nozzle; (20) air exhaust nozzle; (21) instrument ports (several); (22) foam breaker; (23) sight glass with light and steam connection; (24) rupture disc nozzle.**

Figure 8 shows the BIOFLO 110 Bioreactor used for butanol production in Chemical Engineering department.

**Figure 8: BioFlo 110 Bioreactor (7 L)**

In fermentation with strict aseptic requirements it is important to select bioreactor materials that can withstand repeated steam sterilization cycles [20]. On a small scale (1 to 30 L) it is possible to use glass and/or stainless steel. Glass is usually material that vessels are made of. It is useful because it gives smooth surfaces, is non-toxic, corrosion proof and it is easy to examine the interior of the vessel. There are two basic types of fermentor used [20]:

- A glass vessel with a round or flat bottom and a top flanged carrying plate (Figure 9)
- A glass cylinder with stainless-steel top and bottom plates

**Figure 9: Glass vessel with a round bottom and a top flanged carrying plate (BIOFLO 110)**

Pilot scale and industrial scale vessels (Figure 10) are normally constructed of stainless steel or at least have a stainless-steel cladding to limit corrosion [20].

**Figure 10: Stainless steel large scale bioreactor**

## 3.5. Sterilization

As mentioned before, fermentation product is produced by metabolic action of the desirable microorganisms on the nutrient medium present in the vessel. The process must be aseptic; otherwise the fermentation will be spoiled by unwonted products. If the medium is invaded by a foreign microorganism it would have to support the growth of both the production organism and the contaminant, which will result in a loss of productivity [20]. The contaminant itself may degrade the desired product, or its products may act as inhibitors to a wanted product.

Sterilization refers to any process that effectively kills or eliminates microorganisms. The process should be done prior the fermentation, and it includes sterilization of the media, as well as a surface and equipment that will be used in the process. Medium may be sterilized by many processes, such as filtration, radiation, ultrasonic treatment, chemical treatment or heat. Whatever method is chosen for sterilization, it is absolutely not possible to remove all viable cells present in a media. The process itself depends on the type of fermentation to be performed. For continuous type fermentation, media is usually sterilized in a continuous heat exchanger before flowing into vessel for fermentation. In this project we were dealing with batch fermentation, therefore a batch sterilization process will be introduced.

The destruction of microorganism by heating is usually used method in batch sterilization and it may be represented by a first order kinetics equation [4]:

$$-dN/dt = kN \qquad (3.1)$$

Where $N$ is the number of viable microorganism, $t$ is the time of the sterilization treatment, $k$ is the reaction rate constant, or the specific death rate and $t$ is time.

It is important to appreciate that we are considering the total number of microorganisms present in the volume of medium to be sterilized, not the concentration.

When integrated, equation (3.1) gets following expression:

$$N_t / N_0 = e^{-kt} \qquad (3.2)$$

Where $N_0$ is the number of viable organisms present at the start of the sterilization treatment, $Nt$ is the number of viable organisms present after a treatment period *t.*

On taking natural logarithms, equation (3.2) is reduced to:

$$\ln ( N_t / N_0 ) = - kt \qquad (3.3)$$

The graphical representation of equation (3.3) is illustrated in Figure 11.

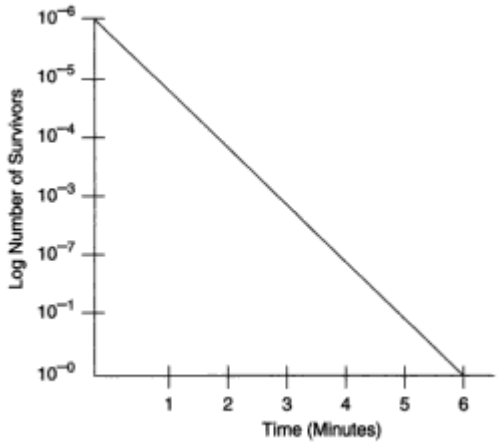


**Figure 11*:* Natural logarithm of the proportion of survivors of a population of microorganisms subjected to a lethal temperature over a time period**

It may be seen that viable microorganism number declines exponentially over the treatment period. The kinetics description makes prediction that an infinite time is required to achieve absolutely sterile conditions ($N_t = 0$) [20]. A frequently adopted risk of contamination is 1 in 1000, which indicate that $N_t$ should equal $10^{-3}$ of a viable cell [20].

The batch sterilization is usually performed in an autoclave, which is a device to sterilize equipment and supplies by subjecting them to high pressure steam at 121 °C or more. Media is poured into the fermentation vessel, closed and then sterilized on appropriate temperature for a certain period of time. The highest temperature which appears to be appropriate for batch sterilization is 121 ° C. The procedure should be designed such that exposure of the medium is kept on minimum. The length of the sterilization is usually 20 to 25 minutes. After the cooling period, it is assumed that the vessel and the medium in it are not contaminated and fermentation process may begin. Figure 12 shows a fermentation vessel prior the sterilization.

**Figure 12: Fermentation vessel ready to be sterilized**

## 3.6. Instrumentation and Control

The success of fermentation process depends on the environmental conditions for biomass and product formation. Microorganisms are live organisms that require specific conditions in order to maintain normal metabolic activity. Prior to actual fermentation, one should be familiar with those requirements, as well as to understand what is happening in a fermentation process and how to control it to obtain optimal operating conditions. The provision of such conditions requires data acquisition and analysis of the fermentation so that any deviation can be corrected [20]. Criteria that are usually monitored are listed in Table 2.

