PRODUCTION OF LACTIC ACID USING FACULTATIVE ANAEROBIC BACTERIA: OPTIMIZATION

NURULSURIANI BT AWANG

BACHELOR OF CHEMICAL ENGINEERING (BIOTECHNOLOGY)
UNIVERSITI MALAYSIA PAHANG

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PRODUCTION OF LACTIC ACID USING FACULTATIVE ANAEROBIC BACTERIA: OPTIMIZATION

NURULSURIANI BT AWANG

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JUNE 2015

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature	:
Name of main supervisor	: IR. DR. NORAZWINA BT ZAINOL
Position	: ASSOCIATE PROFESSOR
Date	:

STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

Signature :
Name : NURULSURIANI BT AWANG
ID Number : KE11038
Date :

DEDICATION

I would like to acknowledge the inspirational instruction and guidance of my supervisor, Dr. Norazwina bt Zainol. Also high appreciation gave to my parents, family, lab assistants, my friends and those who involve in labworks.

ACKNOWLEDGEMENT

I would like to thanks the following people and organisations;

- My supervisors Prof. Ir. Dr. Norazwina Bt Zainol for the guidance through an effective well-arranged weekly meeting.
- My family who have been supported me all the time and encouraged me to do well in finishing this thesis.
- My fellow friends who have helped me a lot in the labworks and also have given opinion for better improvement in this thesis.

ABSTRACT

Lactic acid (CH_3-CHOHCOOH) is the most widely occurring carboxylic acid, having a prime position due to its versatile applications in industries. The use of cheap raw material is important as the price for lactic acid in the market is not too high. This research was done to optimize the production of lactic acid using mixed culture of facultative anaerobic bacteria and banana stem waste (BSW) as the substrate. The inoculum used for this research was probiotic milk drink (PMD). The fermentation process was done by varying the temperature and the fermentation time in 250 mL Erlenmayer flask. The temperature and fermentation time were the factors chosen in this research. The temperature range used was 37-41 °C, while the range for fermentation time was 18-22 hours. Fermentation process was done without agitation and the ratio of BSW: PMD was 4:1; 40 ml of BSW was mixed with 10 ml of inoculum. The total experiment runs were 13 runs. Design Expert 7.0 software was used to set the total number of experimental runs and the range for the factors chosen. The samples from the fermentation process were then analyzed using High Performance Liquid Chromatography (HPLC) and the optimum condition was determined using Response Surface Methodology (RSM). The range for the lactic acid concentration from the experimental result was 0.010 mg/mL to 0.0155 mg/mL. Validation experiment was done to validate suggested optimum condition. Design Expert 7.0 software suggested a total of two validation runs. The fermentation time was maintained at 20 hours. The only parameter that was changed in this validation experiment was the temperature which at 38°C and 39°C. The error from the validation experiment was 0.06 %. In conclusion, Central Composite Design (CCD) and RSM enabled the determination of optimal operating conditions for obtaining greater lactic acid production. The optimization of the analyzed responses demonstrated that the best results for lactic acid production (0.16 g/g) were obtained at 39 °C and fermentation time at 20 hours. The yield of lactic acid at the optimum condition was 0.16 g/g while its concentration was 0.014 mg/mL.

