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Biochemical Identification of an Alkalophilic Lactic Acid Bacterium and Testing its Fermentative Capacity

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Biochemical Identification of an Alkalophilic Lactic Acid Bacterium and Testing its Fermentative Capacity

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The project is submitted in partial fulfillment of the requirement for degree of Bachelor Science with Honours in Resource Biotechnology

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DECLARATION

No portion of the work referred to in this report has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning

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Table of contents

Table of contents iii

List of Abbreviations v

List of Figures and Tables vi

ABSTRACT 1

1.0 INTRODUCTION 2

2.0 LITERATURE REVIEW 5

2.1 Alkaline Environments 5

2.2 Alkaliphiles 5

2.3 Lactic Acid 6

2.4 Lactic acid bacteria 7

2.5 Alkaliphile lactic acid bacteria 8

2.6 *Enterococcus faecalis* 8

2.7 Effect of pH 9

2.8 Batch fermentation 9

3.0 MATERIALS AND METHODS 10

3.1 Biochemical identification 10

3.2 Hydrolysis of sago starch 11

3.3 Preparation of stock culture 11

3.4 Inoculum preparation 11

3.5 Fermentation medium 12

3.6 Preparation of agar....12
3.7 Sampling....13
3.8 Spreading method for microorganism culturing....13
4.0 ANALYTICAL METHODS....14
4.1 Determination of dry cell weight....14
4.2 Lactic acid analysis....15
4.3 Reducing sugar analysis....16
5.0 RESULTS AND DISCUSSION....17
5.1 Biochemical identification....17
5.2 Dried cell weight and bacteria growth....21
5.3 Reducing sugar and lactic acid analysis....25
5.4 Culturing of the microorganism by spreading method....29
6.0 CONCLUSION....31
REFERENCES....32
APPENDIX....37

List of Abbreviations

DCW	Dry Cell Weight
OD	Optical density
LA	Lactic acid
LAB	Lactic acid bacteria
YE	Yeast extract

List of Figures and Tables

Table 1: Biochemical response of the bacteria using API20E test kits. 18

Table 2: Effect of the pH on biomass production as dry cell weight (DCW) 21

Table 3: Effect of the pH on lactic acid production reported as NaOH consumed during fermentation and its standard deviation at pH 7 and pH 8. 25

Table 4: First cycle of fermentation at pH7 39

Table 5: Second cycle of fermentation at pH7 39

Table 6: Third cycle of fermentation at pH7 40

Table 7: First cycle of fermentation at pH 8 40

Table 8: Second cycle of fermentation at pH 8 41

Table 9: Third cycle of fermentation at pH 8 41

Figure 1: Optical isomers of L-(+)-lactic acid and D-(-)-lactic acid (mirror image) 6

Figure 2: API 20E kit used for biochemical identification of bacteria. 10

Figure 3: Biochemical identification of the strain using API20E test kits. 18

Figure 4 : Dry cell weight of bacteria during the fermentation for both pH 7 and 8. 22

Figure 5: Effect of the pH on biomass production during fermentation. 23

Figure 6: Specific growth rate of cell during fermentation at pH 7 and 8. 24

Figure 7: Effect of the pH on lactic acid production. 26

Figure 8: Effect of the pH on the metabolism of the strain as glucose consumption. 26

Figure 9: Effect of the pH on the volumetric productivity of *E. faecalis* in batch fermentation. 28

Figure 10: Effect of the pH on the growth of cell in the initial and final stage of the fermentation (left side: initial stage, right side: final stage). 29

Figure 11: Fermenter used during fermentation 37

Figure 12: Biosensor used for glucose analysis.....38

Figure 13: Inoculation of the strip during biochemical test 38

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ABSTRACT

A newly isolated lactic acid bacteria (LAB) from laboratory of Biofuel R&D (UNIMAS) that has the capability to produce lactic acid (LA) using hydrolysed sago starch as the only carbon source by fermentation process was identified biochemically and it's fermentative capacity was studied by using batch fermentation. The strain was tested to ferment for some carbohydrates contained biochemical kit test API 20E. The isolated LAB was used for LA fermentation production. The production biomass and LA production of the LAB strain were studied with two conditions which were at pH7 and pH8 at temperature of 37°C. From this study, the strain was identified as *Enterococcus faecalis* that is able to produce high concentration of LA at pH 8 which is very useful for industrial application. Fermentation at pH 8 produced a maximum dry cell weight of about 8.48g/L and 130.03g/L of LA, while less production was shown at pH 7 with 5.83g/L and 69g/L. It has been found that the growth of biomass and LA production for *Enterococcus faecalis* are influenced by the pH and the optimum pH for this strain is found to be at pH8.