**Table 2: Process sensors and their possible control functions [20]**

| Category | Sensor | Possible control function |
|---|---|---|
| Physical | Temperature | Heat/cool |
| | Agitator shaft power | Rpm |
| | Foam | Foam control |
| | Weight | Change flow rate |
| | Flow rate | Change flow rate |
| Chemical | pH | Acid or alkali addition |
| | Redox | Additives to change redox potential |
| | Oxygen | Change feed rate |
| | Exit-gas analysis | Change feed rate |
| | Medium analysis | Change in medium composition |

There are three main classes of sensors used in monitoring [20]:

- Sensors that are immersed into the media in a fermentation vessel (pH electrodes, dissolved oxygen electrodes)

- Sensors that operate on samples which are contentiously withdrawn from the bioreactor (exhaust-gas analyzers)
- Sensors which do not come into contact with the fermentation broth or gases (tachometers, load cells)

The temperature in the vessel is one of the crucial parameters that should be monitored and controlled in any process. Microorganisms require optimal temperature for its growth. If the temperature is below optimal, microorganisms will maintain its metabolic activity but the efficiency will suffer. On the other hand, when temperature is above required optimal one for the specific strain, it will result in the metabolic inefficiency or even microorganism's death. The heating of the medium in the vessel is usually controlled by use of water jackets or pipe coils within a fermentor, or electrical heat blankets. Figure 13 shows the inside of water-jacketed vessel, while Figure 14 shows non-jacketed vessel configuration.

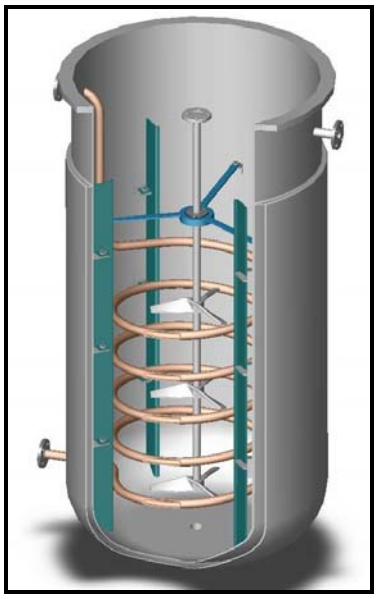

**Figure 13: Water-jacketed vessel**

**Figure 14: Non-jacketed vessel**

The cooling of the media is achieved by running cooling water through the internal cooling coils.

Microbial biomass monitoring in a bioreactor is important since it gives clear understanding of what is going on in the vessel. As mention before, microorganism growth goes through various phases during fermentation, which is dependent on nutrient supplies. Real-time estimation of microbial biomass is usually done indirectly by dry weight samples, cell density (spectrophotometers) and cell numbers (Coulter counters) [20].

In most anaerobic fermentation, it is essential to ensure that the dissolved oxygen concentration does not rise above a specified maximal level. Dissolved oxygen electrodes are used for this application.

In batch culture the pH of an actively growing culture will not remain constant for very long time. In many processes there is a need for pH measurement and control during the fermentation if maximum yield of a product is to be obtained [20].The pH may be controlled by the addition of appropriate quantities of ammonia or sodium hydroxide if too acidic, or sulphuric acid if the change is to an alkaline condition. pH measurement is now routinely carried out using pH electrodes that can withstand repeated sterilization at 121 $^{\circ}$C.

### 3.6.1. Primary Control Unit

The Primary Control Unit (PCU) (Figure 15) is the intelligent interface for entering and displaying process parameters. The PCU is capable of simultaneously controlling up to four individual vessels, and displaying up to 32 parameters in English, French, Spanish, or German [25]. BioCommand® Plus, a PC-compatible data acquisition and control package, is also available for comparing historical data, trend graphing, and validation support [25].

**Figure 15: The Primary Control Unit (PCU)**

# RESULTS

## 1. Introduction

Butanol is produced by the metabolic action of microorganisms through the ABE fermentation. The main products of this process are acetone, butanol and ethanol in a ratio of 3-6-1. *Clostridium acetobutylicum* is the strain widely used for this application, but use of *Clostridium beijerinckii* is getting attention due to the very good results obtained. The bacterium used in this project is *Clostridium beijerinckii ATCC 35702.* It was grown as a batch culture in a small-scale fermentation vessel (5 L working volume) equipped with a heat-blanket in a media comprised of a mixture of many chemicals, including corn syrup as a major food source. All BioFlo 110 fermentation vessels are configured with a 4-baffle stainless-steel insert, dual Rushton agitation impellers, and a high-speed, direct-drive agitation system with mechanical face-seal [26]. Dissolved oxygen and pH probes (Mettler-Toledo) are also included with these fermentors [26]. Samples from the fermenting solution were taken in various stages of the process to allow cell counting which indicates the metabolic action of the bacteria. Changes in pH were recorded throughout the fermentation to indicate metabolic pathways that lead to production of acetone, butanol and ethanol.

## 2. Media Preparation

In order to create a particular environment for bacteria to thrive, a media was created. This consisted of a mixture of many chemicals, including corn syrup as a major food source for the bacteria. The media was prepared in a 4000 ml Erlenmeyer flask on a stir plate to ensure proper dissolving of the ingredients. Around 500 ml of water was placed in the flask initially to prevent clumping. With the stir plate on, all solid

ingredients were added. These included, (for 1.0 liters of media) 0.75 g of $KH_2PO_4$, 0.75 g of $K_2HPO_4$, 0.02 g of $MgSO_4$, 0.01 g of $MnSO_4 \cdot H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 1.0 g NaCl, 10.0 g yeast extract, 2.0 g $(NH_4)_2SO_4$, 18.0 g n-butanol, and enough water to fill the flask up to 1.0 L. The last ingredient is the main food source for the bacteria, 42.0 g corn syrup. All ingredients were measured on an electronic balance using weigh boats. They were multiplied by the number needed for making either 3 or 4 liters. This media was then placed in the refrigerator on 4˚C for further use.

## 3. Sterilization

Prepared media was poured into the vessel in order to be sterilized. This method ensures simultaneous sterilization of the media and the vessel that will be used in the process. According to the vessel manufacturer, dissolved oxygen probe and pH probe are autoclavable; therefore they were immersed in the media and also sterilized. Sterilization was performed in an autoclave on 121˚C for 25 minutes. After autoclaving, a vessel was allowed to cool down for an hour, before reinstalling the system.