ABSTRAK

Asid laktik (CH_3-CHOHCOOH) adalah asid karboksilik yang paling banyak boleh didapati, mempunyai peranan penting disebabkan oleh kepelbagaian kegunaannya dalam makanan, farmaseutikal, tekstil, kulit dan dalam industri kimia yang lain. Penggunaan bahan mentah yang murah penting kerana harga asid laktik di pasaran adalah tidak terlalu mahal. Kajian ini dilakukan untuk mengoptimumkan penghasilan asid laktik dengan menggunakan kultur campuran bakteria anaerobik fakultatif dan sisa batang pisang (BSW) sebagai substrat. Inokulum yang digunakan dalam kajian ini adalah PMD. Proses penapaian dilakukan dengan mengubah suhu dan tempoh penapaian yang dilakukan dalam 250 mL kelalang Erlenmayer. Suhu dan tempoh penapaian adalah faktor-faktor yang dipilih dalam kajian ini. Suhu yang digunakan adalah 37-41 °C, manakala tempoh penapaian yang digunakan adalah 18-22 jam. Tiada putaran dilakukan sepanjang proses penapaian ini dan nisbah BSW:PMD adalah 4:1 dimana 40 mL BSW telah dicampurkan dengan 10 mL inokulum. Jumlah eksperimen yang perlu dilakukan adalah sebanyak 13 kali. Perisian "Design Expert 7.0" telah digunakan untuk menentukan jumlah eksperimen yang perlu dilakukan dan had untuk faktor-faktor yang dipilih. Sampel daripada proses penapaian seterusnya dianalisis dengan menggunakan HPLC dan keadaan optima ditentukan dengan menggunakan RSM. Kepekatan asid laktik yang diperoleh daripada eksperimen adalah antara 0.010 mg/mL sehingga 0.0155 mg/mL. Eksperimen pengesahan telah dilakukan untuk mengesahkan cadangan keadaan optimum. Design Expert 7.0 telah mencadangkan jumlah eksperimen pengesahan sebanyak 2 kali. Masa penapaian telah ditetapkan selama 20 jam. Faktor yang diubah hanyalah suhu iaitu pada 38°C dan 39°C. Peratusan kesilapan yang berlaku dalam eksperimen pengesahan hanyalah sebanyak 0.06%. Kesimpulannya, CCD dan RSM boleh menentukan keadaan optima untuk penghasilan asid laktik. Hasil daripada analisis keputusan eksperimen menunjukkan bahawa keadaan yang terbaik untuk pengeluaran asid laktik (0.16 g/g) ialah pada 39 ° C dan tempoh penapaian selama 20 jam. Hasil asid laktik pada keadaan optimum adalah 0.16 g/g manakala kepekatannya adalah 0.014 mg / mL

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BSW	Banana stem waste
PMD	Probiotic milk drink
HPLC	High Performance Liquid Chromatography
CCD	Central Composite Design
RSM	Response Surface Methodology
PLA	Polylactic acid
GRAS	Generally Recognized as Safe
FDA	Food and Drug Administration

1 INTRODUCTION

1.1 Background, motivation and statement of problem

Lactic acid (2-hydroxypropionic acid or 2-hydroxypropanoic acid) is a chemical compound that plays a role in various biochemical processes and was first isolated in 1780 by the Swedish chemist Carl Wilhelm Scheele. It is an organic acid that can be found widely in nature with the chiral carbon and exist in two enantiomers (Wee et. al., 2005). Food and food-related applications account for approximately 85% of the demand for lactic acid, whereas the non-food industrial applications account for only 15% of the demand. Lactic acid has been used as a preservative and acidulant in food and beverage sector for several decades.

Lactic acid can be produced by microbial fermentation or by chemical synthesis but in recent years fermentation process has become more industrially successful because of the increasing demand for naturally produced lactic acid (Holten, 1971). Lactic acid has been classified by the US FDA (Food and Drug Administration) as GRAS (Generally Recognized as Safe) for use as a food additive (Datta et. al., 1995). At present, 90% of the world production of lactic acid is by bacterial fermentation and the rest is produced synthetically. The chemical synthesis of lactic acid always leads to racemic mixture, which is major disadvantage. Fermentation process comprises the bioconversion of a sugar solution into lactic acid in the presence of a microorganism. Microorganisms that produce lactic acid can be divided into two groups: bacteria and fungi (Wee et. al., 2006). Facultative anaerobic bacteria is an organism which is capable of producing energy through aerobic respiration and then switching back to anaerobic respiration depending on the amounts of oxygen and fermentable material in the environment (Biology Online). Some examples of facultative anaerobic bacteria are the *Staphylococci* (Gram positive), *Escherichia coli* (Gram negative), *Corynebacterium* (Gram positive), and *Listeria* (Gram positive).

Interest in the production of lactic acid is presently growing in relation to its applications in the synthesis of biodegradable polymer materials such as poly lactic acid (PLA) polymers. The US-based NatureWorks is the largest producer of PLA having lactic acid production capacity of 180,000 ton per annum (Chen and Patel, 2011). The

large production capacity means that the demand for lactic acid keeps increasing. So, the production of lactic acid must be in large scale to meet the demand.

