Faculty of Resource Science and Technology

Lactic Acid Fermentation in Alkaline Conditions using *Enterococcus faecalis*

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Bachelor of Science with Honours

(Resource Biotechnology)

2013

Lactic Acid Fermentation in Alkaline Conditions using *Enterococcus faecalis*

Yeoh Hui Di (28686)

This project is submitted in fulfillment of the requirement for the Degree of Bachelor of Science with Honors
(Resource Biotechnology)

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Acknowledgement

First of all, and most importantly, I would like to glory God for His blessings and guidance throughout the projects. I also would like to express my thankfulness to my family especially my mother for her support and advice during undertaking this project.

I also would like to express my gratefulness and sincere thanks to my supervisor, Associate Professor Dr. Cirilo Nolasco Hipolito for spending much of the time for guidance, supervision and patience during the way to complete this project. Besides that, I would also like to express my appreciation to my Co-Supervisor Professor Dr. Kopli bin Bujang for his assistance in any aspect of the project. A special thanks to board of Universiti Malaysia Sarawak for providing the necessary facilities and materials for conducting my study.

I also would like to express my thankfulness to Ms. Rubena Malfia Kalmaldin for her generous help and valuable help and advice especially every time I encountered the problems in this project.

Last but not least, I also would like to thanks to my lab-mates and friends, Lee Shee Yen, Patricia Anee, Seni Empangau and Revathy A/P Sankaran for their support, caring and concern since the beginning of this project.

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List of Abbreviations

^{o}C	Degree Celsius
CFU	Colonies Forming Units
BF	Batch Fermentation
DCW	Dry Cell Weight
g	Gram
g/L	Gram per Litre
L	Litre
hr	Hours
HCl	Hydrochloric acid
HSS	Hydrolyzed Sago Starch
LA	Lactic Acid
LAB	Lactic Acid Bacteria
min	Minutes
μ	Specific Growth Rate
μl	Micro-litre
ml	Milli-litre
NaOH	Sodium Hydroxide
OD	Optical Density
rpm	Revolution per Minutes

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Lactic Acid Fermentation in Alkaline Conditions using *Enterococcus faecalis*

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ABSTRACT

Enterococcus faecalis was used to perform lactic acid fermentation (LAF) in hydrolyzed sago starch (HSS). LAF was studied in alkaline condition which was pH 8 using batch fermentation (BF) modes. From the study, this bacteria can considered as thermo tolerant due to its ability to growth well at 37°C and up to 48 °C and survive at pH 8. BF mode using HSS was performed to study the efficiency in productivity in LAF. The fermentation was performed in 3 L jar fermenter with a working volume of 2 L using HSS and yeast extract as carbon and nitrogen sources respectively. In addition, the kinetics of lactic acid production, glucose consumption, and NaOH consumption for each fermentation were analyzed during the BF. The highest LA production in the fermentation process was 92.52 g/L, with the conversion at range of 92%. Therefore, we can conclude that this isolated strain has potential to be used for industrial application in LA production.

Key words: *Enterococcus faecalis*, lactic acid, lactic acid fermentation, alkaline condition.

ABSTRAK

Enterococcus faecalis *telah digunakan untuk menjalankan asid laktik fermentasi (LAF) dengan menggunakan substrat hidrolisis kanji sagu. LAF dijalankan dalam keadaan alkali iaitu pH 8 telah dikaji dengan menggunakan fermentasi 'batch' (BF). Hasil kajian menunjukkan, strain ini boleh dianggap sebagai bakteria tahan haba dan patogenik kerana keupayaannya untuk hidup pada 37^{o}C hingga 48 ^{o}C dan pH 8. BF dijalankan dengan menggunakan HSS bagi tujuan meningkatkan produktiviti asid laktik dengan menggunakan substrat hidrolisis kanji sagu. Fermentasi tersebut dijalankan dalam 3 L balang fermentasi dengan 2 L jumlah bekerja menggunakan glukosa dan ekstrak yis sebagai sumber karbon and nitrogen secara berturut-turut. Di samping itu, kepekatan asid laktik, baki glukosa, dan baki NaOH juga dianalisis bagi setiap fermentasi semasa BF. Pengeluaran LA yang paling tinggi dalam LAF ialah 92.52 g / L, dengan penukaran sebanyak 92%. Justeru ini, kita boleh membuat keputusan bahawa strain ini mempunyai potensi untuk kegunaan industria bagi menghasilkan LA.*