Prior to sterilization, four objectives were met in preparing the vessel [27]:

- To minimize the pressure differences throughout the sterilization process by ensuring that the air can transfer freely between the inside and the outside the vessel;
- To ensure that minor pressure differences do not expel liquid from the vessel by clamping off all penetrations that go bellow liquid level;
- To protect hydrophobic filters from blockage, which would occur if condensation were allowed to wet and block the filter surface;
- To protect susceptible vessel assembly components from steam damage.

Figure 1 shows the vessel prepared for sterilization.

**Figure 1: Fermentation vessel with the media inside prepared for sterilization**

As may be seen from the Figure 1, all openings were closed to ensure aseptic conditions in the vessel after the sterilization. All filters were protected from the steam by wrapping them with aluminum foil.

After 25 minutes of sterilization and approximately one hour of cooling, the system was reinstalled and prepared for the fermentation.

**Figure 2: System reinstalled after sterilization and ready for fermentation**

## 4. Control Setpoints

Setpoints were keyed into the controller prior to inoculation, and the vessel was allowed to equilibrate prior to inoculation.

- Temperature . . . . . .35°C
- Agitation . . . . . 130 rpm

## 5. Dissolved Oxygen (DO) Control

The DO probe was calibrated at 0% (obtained by briefly disconnecting the cable), and at 100%, (obtained using 130 rpm agitation and airflow). The mixture of 85% $N_2$, 5% $H_2$ and 10% $CO_2$ was bubbling through the media in order to remove oxygen present in the vessel. DO remained at approximately 0.1% until inoculation.

## 6. Inoculation of Bioreactor

Microorganisms were ordered and received from American Type Culture Collection (ATCC). They were placed in an original glass ampoule in which they were shipped. The ampoule was placed in the laminar flow culture hood. 3 ml sterile syringe with hypo needle was filled with sterile reactor media. The cells were retrieved per instruction of ATCC; the end of the ampoule was heated for 1-2 minutes in the flame of the alcohol burner, then water was poured on it with a squirt bottle. The end was struck with the metal file. The half syringe of medium was injected into the vial, cells were re-suspended and fluid re-drawn back into the syringe. Cells were immediately injected into bioreactor trough rubber septum port.

## 7. Results and Discussion

The DO and pH trend graphs reveal the fermentation history. Two fermentation batches were performed during this experiment. The initial pH at the beginning of the fermentation in the first and the second batch was 5.95 and 6.16 respectively. After inoculation, lag time period was noticed in both batches, although the lag phase in second batch was significantly longer. In the lag phase period, microorganisms were not producing, since they were facing a period of adaptation to the environment in the vessel.

### 7.1. Batch 1 Results

Figure 3 shows the changes in pH in the first batch fermentation. After approximately 1800 minutes (30 h), pH started to decline rapidly to about 5.19, which indicates that microorganisms became active. pH started to increase until settling down at

5.4. As mentioned before, ABE fermentation undergoes two phases. In the first phase, microorganisms produce acids and as a response, pH decreases. In the second phase, acids are transformed to acetone, butanol and ethanol, which drive pH back to less acidic state. Figure 3 shows the changes of pH versus elapsed time from the point when microorganisms were injected to the end of the fermentation. It proves that the fermentation has undergone both phases.

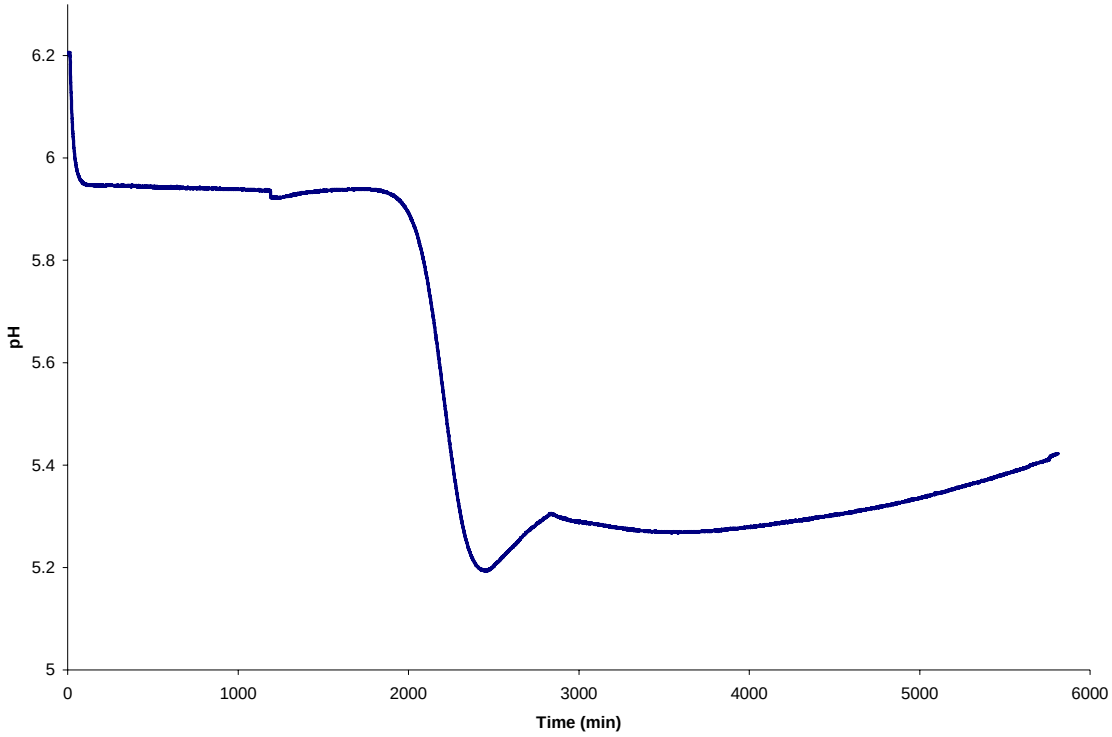


**Figure 3: pH versus elapsed fermentation time in the 1st batch**

The samples from the fermenting solution were taken in various stages of the process, and the amount of cells present in the samples were counted using SmartSpec OD 600 program. The results are shown in Figure 4.