Key words: Lactic Acid, lactic acid bacteria, Lactic acid fermentation, Hydrolysed sago starch

ABSTRAK

Suatu bakteria baru yang diperoleh dari makmal Biofuel R&D (UNIMAS) yang boleh menghasilkan asid laktik menggunakan kanji sagu yang dihidrolisis sebagai sumber karbon melalui proses fermentasi dikenalpasti secara biokimia dan keupayaan fermentasi dikaji dengan menggunakan fermentasi secara "batch". Bakteria tersebut diuji untuk proses fermentasi beberapa karbohydrat yang terdapat dalam API 20E. Bakteria asid laktik yang diperoleh digunakan untuk proses fermentasi untuk menghasilkan asid laktik. Produksi biomass dan asid laktik dikaji menggunakan dua keadaan iaitu pada pH 7 dan pH 8 pada suhu 37°C. Daripada kajian ini, bacteria tersebut dikenal pasti sebagai Enterococcus faecalis yang mampu untuk menghasilkan asid laktik yang tinggi pada pH 8 yang amat berguna untuk kegunaan industri. Fermentasi pada pH 8 memberi berat sel kering maksima dan asid laktik yang tinggi iaitu 8.48g/L dan 130.03g/L manakala pengeluaran berat sel kering maksima kurang ditunjukkan pada pH 7 dengan 5.83g/L dan 69 g/L. Pertumbuhan bakteria dan penghasilan asid laktik bagi bacteria tersebut tinggi pada pH 8. Daripada kajian didapati pengeluaran biomass dan asid laktik bagi Enterococcus faecalis dipengaruhi oleh keadaan pH dan pH optimum bagi bacteria ini adalah pada pH 8.

Kata kunci: fermentasi "batch", asid laktik, bakteria asid laktik, hydrolisis kanji sagu

1.0 INTRODUCTION

2- Hydroxypropanoic acid, $CH_3CHOHCOOH$ or commonly known as lactic acid (LA) is the most broadly occurring natural organic acid (Vishnu *et al.*, 2002). From the past decade, LA has been received much attention due to it's widely use in the industry. Industries that require LA in their products includes pharmaceutical, cosmetic, chemical, textile and food industries (Liu *et al.*, 2010). Conventionally, LA has been used as a preservatives and drug precursor in food and pharmaceutical industries (Zhao *et al.*, 2010). In recent years, the demand for LA is continually increasing. LA can be produced either by synthesis of chemical or by microbial fermentation (Calabia & Tokiwa, 2011).

The commercial production of LA is expensive, this is due to the refined sugar such as glucose and sucrose which is commonly used for the fermentation process (Vishnu *et al.*, 2002). Hence, studies have been performed to substitute the costly carbon sources with raw materials in order to reduce the cost of commercial production of LA. In a study done by Wee *et al.*, (2004) molasses were used for economical production of LA. Other raw materials that commonly used for study are sago, corn, and cassava (Hofvendahl *et al.*, 2000).

According to Li and Cui (2010), there are more than 100 different LA bacteria (LAB) together with filamentous fungi have been used for microbial LA production from renewable resources. The most crucial operational factor that affects the production of LA is pH (Zhang *et al.*, 2007). LAB has different metabolism rate and survival toward the environment. Most LAB has optimum growth at pH 5.5-6.5 and are alkali intolerant (Calabia & Tokiwa, 2011). However there are certain bacteria that can survive and produce LA at alkali condition. Examples of bacteria that can survive and produce LA at pH higher than 7 are *Halalactobacillus halophilus*, *Halalactobacillus miurensis* (Ishikawa *et al.*,

2005), *Halalactobacillus alkaliphilus* (Cao *et al*., 2008) and *Alkalibacterium indicereducens* (Yumoto *et al*., 2008). Most of these bacteria are mesophilic. Bacteria that are able to survive in alkaline condition are termed as alkaliphiles. The discovery of alkaliphiles or alkaliphilic microorganism was fairly recent. Microorganisms from alkaliphilic condition have already created a huge impact in the application of biotechnology for marketing products for consumer (Grant *et al.*, 1990). Example of applications of alkaliphilic microorganisms in biological industry is the production of biological detergents that contain alkaline enzymes such as alkaline cellulases and alkaline proteases (Horikoshi, 1999). Fermentation in alkaline condition will produce different LA. However, less research has been conducted in the bioprocess field like LA fermentation. LA production is significantly affected by the fermentation pH (Mathews & Wenge, 1999). There are very few studies done to examine the stress effect of LAB towards alkaliphilic conditions. Research done by Calabia *et al*., (2011) showed high optical purity of LA up to 98% by using alkaliphilic marine organism.