Kata kunci: Enterococcus faecalis*, asid laktik, fermentasi asid laktik, kaedah alkali.*

1.0 INTRODUCTION

There are increasing demands for Lactic acid (LA) productions in recent years. The Global LA Market aims to achieve 328.9 thousand metric tons in year 2015 and 367.3 thousand metric tons in year 2017 (*Global Industry Analysts Incorporation*, 2012). The high demands for LA productions in thousand metric tons due to the uses for cosmetic products, biodegradable plastics, food additives and even pharmaceuticals formulations (Wee *et al.*, 2006).

LA can be produced biologically by fermentation from different types of carbon sources such as glucose, fructose and maltose (Gao *et al.*, 2011). Production of LA by fermentation can be done in various conditions. The pH is one of the most crucial operational factors that affecting LA production (Zhang *et al*., 2007); and temperature is another crucial operational factors that affecting LA production (Butler, 2004). Based on previous research works, batch fermentation of LA was carried in pH 8 and at 37 ^{o}C by using new isolated alkaliphile LAB identified as *Enterococcus faecalis* gave the best result of LA production (Amirul, 2012; Shirylyne, 2011). Therefore, in my study, LA production was set at pH 8 and 37 ^{o}C by using the same alkaliphile LAB *Enterococcus faecalis* in order to test the lactic acid fermentation (LAF) at laboratory scale.

Fermentation is one of the oldest and remains most economical method of producing products, which is highly acceptable to human (Steinkraus *et al*., 1983). Fermentation at high temperature using thermo-tolerant bacteria can increase the LA productivity (Fernandez *et al*., 2003). There are several potential benefits associated with fermentation at high temperature; one of the benefits is that it is more favourable from an economics point of view due to the higher productivity of fermentation under vacuum (Lee *et al*., 1993). Banat *et al.*, (1998) reported that LAF at high temperature using thermo-tolerant

yeast have benefits such as energy savings through reduced both cooling costs and contamination.

In this study, a strain of thermo tolerant bacteria *Enterococcus faecalis* was used to test its ability to produce LA at high temperature. To improve the productivity, the batch fermentation mode was applied to reuse the cells in subsequent fermentation cycles.

2.0 OBJECTIVES

General: To perform batch fermentation for lactic acid production in alkaline conditions using *Enterococcus faecalis* to improve the productivity.

1. To study the LA production in alkaline conditions using *Enterococcus faecalis*.
2. To determine the kinetics of the fermentation as glucose consumption and LA production of *Enterococcus faecalis* during batch fermentation in alkaline conditions.

3.0 HYPOTHESIS

Ho: *Enterococcus faecalis* is not able to stand alkaline condition for growing and to perform fermentation to produce lactic acid.

Ha: *Enterococcus faecalis* is able to stand alkaline condition for growing and to perform fermentation to produce lactic acid.

4.0 LITERATURE REVIEW

4.1 Lactic Acid

Lactic acid (2-hydroxypropanionic acid), $C_3H_6O_3$ is an organic hydroxyl acid which occur abundantly in nature (Roslina, 2008). It was the first organic acid to be commercially produced by fermentation, with the production beginning in 1881 (Ruter, 1975), (Severson, 1998). LA has melting point of 53^{o}C and boiling point of 122^{o}C with no colour, sour in taste and can dissolve in water and alcohol except chloroform. It is naturally occurring organic acid that can be produced by chemical synthesis or fermentation (John *et al*., 2009). Chemical synthesis production is by using petroleum-based chemicals while fermentation production is by using LAB or fungi. LA molecular shape is enantiomerism and is given by the two optical isomers (Beninnga, 1990). The two optical isomers are L-(+)-LA and D-(-)-LA.