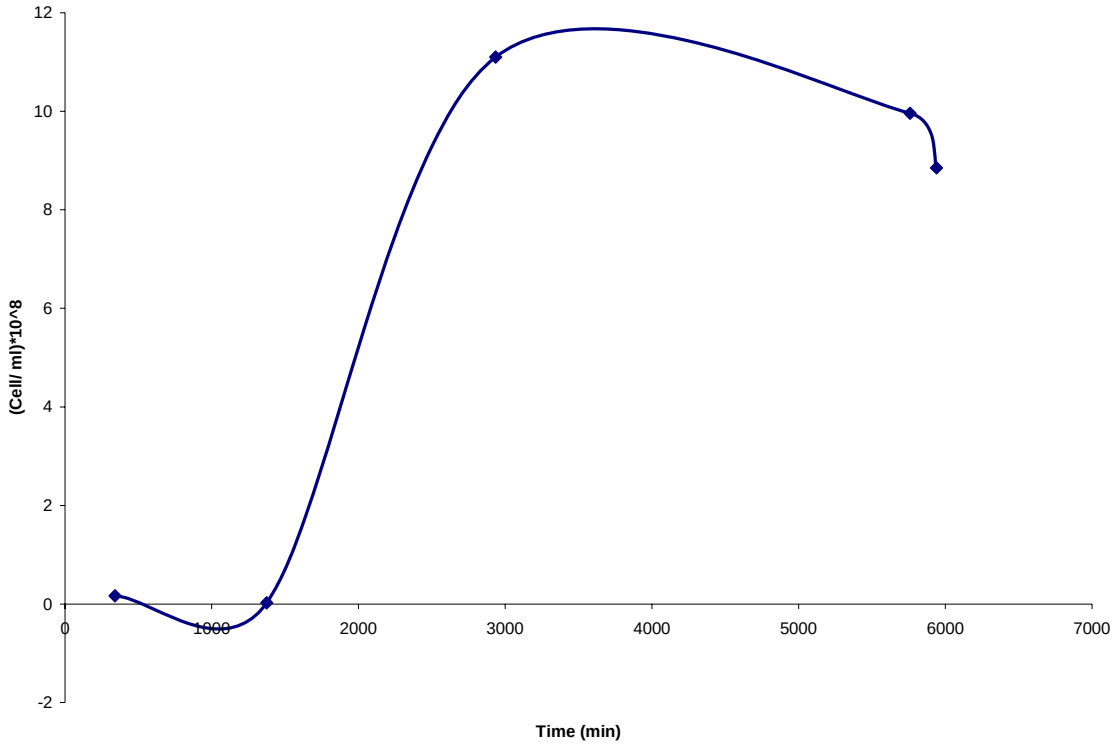


**Figure 4: Cell growth trend in 1st batch**

Figure 5 shows the comparison of growth and pH patterns in the 1st batch.

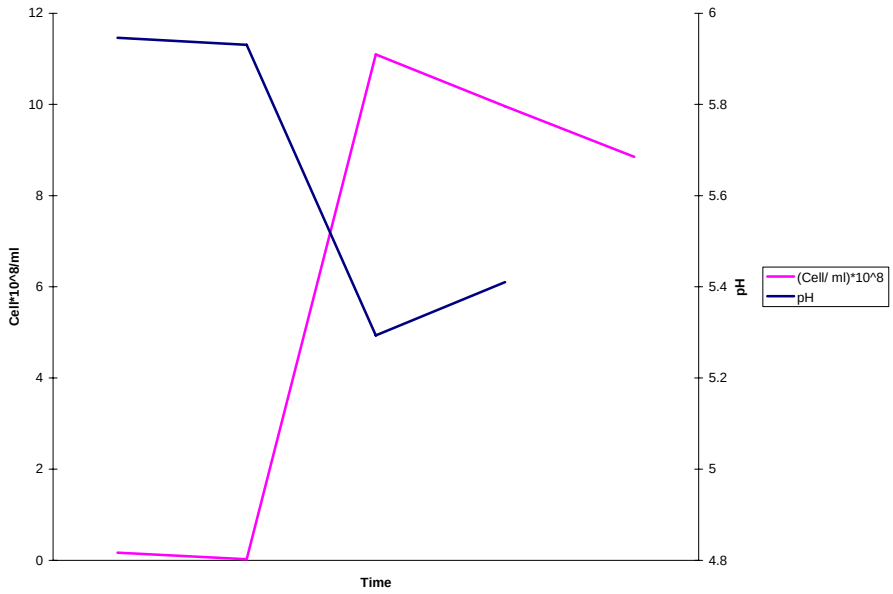


**Figure 5: Cell growth and pH changes in the 1st batch**

As depicted in the figures above, the lag phase lasted about 1400 minutes (23h), when microorganisms reached exponential (log) phase and started to grow. Their metabolism became active and the production started. When comparing Figure 3 and Figure 4, it can be seen that the exponential phase corresponds to a rapid change of pH from 5.95 through 5.19 to the pH value of 5.3. After that, microorganisms reached a stationary phase when pH slightly increased from 5.3 to an end value of 5.4. During this process, it is assumed that the acids were converted to end products (acetone, ethanol, and butanol), which has driven pH to a less acidic environment.

During the process, DO was maintained close to zero by constantly bubbling the mix of gases in order to obtain anaerobic conditions. The minimum concentration achieved was approximately 0.17%. Once the exponential phase started, DO value rapidly changed to 0% and remained the same until the end of the fermentation. Figure 6 shows the trend of the DO change compared to changes in pH.