Concerning with the recent isolation of *Enterococcus faecalis*, the capability of the bacteria in LA production is unspecified, thus it is necessary to study on this bacteria for better understanding of their biochemical features and also the maximum productivity and yield of LA that can be produced by this bacteria.

Objectives

- To identify the isolated LAB by using biochemical test.
- To show that the isolated LAB able to produce high concentration of LA at pH 8 and temperature of 37°C.

Hypothesis

The LAB is able to produce high concentration of LA at alkaline condition especially at pH 8 or higher.

Ho: $\mu_1=\mu_2$ The production of LA at pH is the same at pH 7 and pH 8

Ha: $\mu_1\neq\mu_2$ The production of LA at pH is different at pH 7 and pH 8

2.0 LITERATURE REVIEW

2.1 Alkaline Environments

The presence of carbonate springs, alkaline soils and lakes create the natural existence of alkaline environments. Alkaline condition can be distinguished by the high value of pH which is equal to or more than 8. The most stable naturally occurring alkaline environments on earth that most studied are soda lakes and soda deserts. They have pH values higher than 10 (Tiagoa *et al.*, 2005). Alkaline environments are characterized by high concentrations of Na_2CO_3 (usually as $Na_2CO_3.10H_2O$ or $Na_2CO_3.NaHCO_3.2H_2O$). Natural occurring stable alkaline environment can be divided into two classes which are high Ca^{2+} environments and low Ca^{2+} environments. Examples of environment with low Ca^{2+} are soda lakes and deserts which are dominated by sodium carbonate while groundwater bearing high CaOH is an example environment that contain high Ca^{2+} (Ulukanli & Digrak, 2002).

2.2 Alkaliphiles

The term of alkaliphiles or sometimes known as alkalophiles are referred to organisms that grow optimally at pH higher than 7. Due to the wide distribution of alkalophilic microorganisms, they can be found in most of environment, even in environments not in alkaline condition (Grant, Mwatha, & Jones, 1990). Alkaliphiles are used widely for the manufacture of consumer product for example production of alkali tolerant enzymes. Currently these enzymes are used in detergent compositions and leather tanning. These enzymes are also said to be useful for food, waste treatment and textile industries.

2.3 Lactic Acid

2-hydroxypropionic acid or 2-hydroxypropanoic acid is an organic hydroxyl acid which is commonly known as LA and its chemical composition is $C_3H_6O_3$. The occurrence of LA in nature is widespread. The function of LA in animals and humans is supplying energy to muscle tissues (Reddy *et al*., 2008). LA was first discovered and isolated from sour milk by Carl Wilhem Scheele a Swedish chemist in the year 1780. Production of LA begins in the year of 1881, by fermentation process. It was the first organic acid to be commercially produced using fermentation (John *et al*., 2009).

LA is water soluble, non-volatile, and odourless organic acid. There are two optical isomers of LA which are L- (+) and D- (-) (mirror image) as shown in Figure 1 (Reddy *et al*., 2008). Humans and other mammals produce L (+) isomer whereas in bacteria they produced both D (-) and L(+) enantiomers (Garlotta, 2001). LA is a naturally occurring organic acid that can be produced by chemical synthesis or fermentation. Fermentation using microbes produces stereospecific L(+), D(−) and DL mixture of LA whereas chemical synthesis produces racemic DL-LA. LA is a multi-functional versatile organic acid that has wide range of application, is the first biotechnological production (Reddy *et al*., 2008). LA is used in food, pharmaceutical, leather, textile industries and also as chemical feed stock.

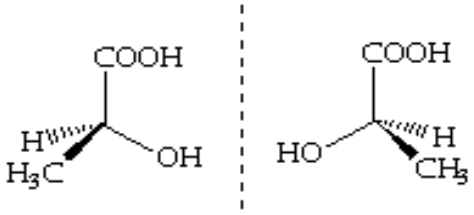

Figure 1: Optical isomers of L-(+)-lactic acid and D-(-)-lactic acid (mirror image)

Polymer producers together with other industrial users generally require large quantity of LA at low cost thus, cheap raw materials is essential for sufficient economic production of LA. Cheap raw materials that have been used for LA production are starchy and cellulosic materials and molasses (John *et al*., 2009).