4.1.1 Applications of Lactic Acid

LA has been widely used in a various industries. The usages of LA in various industries are as below:

(a) Food industry

LA is naturally present in many food stuffs and generally recognized as safe (GRAS) (Datta *et al*., 1995). Is it formed by natural fermentation in products such as cheese, soy sauce, sourdough, pickled vegetables and meat products. Besides that, LA also widely used in bakery products, beverages, meat products, confectionery, dairy products, salads dressings and ready meals. LA in food industry usually acts as either pH regulator or preservative. It is also used as a flavoring agent.

(b) Pharmaceutical industry

The primary functions of LA in pharmaceutical are as pH-regulator, metal suspension, chiral intermediate and natural body constituent in pharmaceutical products. In polylactic acid, biocompatible thermoplastic polyester with high strength, it can also be used for dental and drug delivery systems, surgical sutures and implantable surgical devices (Di Lorenzo, 2005).

(c) Leather and textile industry

LA is also used in leather and textile industry where acidity is required and where its properties offer specific benefits. The examples are the manufacture of leather and textile products, computer disks and car coating.

(d) Animal feed

LA is a commonly used as an addictive in animal nutrition. It has health promoting properties which can enhance the performance of farm animals. LA can be used as an addictive in food and/or drinking water.

(e) Biodegradable plastics

LA is the principal building block for Poly LA (PLA). LA is used in a very large amount of chemical intermediate for biodegradable PLA, a typically high crystalline polymer with attractive market prospect (Yu *et al*., 2008). PLA is a bio-based and bio-degradable polymer that can be used for producing renewable and compostable plastics and also for food packaging, garbage bags, and agricultural plastic sheeting. This is due to its mechanical properties (Ohara, 2003; Thomsen, 2005). This kind of plastic is a good option for substituting conventional plastic produced from petroleum oil because of low emission of carbon dioxide also apart reduces the increasing amount of solid waste nowadays.

4.2 Alkaliphile Lactic Acid Bacteria

Alkaliphile LAB are gram-positive bacteria that can ferment and produce LA. LAB cells are ovoid; present as singly, in pairs or in short chains. LAB are either anaerobes, micro-aerophilic or aero-tolerant (Wood & Holzapfel, 1995). LAB produce LA as a sole or main end-product from the energy-yielding fermentation of glucose. Meanwhile, LAB are classified as homofermentative and heterofermentative (John & Nampoothiri, 2007). Homofermentative LAB produce only single product about 95% of LA while heterofermentative LAB produce not only LA and also produce other by-products such as carbon dioxide, ethanol and acetic acid.

Alkaliphile LAB, *Enterococcus faecalis* is used in LA fermentation. *Enterococcus faecalis* is predominant among the enterococci. *Enterococcus faecalis* can survive in alkaline conditions but needs complex nutrient requirements for survival, such as vitamins, pyridoxine, amino acids and nicotinate (John & Nampoothiri, 2007). *Enterococcus faecalis* is categorised as homofermentative LAB, therefore the predominant end product of glucose fermentation is L(+)-lactic acid. Moreover, *Enterococcus faecalis* can grow in good conditions either at 10°C or 45°C. *Enterococcus faecalis* has multiple antibiotic resistances (John & Nampoothiri, 2007), therefore it has advantage to reduce contamination in the LA fermentation. Contamination is easier to occur during fermentation. This situation leads to the production of another by-product from unwanted microorganisms and wasting of substrate. Therefore, contamination problem can be minimised by using the *Enterococcus faecalis* that isolated from the Biofuel R&D Laboratory (UNIMAS) for LA production compared to commonly used LAB (Calabia *et al.*, 2011).

4.3 Batch Fermentation

Figure 1 shows the microbial growth phase in batch culture system. The batch growth can be divided into four phases which are lag, exponential or log, stationary and death.

