LACTIC ACID PRODUCTION USING ALKALIPHILE LACTIC ACID BACTERIUM

AMIRUL BIN MAHMUD (23121)

Bachelor of Science with Honours
(Biotechnology Resource)
2012
LACTIC ACID PRODUCTION USING ALKALIPHILE LACTIC ACID BACTERIUM

AMIRUL BIN MAHMUD (23121)

This project is submitted in partial fulfilment of the Final Year Project (STF 3015) (Resource Biotechnology)

Supervisor: Assoc. Prof. Dr. Cirilo Nolasco Hipolito
Co-Supervisor: Prof. Dr. Kopli Bin Bujang

Resources Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
DECLARATION

I hereby declare that no portion of the work referred in this project has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

______________________
(Amirul Bin Mahmud)
Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak
ACKNOWLEDGEMENTS

This research project is completed with the help of many people. First of all, I would like to convey my supervisor Assoc. Prof. Dr. Cirilo Nolasco Hipolito and co-supervisor, Prof. Dr. Kopli Bin Bujang whose guidance, stimulating suggestions and encouragement helped me in all the time of research for and writing of this dissertation.

I am also indebted to my family member especially my parent and siblings who supported me. Special thanks also to the lab assistant and postgraduate students who have been very helpful in providing technical support and assistance for this project.

Last but not least, my sincere appreciation is also extends to all my friends and others who have provided assistance, offering advices and critics, including crucial input for my planning and findings. The guidance and support received from all was vital for the success of this project.
TABLES OF CONTENTS

DECLARATION.........................................................................................................................i

ACKNOWLEDGEMENTS ........................................................................................................ii

TABLES OF CONTENTS ......................................................................................................... iii

LIST OF ABBREVIATIONS ...................................................................................................... v

LIST OF TABLES AND FIGURES ............................................................................................ vi

ABSTRACT .....................................................................................................................................1

1.0 INTRODUCTION ................................................................................................................. 2

2.0 LITERATURE REVIEW ........................................................................................................ 4
  2.1 Carbon source .................................................................................................................. 4
  2.1 Lactic acid ...................................................................................................................... 4
  2.3 Lactic acid bacteria (LAB) ............................................................................................ 5
  2.4 Effect of pH ................................................................................................................... 6
  2.5 Batch fermentation ........................................................................................................ 6
  2.6 Lactic acid fermentation at alkaliphilic condition ............................................................ 7

3.1 MATERIALS ...................................................................................................................... 8
  3.1.1 Bacterium ................................................................................................................ 8
  3.1.2 Culture media .......................................................................................................... 8
  3.1.3 Fermentation media ............................................................................................... 8
  3.1.4 Fermenter .............................................................................................................. 8
3.2 METHODS.......................................................................................................................... 9
  3.2.1 Refreshing bacterium ........................................................................................................ 9
  3.2.2 Inoculum preparation ...................................................................................................... 9
  3.2.3 Batch fermentation system .......................................................................................... 9
  3.2.4 Sampling ...................................................................................................................... 9

4.0 ANALYTICAL METHODS ................................................................................................. 10
  4.1 Determination of dry cell weight .................................................................................... 10
  4.2 Lactic acid determination .............................................................................................. 10
  4.3 Reducing sugar analysis ................................................................................................. 11

5.0 RESULTS .......................................................................................................................... 12
  5.1 Dried cell weight and bacteria growth ........................................................................... 12
  5.2 Reducing sugar and lactic acid analysis ......................................................................... 14
  5.3 Culturing of the microorganism by Spreading method ............................................... 18
  5.4 Overall result ................................................................................................................ 19

6.0 DISCUSSION ................................................................................................................... 21

7.0 CONCLUSION .................................................................................................................. 26

REFERENCES ......................................................................................................................... 27
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>Lactic acid</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>DCW</td>
<td>Dry cell weight</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
</tbody>
</table>
LIST OF TABLES AND FIGURES

Table 1: Dry cell weight of the fermentation broth at pH 9 and 10 ..............................................12

Table 2: Comparison of fermentation result at pH 9 and pH 10 .....................................................19

Figure 1: Optical isomers of L-(+)-lactic acid and D-(-)-lactic acid (mirror image) .....................5

Figure 2: Optical density (OD) and the growth rate of the bacteria during fermentation for both pH 9 and 10. ........................................................................................................13

Figure 3: Kinetics of lactic acid fermentation using glucose as substrate at pH 9 .......................16

Figure 4: Effect of the pH on the Optical density (OD) and the growth rate of the bacteria. ......17