2.4 Lactic acid bacteria

Lactic acid bacteria (LAB) are gram-positive bacteria, non-sporulating, acid tolerant (Liu *et al*., 2005) present in the shape of rod or cocci, aero-tolerant anaerobes and frequently occur in chains. LAB are heterotrophic organisms. Normally they have complex nutritional requirements because they short of many biosynthetic capabilities (Reddy *et al*., 2008). LAB can be divided into homofermentative and heterofermentative. The difference between these both is the end product of the fermentation. For homofermentative LAB, they convert sugar completely into LA whereas for heterofermentative LAB together with LA other by-products such as acetic acid, ethanol and carbon dioxide are produced (Li & Cui, 2010). In natural, LAB can be found in plant, meat, cereal and dairy fermentation environments. LAB have a long tradition of use in industrial and artisan food and feed fermentation. In this industry, LAB are used as starter cultures for fermenting raw materials of vegetables or animal origin (Liu *et al*., 2005). An example of homofermentative bacteria is *Lactococcus lactis* (Nolasco-Hipolito *et al*., 2002) and *Lactobacillus virdescens* 7-7 is an example of heterofermentative bacteria (Drinan *et al*., 1976). LAB degrade glucose molecule by reduction reaction in anaerobic condition.

2.5 Alkaliphile lactic acid bacteria

Alkaliphile LAB are bacteria that can produce LA by fermentation process. These bacteria can be found either as Gram positive or Gram negative. Most of alkaliphilic LAB are present as aerobes or facultative anaerobes (Ntougias & Russell, 2001). Based on a study done by Ishikawa *et al*., (2011), he stated that "all species of the genus *Alkalibacterium* possess halophilic and alkaliphilic properties and produce LA as the main product of glucose fermentation".

2.6 *Enterococcus faecalis*

Enterococcus genus is a subdivision of the streptococci (Law-Brown & Meyers, 2003). In a study Brown & Meyers (2003), physiologically relate enterococci to streptococci based on their abilities to grow at 10 and 45°C, at pH 9.6 and in the presence of 6.5% NaCl and 40% bile. According to Devriese *et al*., (1990) said that not all enterococci capable to grow at such high pH, salt and bile concentration. *Enterococcus faecalis,* is a normal bacterium which is a gram- positive coccus and anaerobic facultative (Lee *et al*., 2006). E. *faecalis* is considered a species that is included as one of the Lactic Acid Bacteria (LAB). Ohhira, (1999)had done a study on E. *faecalis* with other LAB and in his experiment he found that this strain had been active at 45°C with high LA production compared to other LAB. In another study, researchers have found that E. *faecalis* RKY1 is an efficient lactic acid bacterium for the production of LA from molasses by using batch fermentation, because it could produce LA with high concentration and high productivity from non-pre-treated molasses (Wee *et al.*, 2004).

2.7 Effect of pH

In all fermentation process, pH plays an important role because it can affect the rate of growth and also the production rate. Metabolic reaction of bacteria in processing substrate varies according to pH. Each bacterium has its own optimum pH to process substrate. According to (Nannen & Hutkins, 1991) in a study stated that intracellular pH or cytoplasmic pH of the bacteria has the greatest effect on cellular activity whereas the pH of medium effects indirectly on the cellular activity. There are various ways to control pH of the fermentation process. Examples of ways to control pH are it can be set at the beginning and left to decrease through the acid production, can be controlled by base titration, or by extraction, adsorption and electrodialysis of LA. Only certain bacteria able to survive in high pH, and from this number only few bacteria can do fermentation at alkaline condition. Based on the studies of researchers on the effect of pH on LA they had discovered the optimum pH for LA production varies between pH 5-7 (Hofvendahl & Hagerdal, 2000).

2.8 Batch fermentation

Batch fermentation is a fermentation method which is done in anaerobic condition whereby all the processes done in a closed system without contact from outside. It is a basic fermentation process. The advantages of this method are the concentration and yield of LA are higher, the system is easy to set up and it can be operated with limited knowledge. The performance of process can be improved iteratively learning from earlier batch runs. Besides that, batch fermentation does not need to modify the hardware when the facilities used for many products.