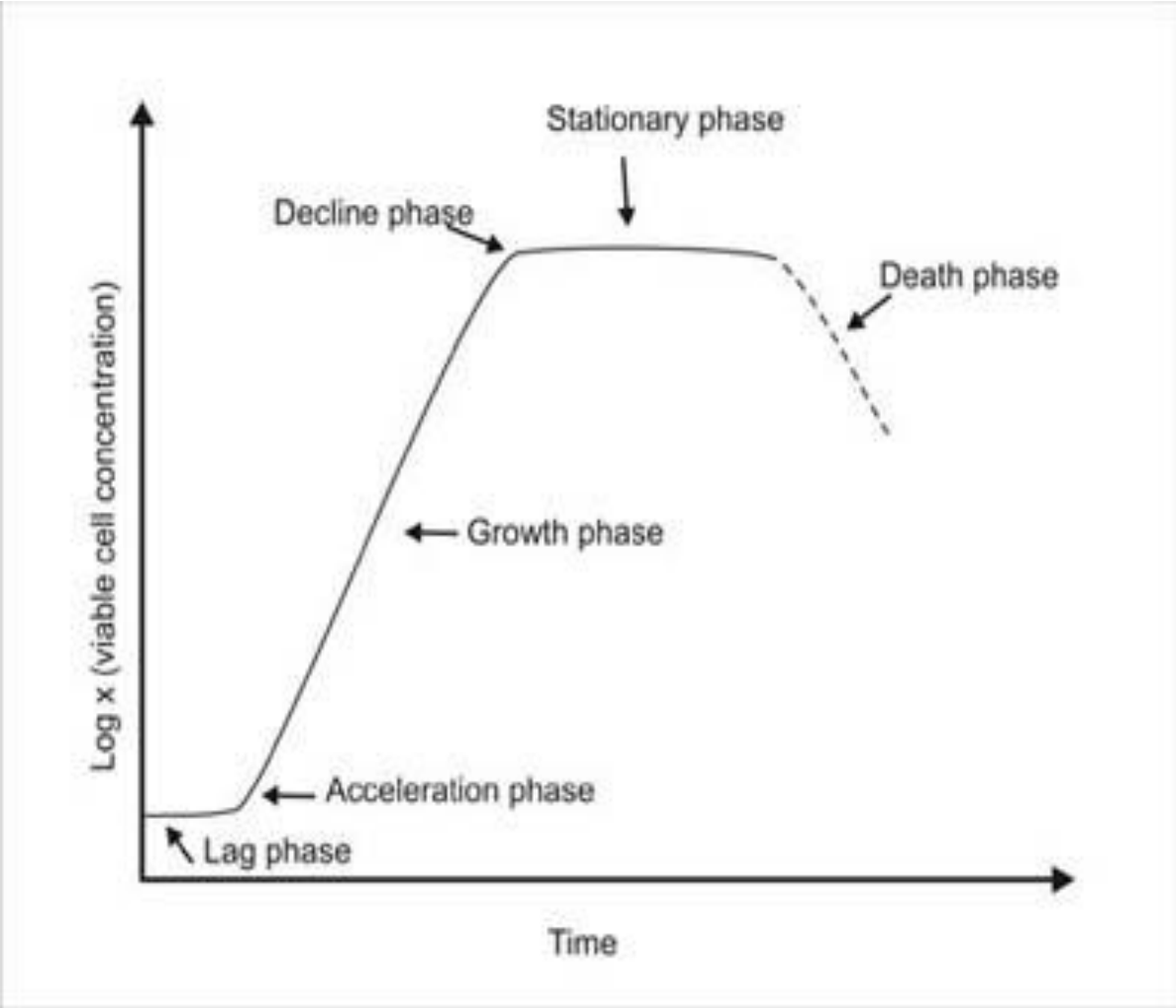

Figure 1: Microbial Growth Phase in Batch Culture System

Batch fermentation is a method for fermentation in anaerobic condition. All processes are done in closed system without contact from outside during the process run. In former case, in batch fermentation, all ingredients used in the operation are fed to the processing vessel at the batch run (Cinar *et al*., 2003). Scragg (1991) pointed out that it is operated under the optimum conditions of temperature, pH and redox potential. In their advantages, this system is easier to set up (Noor Aini, 2004) and can operate with limited knowledge.