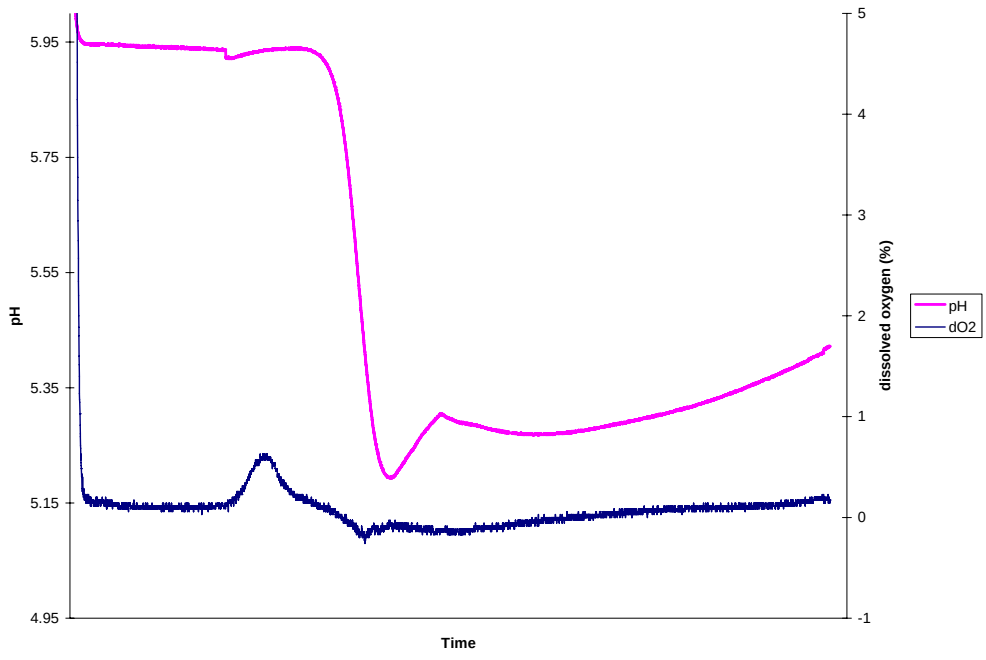


**Figure 6: DO trend compared to pH changes in 1st batch**

There is one possible explanation of this phenomenon. Once the microorganisms became active, they started to produce different products. Common by-products of ABE fermentation are carbon-dioxide and hydrogen. The produced $CO_2$ and $H_2$ additionally lowered the concentration of oxygen in the vessel, which is one more indicator of the metabolic activity.

## 7.2. Batch 2 Results

As previously mentioned, the second batch showed slightly different trends than the first one. Samples taken from the first batch were used to collect and maintain vial cells that can be reused. Cells were frozen until ready to be used as an inoculum for the second batch. The adaptation (lag) phase lasted more than a week, and it was assumed that something went wrong during the preparation and sterilization that affected the process. Therefore, the system was turned off and left to settle down at room temperature of 25°C. After a couple days it was noticed that the fermentation actually took place. The system was turned on again, and data-collection restarted with the temperature remaining at 25°C.

Through further research, I learned that Bacteria of *Clostridium* species can be found in two distinct states. In the vegetative state, the bacterium is metabolically active and uses available nutrients to grow. On the other hand, when nutrients are scarce, a developmental program of endospore formation (sporulation) is initiated, resulting in the production of a highly resistant spore [28]. In the spore state, the bacterium is metabolically dormant, and its genetic material, protected in the core of the spore, can endure a variety

of challenges, including radiation, heat and chemicals [28]. The microorganisms from the first batch were stored in a freezer until inoculated and that resulted in sporulation.

After the second batch was inoculated, microorganisms needed more time to liberate and become active, which extended adaptation (lag) phase. The initial pH after the inoculation in the bioreactor was 6.16. The fermentation took place during the weekend at the temperature of 25°C, so data was collected after it started when pH already reached 5.4 (it is labeled in Figure 7 below as Time=0 min). Previous data is unavailable. Figure 7 shows the changes of pH versus fermentation time, while Figure 8 shows the trend of DO change compared to changes in pH.