Figure 5: Spreading method fermentation broth after complete fermentation of pH 9(right) and pH 10 (left) ......................................................................................................................18
Lactic Acid Production Using Alkaliphile Lactic Acid Bacterium

Amirul bin Mahmud

Resource Biotechnology Programme
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

Batch fermentation of glucose to lactic acid was conducted using new isolated bacteria from laboratory of Biofuel R&D (UNIMAS) at alkali pH of 9 and 10. Commercial glucose was used to produce lactic acid and the effect of alkali pH on biomass, lactic acid production, and presence of contamination were investigated. Batch fermentation system was done at 37 °C and agitated at 250 rpm with controlled pH environment. Fermentation at pH 9 gave high production biomass and lactic acid of about 1.80 g/L and 45.21 g/L while less production is shown at pH 10 with 1.27 g/L and 42.66 g/L. Spread plate of both fermentation show growth only single colony which no contamination. Consequently, it shows that high pH affect cell growth, lactic acid production and prevents contamination. Complete fermentation at pH 9 show is more favourable with high rate production of lactic acid in 42 h compared to at pH 10 in 72 h.

Key words: high pH, batch fermentation, lactic acid, alkaliphile lactic acid bacteria, glucose

ABSTRAK

Fermentasi secara “batch” daripada glukos kepada asid laktik dilakukan oleh bakteria baru yang diperoleh dari makmal Biofuel R&D (UNIMAS) pada pH alkali 9 dan 10. Glukos komersial digunakan untuk menghasilkan asid laktik dan perlaksanaan kajian untuk mengesan pH beralkali terhadap biomass, produksi asid laktik dan kehadiran pencemaran. Fermentasi secara “batch” dilakukan pada suhu 37 °C dengan kadar putaran 250 rpm dalam suasana persekitaran pH yang terkawal. Fermentasi di pH 9 menghasilkan produksi biomass dan asid laktik yang tinggi berjumlah 1.80 g/L dan 45.21 g/L secara keseluruhannya manakala produksi yang rendah ditunjukkan di pH 10 dengan jumlah 1.27 g/L dan 42.66 g/L secara keseluruhannya. Plat penyebaran untuk kedua-dua fermentasi menunjukkan hanya satu koloni tumbuh yang menandakan tiada pencemaran. Ini menunjukkan pH tinggi memberi kesan terhadap tumbarnasaran sel, produksi asid laktik dan menghalang pencemaran dari berlaku. Keseluruhan fermentasi di pH 9 adalah lebih bagus dengan sebab kadar produksi asid laktik adalah selama 24 jam berbanding di pH 10 selama 72 jam.

Kata kunci: pH tinggi, fermentasi “batch”, asid laktik, bakteria asid laktik beralkali, glukos
1.0 INTRODUCTION

There was increasing demand for lactic acid (LA) production in recent years. The global LA market to reach 328.9 thousand metric tons by 2015 (Global Industry Analyst Inc., 2011). These rise in demand is from the existed of end-use market and the emergence of new product application. The product includes leather and cosmetic (John et al., 2007), natural preservatives in food (Nithya et al., 2012) and the new emergence application is such drug delivery carrier in tissue engineering (Makadia and Siegel, 2011). LA can be produced biologically by fermentation from different type of carbon source such glucose, fructose and maltose (Gao et al., 2011). Production of LA by fermentation can be in various conditions. The pH is one of the most crucial operational factors that affecting LA production (Zhang et al., 2007). It is the key environmental parameters that affect fermentation process (Yuwono and Kokugan, 2007). The majority of lactic acid bacteria (LAB) have optimum growth at pH 5.5 - 6.5 and alkali intolerant (Calabia, 2011). There are certain bacteria that can survive and produce LA at high pH condition of more than 7. These bacteria mostly are mesophilic include Halalactobacillus halophilus (Ishikawa et al., 2005), Halolactobacillus miurensis (Ishikawa et al., 2005), Halolactobacillus alkaliphilus (Cao et al., 2008) and Alkalibacterium indicereducens (Yumoto et al., 2008). Contamination of other organism is one the major problems during LA fermentation. The common issue of main product fermentation which shows that there is still other by-product is formed other than LA due to contamination. The use of new isolated alkaliphile LAB from Biofuel R&D laboratory (UNIMAS) has advantage to reduce these problems. Contamination problems can be minimized by using LAB for LA production compare to commonly used LAB (Calabia, 2011). Therefore, the objective of this study is to examine the production of LA at alkaline pH. Special interest was focused on
alkaliphile LAB that has an ability to survive at pH at least or higher than 9. The aims of this study were:

i. Establish LA fermentation process in batch fermentation system with the same productivity or better than mesophilic LAB

ii. Performed batch LA fermentation with less risk of contamination with unwanted microorganism
2.0 LITERATURE REVIEW