As shown in Figure 1 (Cunningham *et al.,* 2001), after inoculation, the culture enters lag phase. During lag phase, the sizes of the cells are increased but not their number. After that, the cultures enter log or exponential growth phase in which the cells divide at a maximal rate and their generation time reaches maximum. Then, in stationary phase, the growth is stationary due to the depletion of nutrients and the accumulation of inhibitory end products in the medium. Eventually, the stationary phase of bacterial population culminates into death phase in which the viable bacterial cells begin to die.

The performance process can also be improved iteratively learning from earlier batch runs. Besides that, the initial conditions can be standardized and the operating conditions can be easily control without risk of contamination to the culture if proper sampling procedures are applied. Other than that, batch fermentation does not need to modify the hardware when the facilities used for many products and this system is good enough but increased demand of LA motivated the search for new methods.

4.4 Effect of Temperature in Lactic Acid Fermentation

Temperature is one of the most important factor that affecting bacterial growth. Each 10°C increase in temperature approximately doubles chemical reaction rate. However, this relationship only holds for a defined temperature range. For instance, growth rate fails to increase cell death at low temperature. Hence, the fermenter should have an adequate provision for temperature control.

Butler (2004) pointed out that most cells in culture grow best at 37°C and at pH 7.4. When the cells were subjected to a temperature slightly lower than the optimum temperature 37°C, then the growth rate was reduced but the cells were not damaged. However, higher temperatures of 39°C to 40°C will destroy the cells. This, it is very important to be sure that the temperature does not increase in the fermenter. It was found at 37°C was the optimum temperature for growing and LA production for the isolated strain (Shirylyne, 2011). The faster reproduction of cells fermented at 37°C was proven by the shorter phase. As previously mentioned, the cells increase in size and weight rather than increase in numbers during lag phase. Hence, it was a non-productive period of a fermentation process. It was often desirable to minimise or control the duration of the lag phase.

4.5 Lactic Acid Fermentation at Alkaline Conditions

pH is crucial in fermentation process as it affects growth rates and production rate. Both effects are influenced by the breakdown of substrates and the transport of both substrate and product through cell wall (Noor Aini, 2004). Variations in the pH create different metabolism through bacteria in processing substrate. Under anaerobic condition, certain bacteria produce neutral products during growth at low pH while certain bacteria will switch to the production of acid products (Scragg, 1991). High pH allows only certain bacteria to survive and from that, there are certain bacteria have ability to undergo LA fermentation at alkaline conditions. Alkaline pH can affect cell wall by irreversible damage to surface-located proteins (Lengeler *et al*., 2009) and LAB maintain their inner pH by controlling potassium ions in and out through membrane.

5.0 MATERIALS AND METHODS

5.1 Yeast Preparation and Culturing

Enterococcus faecalis isolated from the Biofuel R&D Laboratory (UNIMAS) was used in this study and it was kept at -84^{o}C in yeast extract-glucose medium in 2 ml eppendorf vials. One vial containing 1 ml of frozen *Enterococcus faecalis* was thawed at room temperature and refreshed in 5 ml culture medium containing 20 g/L glucose, 5 g/L yeast extract. The broth culture was incubated for 9 hr at 32^{o}C. Sub-culturing was performed every 2 weeks, for activating the bacteria.

5.2 Sago Starch Hydrolysis

The hydrolysis of sago starch has been reported elsewhere (Carvaval-Zarrabal *et al.,* 2008). The hydrolysis of sago starch was performed using enzymes from Novozyme with the conditions reported by the manufacturer. Briefly, 200 g of sago starch (dry basis) was suspended and dissolved in distilled water. The final volume adjusted to 2 L. The pH of the suspension was adjusted to 6.5 and 0.5 µl of enzyme Termamyl SC (Novozyme Co.) per gram of starch was added for liquefaction of starch at 90-95^{o}C for 2 hr. The saccharification process was performed by adding 0.6 µl Dextrozyme (Novozyme Co.) per gram of starch at pH 4.5, heated at 60-63°C for 24 hr and agitated at 200 rpm. After 24 hr, LSS became hydrolyzed sago starch (HSS).