3.0 MATERIALS AND METHODS

3.1 Biochemical identification

The isolated bacterium that was obtained from laboratory of Biofuel R&D (UNIMAS) was biochemically identified using API20e kit (bioMérieux, Inc, France) as shown in the figure 2. 10ml of culture media was centrifuged at 10,000 rpm for 3 minutes. The supernatant was thrown and the pellet was washed with 0.7% of NaCl for three times. The pellet was then transferred in 10ml of sterilized distilled water. Using pipette the bacteria suspension was distributed into the tubes of the strips. The strip was then incubated at 37°C for 24 hours. After the incubation period, the strip was read by referring to the Reading Table that was given together with the package.

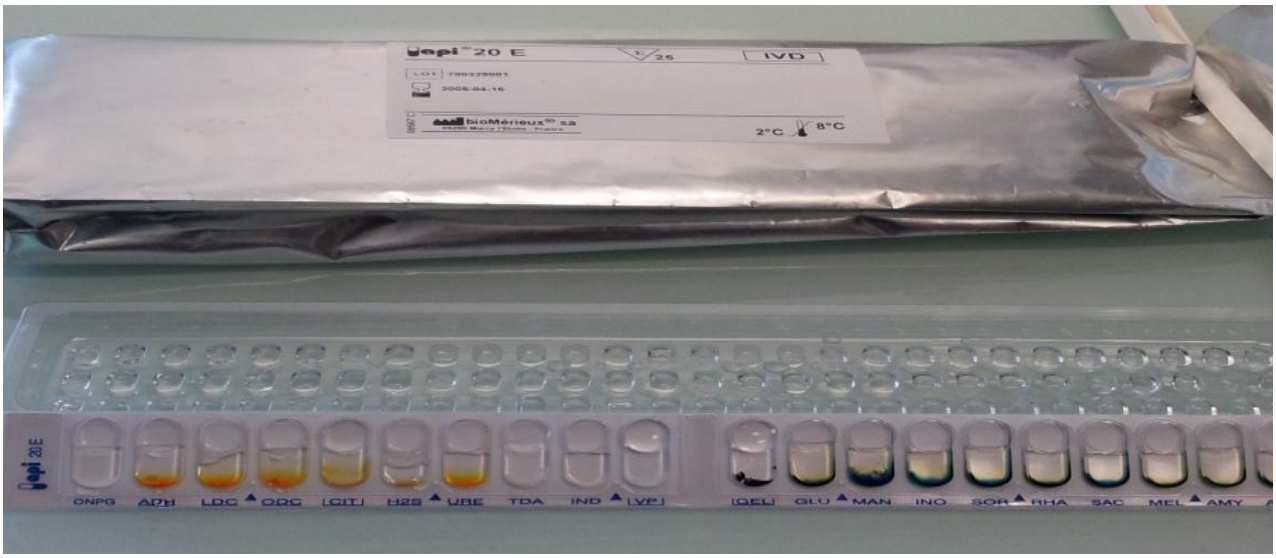

Figure 2: API 20E kit used for biochemical identification of bacteria.

3.2 Hydrolysis of sago starch

120g of starch was weighed in a beaker and 1000ml of water was added and stirred. pH was adjusted to 6.5 by using NaOH. The beaker was then placed on a plate stirrer and 115µl of Termamyl enzyme was added and stirred using magnetic stirrer for liquefaction process. The temperature was set at 150°C for 2 hours until solution gets liquefaction (gelatinization). After two hours, the solution was cooled down and the pH was adjusted to 4.5 by adding H_2SO_4. The temperature was then set to 70°C and 115µl of Amyloglucosidase enzyme was then added into the solution for the saccharification process and was left for 24 hours.

3.3 Preparation of stock culture

4 gram of glucose and 1g of Yeast extract (YE) was weighed in a beaker and was mixed with 200ml of water and stirred until dissolved. 20ml of the solution was then transferred into universal tubes and was autoclaved at 121°C for 20 minutes. 200µl of the freeze stock culture that was obtained from the laboratory was added into each universal tubes and were stored in the refrigerator.

3.4 Inoculum preparation

4 gram of glucose and 1g of YE was weighed in a beaker and was mixed with 200ml of water and stirred until dissolved. It was then transferred into 250ml bottle and was sterilized at 121°C for 20 minutes. YE was also prepared by weighing 5g of YE and it was stirred with 100ml of water and then sterilized.