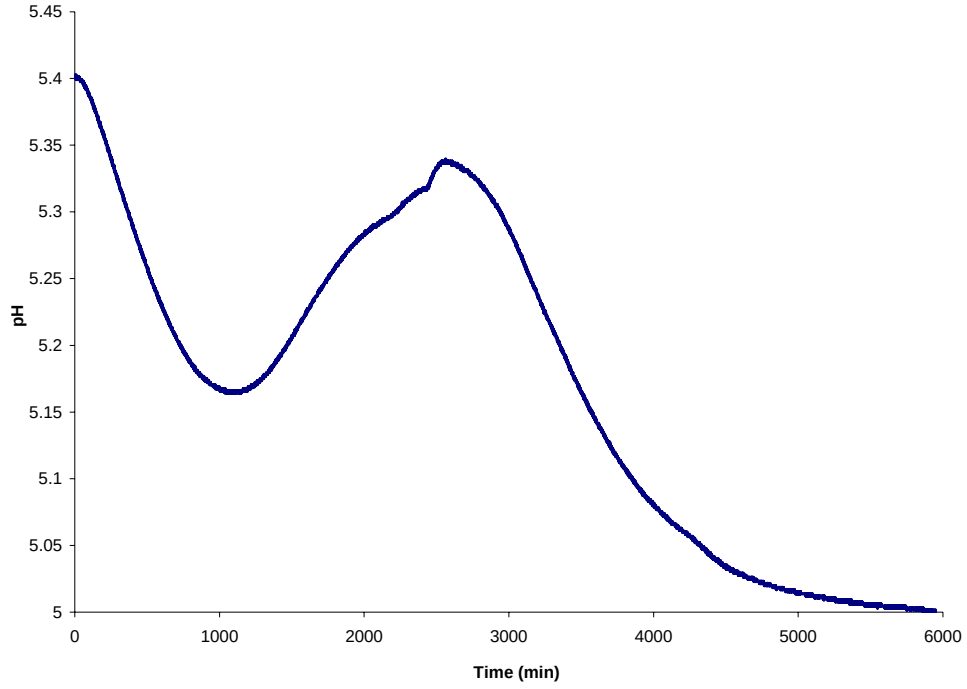


**Figure 7: pH versus elapsed fermentation time in the 2nd batch**

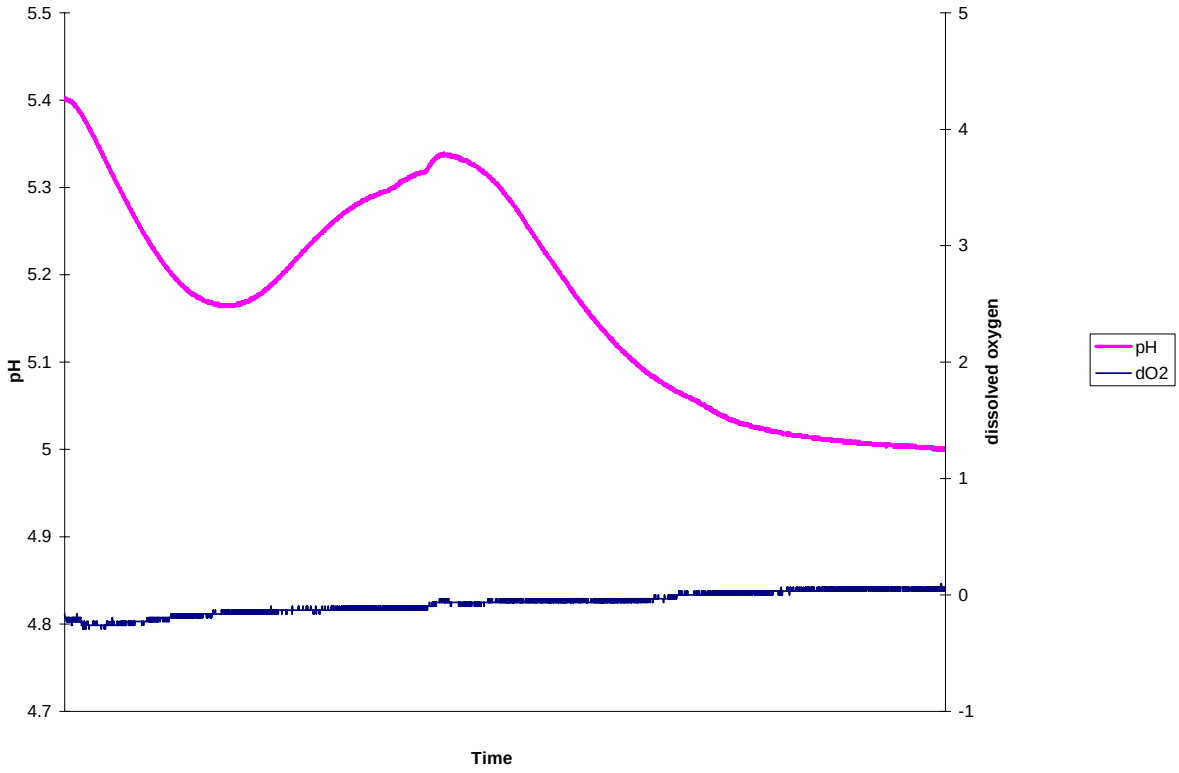


**Figure 8: DO trend compared to pH changes in the 2nd batch**

Cell concentrations in various stages of the process are shown in Figure 9.

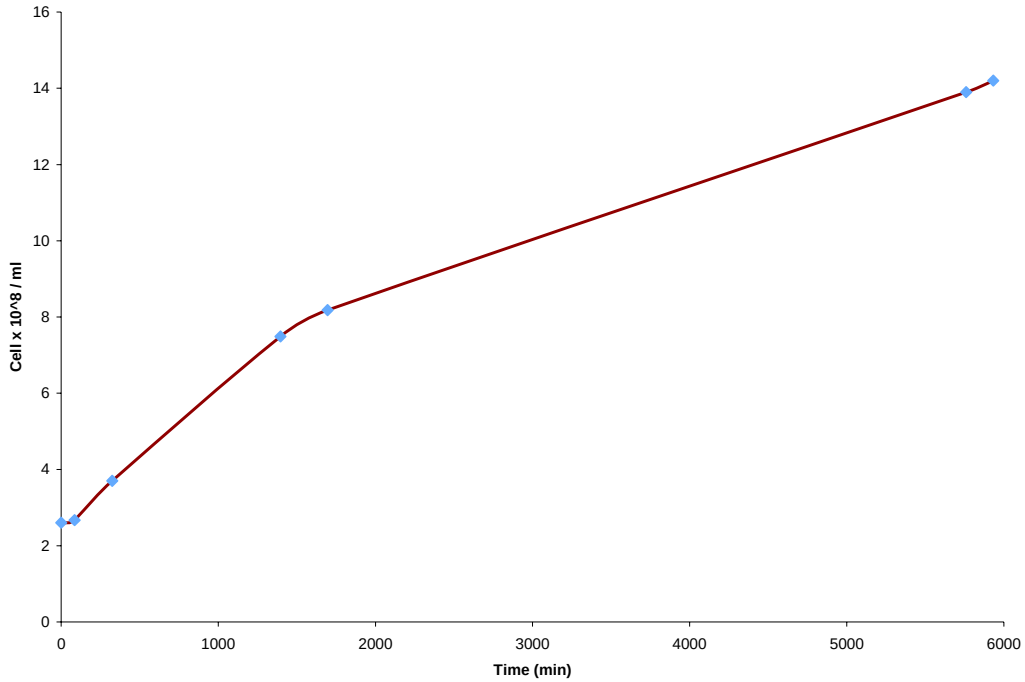


**Figure 9: Cell growth trend in the 2nd batch**

Figure 10 shows the comparison of the cell growth and pH patterns in 2nd batch.