5.3 Inoculum Preparation

One tube containing refreshed active culture of *Enterococcus faecalis* after 9 hr incubation was inoculated into 250 ml of Erlenmeyer flasks which contains 20 g/L glucose and 5 g/L yeast extract. The cells were cultivated on an orbital incubator shaker GYROMAX^{TM} 706 at 100 rpm at 37^{o}C for 17 hr. After 17 hr, the culture broth was centrifuged on high speed refrigerated centrifuge Kubota model CR21G at 4000g_c for 5 minutes to harvest the cells.

5.4 Alkaline Conditions on the Growth of *Enterococcus faecalis*

The fermentation was carried out in 3 L jar fermenter, fully computer controlled system. The parameters such temperature, pH, agitation, cell concentration were monitoring on-line in real time. The LA fermentations were carried out in batch fermentation mode to study the alkaline conditions on the growth of the isolated *Enterococcus faecalis*. For these experiments, HSS and yeast extract were used as carbon and nitrogen sources at level of 100 g/L and 5 g/L respectively. The temperature tested was 37°C; the agitation was controlled at 200 rpm with the pH 8 by addition of 10 M NaOH. The inoculums size in these experiments was set at an optical density (OD) in the range of 0.10 g/L for the initial fermentation. All the experiments were carried out by four times to report the mean of the results obtained the parameters monitored.

5.5 Analytical Methods

During the batch fermentation, 10 ml of broth samples were collected every 4 hr.

5.5.1 Lactic Acid Analysis

The LA production was assayed based on sodium hydroxide (NaOH) consumption, this consumption in gram into grams of LA, due the stoichiometric of the reaction. 10 M NaOH was pumped into the fermenter when LA was produced which indirectly dictates LA production. The amount of LA was determined as in the following equation:

( )

Where: W_i = Initial weight of NaOH (g)
W_f = Final weight of NaOH (g)
V = Final volume at the end of the fermentation (l)

Conversion factor of 0.69 was obtained by:

From the chemical equation,

$$C_3H_6O_3 \text{ (LA)} + NaOH \rightarrow C_3H_5O_3Na \text{ (Sodium lactate)} + H_20$$

Molar mass: NaOH = 40 g/mol, LA = 90g/mol Ratio: NaOH (1): LA (2.25)

1M = 40 g/mol, 10M = 400 g/mol → Density (ρ) = 1.3 g/ml

1 g if 10M NaOH has x ml → 1.3 g/ml = — → x ml = — → 0.7564 ml

By applying the formula of density, (ρ) = ———

From the ratio of NaOH to LA is 1: 2.25, Thus factor, F = 0.3077 (NaOH) x 2.25 (LA)

Therefore, **F = 0.69.**

5.5.2 Analysis of Glucose Concentration and OD

During BF, the residual glucose concentration was determined by injected 30 µl of the samples with the dilution of 100 x with the distilled water into Biosensor unit. Also, OD of glucose was determined by Shimadzu UV-Vis Spectrophotometer model UV Mini-1240 at the wavelength 575 nm.

5.5.3 Determination of Biomass Concentration by DCW

The broth sample was diluted to certain OD with sterile tap water. 250 ml of the broth sample was put in 250 ml centrifuge bottles. The broth was centrifuged at 10,000 rpm for 10 min. Then, the supernatant was discarded and 10 ml of 0.2 M HCl was added in the tube. The cells were resuspended and centrifuged again at 10,000 rpm for another 10 min. Next, the supernatant was discarded and 10 ml of HCl was added again. After that, the tube was centrifuged again at 10,000 rpm for 10 min. Later, the supernatant was discarded again and distilled water was added in the tube, to resuspend the cells. Then, the resuspend cells that contained in the centrifuge tube were dried in oven at 105 ^{o}C for 16 hr until the weight was constant. DCW was determined as follow:

( ) —

Where: A = Weight of cells (g)

M = Volume of sample (l)