3.5 Fermentation medium

Batch fermentation method was used. Fermentation process is be done by using 2000 ml shaking flask with 1000 ml working volume. Before starting fermentation, the fermenter need to be sterilized by adding water into it and autoclaved it for 20 minutes at the temperature of 12°C. To start fermentation, 1000ml of the sterilized hydrolyzed sago starch was added into the fermenter together with the 100ml YE. The 200 ml culture media with the bacteria *Enterococcus faecalis* previously incubated for 24 hours was also added into the fermenter. Fermentation is performed in shaker with agitation of 200 rpm. Fermentation temperature was set to 37°C. The initial pH for fermentation was set to pH 7. The fermentation was done by three repetitions of the experiments.

3.6 Preparation of agar

Agar was prepared by using nutrient agar. 9.6g of nutrient agar was weighed and 450ml of distilled water was added and stirred. It was then put in microwave for 10 minutes to dissolve fully. 15ml of the solution was then poured into universal tubes and sterilized at 121°C for 20 minutes. After sterilizing, the universal tubes were then transferred into oven at 60°C for 1 hour. Then it was poured into petri dish and sealed it was left to solidify. All this must be done in the laminar hood to prevent contamination.

3.7 Sampling

15 ml of fermentation medium was taken from the fermenter for the sampling purpose. Sampling was done in every 4 hours for the first 12 hours and then every 6 hours for the next 12 hours and then for next every 24 hours until the glucose is exhausted. From the 15 ml of the sample taken, 10 ml of it was centrifuged and for the determination of dry cell weight, while the supernatant obtained was used for glucose analysis and also for the spectrophotometric reading to determine cell concentration during fermentation process. From the remaining 5 ml of the sample, 1 ml of it was used for culturing of microorganism by spreading method and the rest was used for the spectrophotometric reading where the optical density reading of sample without cell was obtained.

3.8 Spreading method for microorganism culturing

Nutrient agar was prepared for spreading method. 5 gram of nutrient agar was mixed with 250 ml of water and it was heated in the microwave oven for 10 minutes for it to be dissolved. 1 ml of sample that was obtained during fermentation was used for the culturing of this bacterium to obtain colony forming unit (CFU). Sample was submitted to 10-fold dilution by mixing 100 µl of sample with 900 µl of sterilized distilled water. The sample dilutions was then streaked on nutrient agar and incubated at 37°C for 48 h in an incubator in static conditions. After 48 hours, the number of colony was counted and the size of the colony was observed.

4.0 ANALYTICAL METHODS

4.1 Determination of dry cell weight

Remaining fermentation broth was centrifuged at 8000rpm for 10 minutes at 27°C. The supernatant was collected to measure the left over volume. The cell was washed with 0.2M hydrochloric acid (HCl) and centrifuged again. The supernatant produced was discarded. The pallet was dried at 60°C for 24 hours. The dry cell weight was calculated every time sample was taken and the analysis of biomass production was done. The dry cell weight formula used is:

$$\text{DCW (g/L)} = \frac{(Weight\ of\ centrifuge\ tube + Cells)g - (Weight\ of\ centrifuge\ tube)g}{(Sample\ volume)mL} \text{x}10^3$$

4.2 Lactic acid analysis

From the experiment, 10M NaOH was used to neutralize and maintain the pH in the experiment conducted. The value of LA production was calculated from the NaOH conversion factor due to indirectly NaOH consumption in formation of LA by the cells.

$$C_3H_6O_3 \text{ (LA)} + NaOH \longrightarrow C_3H_5O_3Na \text{ (Sodium lactate)} + H_2O$$

Conversion factor of 0.69 was obtained by:

From the chemical equation of LA and NaOH:

Molar mass: NaOH = 40g/mol, LA= 90 g/mol

Ratio: NaOH/LA= (2.25)

1M= 40g/mol, 10M= 400g/mol ⟶ density (ρ) = 1.3 g/ml

1 g of 10M NaOH has x ml ⟶ 1.3 g/ml = $\frac{1g}{x\ ml}$ ⟶ $x\ ml = \frac{1\ g}{1.3\ g}$ ⟶ 0.7564 ml

By applying the formula of density, (ρ) = $\frac{g_{mass}\ (g)}{volume\ (ml)}$

$$NaOH_g = \left(\frac{0.4\ g}{1\ ml}\right)(0.7564ml) = 0.3077g$$

From the ratio of NaOH to LA is 1: 2.25

$$\text{LA}_g = \left(g_{NaOH/ml}\right)\left(\frac{LA_{mol/g}}{NaOH_{mol/g}}\right) = 0.3077\left(\frac{90}{40}\right) = 0.69$$

Thus factor is, F = 0.69