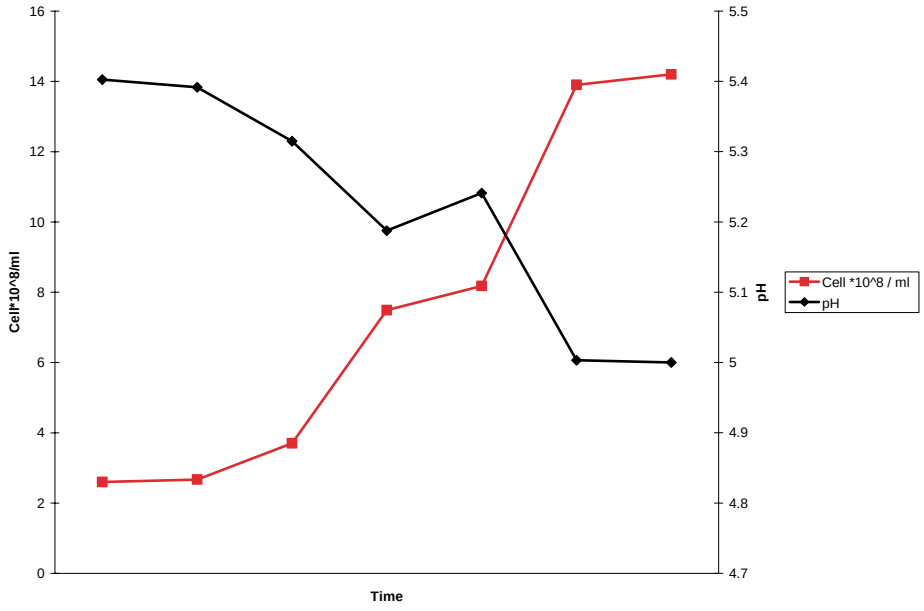


**Figure 10: Cell growth and pH changes in the 2nd batch**

## 7.3. Comparison of the Two Batches

Due to the error occurred during the second batch, it was very hard to compare results achieved in two batches. First batch was operating at the temperature that was optimal for the chosen microorganism (35°C) strain and showed expected growth patterns and results. The second batch showed very long lag (adaptation) phase as a result of sporulation. Fermentation took place at a lower temperature of 25°C, and the data was being collected after the system reached exponential (log) phase. The lack of previous data does not allow proper comparison of the two batches. However, Figure 11 shows the comparison of pH changes in the first and second batch, starting from the log phase when pH reached 5.4 in both batches.

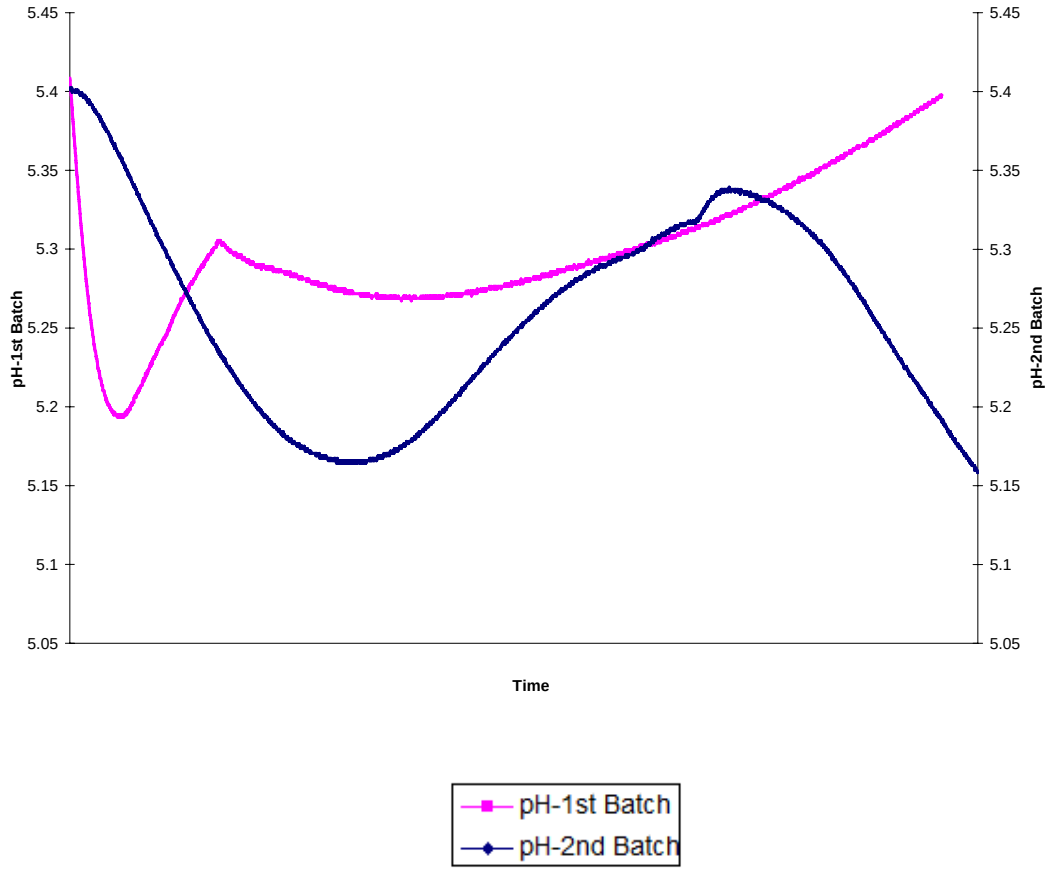


**Figure 11: pH changes in the 1st and the 2nd batch**

It can be noticed that the same patterns occurred in both batches. pH changed from 5.4 to approximately 5.15 as the response to acid production. After some time, pH started to increase in both batches which indicated conversion of acids to the main product. However, it can be seen from the graph that the pH in second batch started to decrease again. There are various reasons that may affect the process. Mediums for both batches are prepared using the same recipe, but the human error during the preparation could be responsible for different microorganism's response to nutrients.

Overall, both fermentation batches proved that a metabolic action did take place and that microorganisms were productive.