2.1 Carbon source

Carbons sources such as glucose are obligate substrate element for fermentation process by bacteria. General formula for glucose is C$_6$H$_{12}$O$_6$. Bacteria are more effective to produce LA from the source of glucose. Roslina (2008) stated that purest product of LA is obtained when a pure sugar (glucose) is fermented. A wide variety of carbon sources is capable of producing LA including molasses, fruit waste, glucose, sucrose, fructose, and lactose (Roslina, 2008). Carbohydrates are sources of carbons and can be easily convert into glucose by chemical synthesis. LAB has ability to produce LA by 98.3% (Calabia, 2011).

2.1 Lactic acid

Lactic acid (2-hydroxypropanionic acid), C$_3$H$_6$O$_3$ is an organic hydroxyl acid whose occurrence in nature is widespread (Roslina, 2008). It was the first organic acid to be commercially produced by fermentation, with production beginning in 1881 (Ruter, 1975 and Severson, 1998). LA has melting point of 53 °C and boiling point of 122 °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, 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 (Harm Benninga, 1990). The two optical isomers is L-(+)-LA and D-(−)-LA (mirror image) as shown in Figure 1.
Figure 1: Optical isomers of L-(+)-lactic acid and D-(−)-lactic acid (mirror image)

2.3 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are gram-positive bacteria, not forming spore, not respiring, shape of rod or cocci, live in anaerobic condition and have ability to excrete LA as by product of fermentation. Brian et al., 1995 defined LAB by the formation of LA as a sole or main end-product from carbohydrate metabolism. LAB is classified into (1) homofermentative and (2) heterofermentative (John and Nampoothiri, 2007). Homofermentative LAB is produce only single product of LA while heterofermentative produces not only LA, but produces other by-products such ethanol, diacetyl, acetic acid and carbon dioxide. Specificity of LA produce is depending on type of the bacteria. An example of homofermentative bacteria are Lactococcus lactis (Nolasco-hipolito et al., 2002), Lactobacillus delbrueckii (John et al. 2006,; Kadam et al., 2006) and Lactobacillus helviticus (Tango and Ghaly, 2002). Heterofermentive bacteria are such as Lactobacillus viridescens 7-7, Lactobacillus fermenti D1 and Leuconostoc lactis S3 (Drinan et al., 1975). There are other LAB with genus Enterococcus include Streptococci that can tolerate with 6.5% content sodium chloride and alkaline pH of 9.6 (Mavhungu, 2006). Substrate for LAB to do fermentation can be carbohydrates based product or glucose. LAB degrade glucose molecule by reduction reaction in anaerobic condition.
2.4 Effect of pH

The 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 at high pH (Scrugg, 1991). High pH allows only certain bacteria to survive and from that, there is certain bacteria have ability to undergo LA fermentation. High pH can affect cell wall by irreversible damage to surface-located proteins (Lengeler et al. 2009). Different alkaliphile lactic acid bacteria have specialised cell-wall peptidoglycan that resist different alkaline condition. For example, alkaliphilic marine LA bacterium that has type A4β, Orn-D-Glu while *alkalibacterium* has A4β, Orn-D-Asp (Ishikawa et al., 2003). In normal LA fermentation, pH is always decreased with increasing time because of acid properties in LA and is adjusted with addition alkaline substrate (Mass et al., 2008).

2.5 Batch fermentation

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, 2003). In their advantages, this system is easier to set up (Noor Aini, 2004) and can operate with limited knowledge. The performance process can also be improved iteratively learning from earlier batch runs. Other than that, batch fermentation does not need to modify the hardware when the facilities used for many products.
2.6 Lactic acid fermentation at alkaliphilic condition

Researchers around the world are looking for the ideal process to increase the productivity of the systems in fermentation technology. One of the main issues is the contamination with other microorganisms. This situation leads to produce another by-product from unwanted microorganisms and wasting of substrate. Therefore different methods have been implemented to overcome these issues. In these projects, the production of LA was done in alkaliphilic conditions. There are very few studies done to examine the stress effect of LAB towards alkaliphilic environments. Several LAB has been identified that can tolerate high pH and produced large amount of LA such Enterococcus hirae that has an ability produce L-lactic acid as major end product at pH 9.6 (Collins and Farrow, 1985). Ishikawa et al., 2009 reported that Alkalibacterium olivapovliticus has optimum growth between 9 - 11.5 and produce LA as the main product of glucose fermentation. These include other Alkalibacterium species such A. olivapovliticus, A. psychrotolerans, A. iburiense and A. incidireducens. The latest research on alkaliphile LAB is by using alkaliphilic marine organism and result in high optical purity of LA as 98% (Calabia et al., 2011).
3.1 MATERIALS

3.1.1 Bacterium
An alkaliphile LA bacterium isolated in the laboratory of Biofuel R&D (UNIMAS) was used in this research experiment. The prepared stock culture is stored in glycerol of 20% at ultra-low temperature of -84 °C. The stock culture was kept in 10 ml media containing 20 g/L glucose and 5 g/L yeast extract and incubated at 37 °C for 18-24 hs. One vial containing 1 ml of frozen bacteria 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 h at 32 °C. Sub-culturing was performed every 2 weeks.

3.1.2 Culture media
Alkaliphile LA bacteria was cultured in medium containing 20 g/L glucose and 5 g/L yeast extract with 10 ml volume. 10% inoculum volume was use for every fermentation process.

3.1.3 Fermentation media
Fermentation media consist of commercial glucose as carbon source and nutrient broth as nitrogen source for the bacteria.

3.1.4 Fermenter
All nutrients needed for fermentation process are dissolved in the 1L fermenter and was autoclaved. The pH fermentation was conducted at 9 and 10 separately. The temperature was fixed at 37 °C. The agitation was set constant at 250 rpm.
3.2 METHODS

3.2.1 Refreshing bacterium

1.5ml freeze stock (-84 °C) culture was refresh by transfer into tube containing 10 ml glucose and 5 g/l of yeast extract and was incubate at 32 °C for 18-24 h.

3.2.2 Inoculum preparation

The medium content for the pre-culture is 20 g/L of commercial glucose and 8 g/L of nutrient broth. In each 7 ml pre-culture was take out from refreshed cell for preparation of 70 ml inoculum (10% inoculum). Solution media was sterilized at 121 °C for 15 minutes before culture was put inside.

3.2.3 Batch fermentation system

Fermentation process was done in 1 L fermenter with 700 ml working volume. Fermentation was performed at temperature of 37 °C with agitation rate of 250 rpm. The initial pH was increased slowly to pH 6.5 until the 0.100 optical density (OD) was reached and later it was increased to pH 9. The pH was controlled manually by pump 10 M Sodium hydroxide (NaOH) with the pH range of 1.5 for every pump. Experiment was repeated with the next pH of 10.

3.2.4 Sampling

For the first 12 h, sampling was done in every 3 h. After 12 hs, sampling was done every 6 h until the glucose consumed by the bacteria.
4.0 ANALYTICAL METHODS

4.1 Determination of dry cell weight

Remaining fermentation broth is centrifuged at 8000 rpm for 5 minutes at 4 °C. The supernatant was collected to measure the left over volume. The cell is washed by suspended with 0.2 M Hydrochloric acid (HCl) and centrifuged at 8000 rpm for 5 minutes at 4 °C. The supernatant produce was discarded. Washed process was repeated for 3 times. The pellet was dry at 60 °C for 24 h. Graduated centrifuge tube reweigh for DCW determination using formula below:

\[ \text{DCW} = \frac{A}{B} \times 10^3 \quad \text{Eq. 1} \]

Where

\( A = \) Weight of centrifuge tube + cells (g)

\( B = \) Weight of centrifuge tube (g)

\( C = \) Sample volume (mL)

4.2 Lactic acid determination

LA produce was analysed using High Performance Liquid Chromatography (HPLC) installed with organic column. 20 µL sample volume injected into the system and with detection temperature 65 °C at 0.6 mL/min flow rate and 0.005 mM H₂SO₄ use as mobile phase. Lactic acid concentration was automatically calculated by computer after inject with lactic acid internal standard.
4.3 Reducing sugar analysis

Reducing sugar was analysed using High Performance Liquid Chromatography (HPLC) installed with organic column. 20 µL sample volume injected into the system and with detection temperature 65 °C at 0.6 mL/min flow rate and 0.005 mM H$_2$SO$_4$ is used as mobile phase. Reducing sugar concentration was automatically calculated by computer after inject with glucose internal standard.
5.0 RESULTS

5.1 Dried cell weight and bacteria growth

The dry cell weight was done to observed total of biomass production during fermentation. The remaining broth after complete fermentation is centrifuged to collect the cell pallet. The cell pellet washed with HCl and dried in oven for calculation of dried cell weight:

From Eq. 1, the dry cell weight of the cell pallet at pH 9 and 10 that was obtained is:

<table>
<thead>
<tr>
<th></th>
<th>pH 9</th>
<th>pH 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Cell Weight (g/L)</td>
<td>1.8</td>
<td>1.27</td>
</tr>
</tbody>
</table>

From Table 1, the dried cell weight that was produced during fermentation at pH 9 was 1.80 g/L and pH 10 was 1.27 g/L. pH 9 shows high value of cell compare to pH 10. The dry cell weight differences between pH 9 and 10 is 0.53 g/L.
Figure 2: Optical density (OD) and the growth rate of the bacteria during fermentation for both pH 9 and 10. Symbols: OD: (●), pH 9; (■) pH 10; μ: (▲), pH 9; (○), pH 10.

Figure 2 shows turbidity of the bacteria during fermentation at pH 9 and 10. Fermentation at pH 9 shows high OD value compare to pH 10. From the start of the fermentation, the OD value of pH 9 has rapid increase to 0.823 in 9 h while at pH 10 it steady increase to 0.692 in 36 h. The highest OD recorded was 0.907 at pH 9 while 0.705 at pH 10. The highest OD recorded at pH 9 was 30 h while pH 10 was 42 h. The cells slowly decrease the OD value after 30 h of fermentation at pH 9 while 42 h at pH 10. Fermentation complete in 42 h at pH 9 and 72 h for pH 10 with the last OD value recorded was 0.868 and 0.646 respectively. The μ of the bacteria start increase rapidly from 0 h to 6 h for both fermentations at pH 9 and 10. At pH 10
fermentation show rapid growth until 6 h while pH 9 fermentation show rapid growth was continued to 9 h. Slow growth observed at 9 h at pH 10 while pH 9 the growth are almost none. Move to 42 h, pH 9 have no growth starting 18 h but pH 10 has steady slow growth rate. No growth was observed at 42 to 72 h in pH 10 while pH 9 has complete fermentation with no other cell growth during this 42 h time.

5.2 Reducing sugar and lactic acid analysis

From the experiment, 10M NaOH was used to neutralize and maintain the pH in each experiment conducted. 51 g and 47.5 g of 10M NaOH was pump into fermentation at pH 9 and pH 10 respectively. From the value, theoretically LA production was calculated from the NaOH conversion factor due to indirectly NaOH consumption in formation of lactic acid by the cell.

\[
C_3H_6O_3 \text{(Lactic acid)} + \text{NaOH} \rightarrow C_3H_5O_3Na \text{(Sodium lactate)} + H_2O
\]

Conversion factor of 0.69 was obtained by:

From the chemical equation of LA and NaOH:

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

Ratio: NaOH (1) : LA (2.25)

1M = 40 g/mol, 10M = 400 g/mol → density \((\rho)\) = 1.3 g/ml

\[
1 \text{ g of 10M NaOH has } x \text{ ml} \rightarrow 1.3 \text{ g/ml} = \frac{1 \text{ g}}{x \text{ ml}} \rightarrow x \text{ ml} = \frac{1 \text{ g}}{1.3 \text{ g}} \rightarrow 0.7564 \text{ ml}
\]

By applying the formula of density, \(\rho = \frac{\text{mass (g)}}{\text{volume (ml)}}\)
From the ratio of NaOH to LA is 1 : 2.25

Thus factor, \[ F = 0.3077 \times 2.25 \]

\[ F = 0.69 \]

From the calculation of the LA conversion factor from NaOH, the factor 0.69 was used to know theoretically the production of lactic acid using the weight of 10M NaOH that was pumped in during fermentation. Every sampling h, the weight of NaOH pump in was recorded to construct theoretically the graph curve of reducing sugar against lactic acid production. The weight of NaOH is multiply with LA conversion factor to obtain the concentration of lactic acid that was produced. Reducing sugar calculated by subtract weighted glucose with the value of lactic acid produced.
Fermentation at pH 9 and 10 was done to construct the model parameter as a function of pH in fermentation of alkaliphile LAB. **Figure 3** show the theoretical lactic acid production curve was plotted as parameter estimates of lactic acid fermentation by using LA conversion factor of 0.69.

During fermentation, sample was obtained in given set time for observation of reducing sugar and lactic acid analysis. Sample was analysed using HPLC machine installed with organic membrane to plot the graph:

**Figure 3:** Kinetics of lactic acid fermentation using glucose as substrate at pH 9. Symbols (■), Glucose; (◆), Lactic acid.