### 7.1. Solvent Analysis

Solvents were determined using a Varian CP-3800 Gas Chromatograph equipped with a TCD (thermionic specific) detector and a 1/8 in. packed column designed to handle the high concentrations that could be present in the samples [29]. The initial and final temperatures were 150°C. Both injector and detector temperatures were 220°C. Helium at 44 ml/min was the carrier gas. Samples (1µl) were injected into GC and external standards were used to calculate the actual concentration of solvents. The products obtained during the fermentation in the 1st batch (acetone and butanol) are shown in Figure 12.

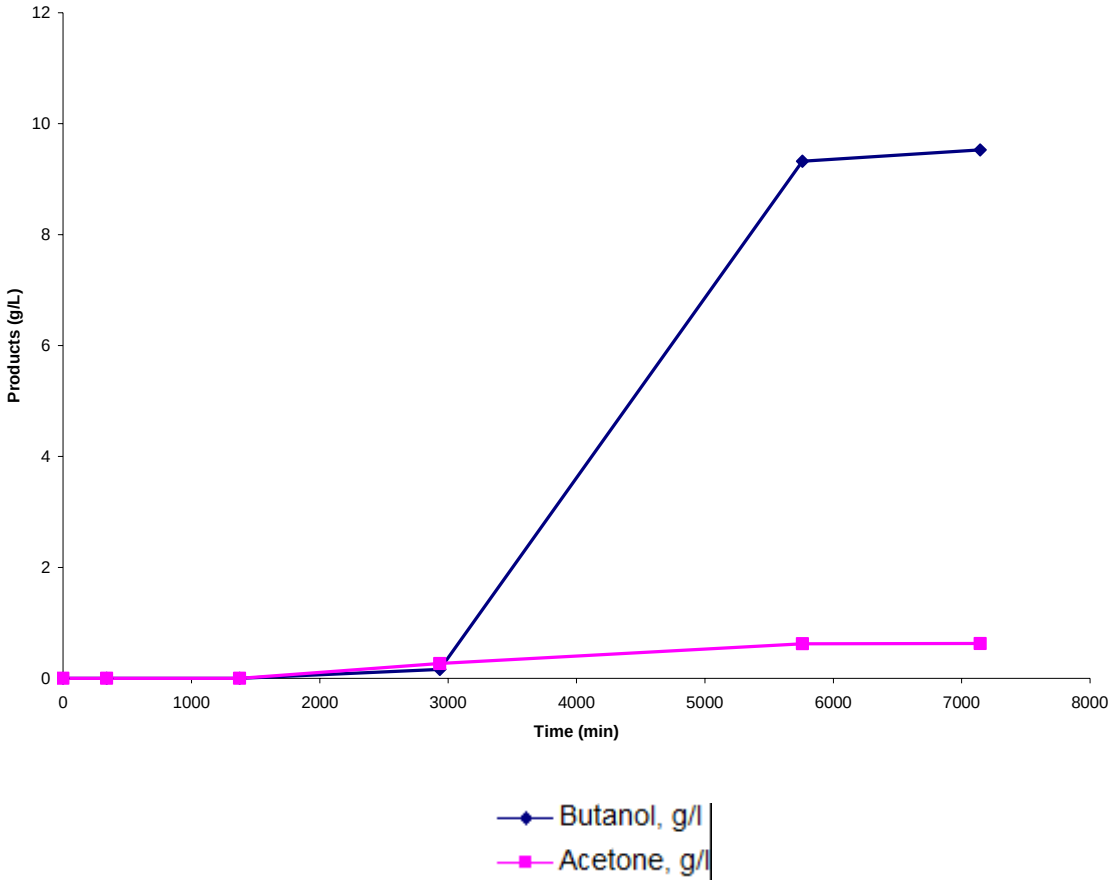


**Figure 12: Production of acetone and butanol from *C. beijerinckii ATCC 35702***

## 8. Conclusion

Using corn syrup as a food source for *Clostridium beijerinckii ATCC 35702* delivered a butanol production of approximately 9.53 g/L after 5 days of fermentation. The pH of the solution after autoclaving and inoculation was about pH 6, which initially decreased, as the sugars were utilized and acids were produced. After two days of the fermentation process, pH began increasing as butanol reached a maximum of 9.53 g/L. Solvents were measured using a Gas Chromatograph.

Butanol production depends a lot on the microorganisms used in the process, as well as on the media prepared for them. The conventional substrates for ABE fermentation are starch or molasses. However, new substrates are introduced, and they include wheat straw, corn fiber, liquefied cornstarch, Jerusalem artichokes, cheese way, apple pomace and algal biomass, in addition to various other substrates derived from cellulose [30]. The typical ABE fermentation using molasses as a main source of sugar usually results in broth containing 13.7 g/L butanol, 5.4 g/L acetone and 1.5 g/L ethanol [30]. Jerusalem artichokes contain sugars that need to be hydrolyzed by acids or enzymes prior to the fermentation. Yield is typically 24 g/L of total solvents [30]. Cheese way also needs pretreatment prior to the fermentation. It contains low sugar, but it is still suitable for ABE fermentation. The solvent production is in the range of 5-15 g/L [30]. Algal fuel forms a suitable fermentation substrate with yield up to 16 g/L [30]. More recently, several other substrates have been used, such as liquefied cornstarch (yield of 81.3 g/L ABE solvents under fed batch mode), wheat straw (yield 12 g/L ABE solvents), and corn fiber hydrolysate (yield of 9 g/L ABE solvents) [30].

The problem faced during this experiment was a long lag phase due to sporulation of microorganisms. In order to overcome this problem in the future and shorten the adaptation (lag) phase, microorganisms should be treated prior the inoculation. Treatment considers exposing microorganisms to a temperature of 80°C for some period of time (usually 10 min). After the treatment, they will be activated which will shorten adaptation time and cause microorganisms to become active more quickly.

Overall, it can be concluded that the fermentation process using this particular strain of bacteria and corn syrup was successful. Microorganisms reacted well on a food source provided, and showed a growth trend expected for batch fermentation. Butanol, as the primary expected product of this fermentation, was successfully produced with a maximum amount of 9.53 g/L.

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