A mixed culture encompasses more than one species. In this culture type, the existence of symbiotic relationship among various bacteria has been demonstrated (Moon and Reinbold, 1976). Mixed culture is much more cheap and easily can be obtained in the market than pure culture. Mixed culture of lactic acid bacteria are currently used in dairy industries for cheese and fermented milk manufacture. The example of mixed culture bacteria are in the commercial mixed culture probiotic milk drink (PMD) such as Nutrigen, Vitagen, Yakult, Solivite and more. Commercial mixed culture probiotic milk drink contains lactic acid bacteria known as *Lactobacillus sp.*

Banana stem waste is used in this study as the raw material in the fermentation of lactic acid. Lactic acid bacteria are traditionally fastidious microorganisms and have complex nutrient requirements (Fitzpatrick & O Keeffe, 2001). Refined sugars such as glucose or sucrose have been more frequently used to produce lactic acid (Hofvendahl & Hahn, 1997). However, these are economically not feasible due to high cost of pure sugars whereas the product (lactic acid) is relatively cheap. The production of lactic acid through fermentation technology in industry is mainly dependent on cost of raw material to be used. Therefore, it is mandatory to have a raw material for industrial production of lactic acid with several characteristics such as low cost, minimum level of contaminants, rapid fermentation rate, high lactic acid production yields, little or no by-product formation and year-round availability (Ryu et al., 2003).

Optimization of lactic acid production is important so that the best condition to produce high yield of lactic acid can be determined. Optimization of the process condition is important because it can affect the formation, concentration and yield of a particular fermentation end product thus affecting the overall process economics (Schmidt, 2005). The optimum condition can produce high yield of lactic acid using cheap raw material besides can reduce the cost. There have been numerous investigations on the development of biotechnological processes for lactic acid production. The ultimate objectives are to enable the process to be more efficient and economical. Lactic acid is produced in fermentation process by using facultative anaerobic bacteria such as *Lactobacilli sp*. Central Composite Design (CCD) in Response Surface Methodology

(RSM) is used in this study to determine the optimum condition for production of lactic acid. The parameters that are used in this study are fermentation time and temperature.

1.2 Objectives

The following are the objectives of this research:

- To produce lactic acid by using mixed culture from PMD.
- To optimize the performance of the process based on the selected parameters.

1.3 Scope of This Research

The scopes of this research include collection of banana stem in Gambang area, preparations of banana stem waste and experimental design using Central Composite Design (CCD) in Response Surface Methodology (RSM). This research was done in shake flask. The source of inoculums used in this research was from PMD in natural flavour. PMD was bought at supermarket in Gambang area. The banana stems collected were pressed using sugarcane juicer machine. The juice of the banana stem was used as the substrate in the fermentation process. Design Expert Software was used in this study analyze the sample from the fermentation process and hence to find the optimum condition in producing lactic acid. By using CCD, the condition for fermentation process was determined. After that, fermentation of banana stem juice and probiotic milk drinks (PMD) was done by varying the temperature and fermentation time. 13 runs of experiment were done at different temperature and fermentation time in the range of 37-41 °C and 18-22 hour. Then, the products of the fermentation were collected and analyzed using HPLC to determine the concentration of lactic acid. Finally, the data was analysed to find the optimum condition of lactic acid production by using RSM in Design Expert Software.

LITERATURE REVIEW

2.1 Overview

The demand for lactic acid is increasing year by year. The main markets for lactic acid have been in food, pharmaceutical and cosmetics industry, but presently the main growing application of lactic acid is in the production of biodegradable and renewable raw material based poly lactic acid (PLA) polymers. Production figure of 260,000 ton as 100 % lactic acid for conventional (excluding PLA) markets in 2008 and forecast over 1 million ton annual production of lactic acid for conventional markets in 2010 (Jem, 2010). Worldwide demand on the uses of lactic acid in food related industries almost reached about 85% (Sheeladevi *et al.*, 2011).

2.2 Bacteria Producing Lactic acid

2.2.1 Pure Culture

Pure culture is a laboratory culture containing a single species of organism. A pure culture is usually derived from a mixed culture (one containing many species) by transferring a small sample into new, sterile growth medium in such a manner as to disperse the individual cells across the medium surface or by thinning the sample many fold before inoculating the new medium. Both methods separate the individual cells so that, when they multiply, each will form a discrete colony, which may then be used to inoculate more medium, with the assurance that only one type of organism will be present. Isolation of a pure culture may be enhanced by providing a mixed inoculum with a medium favouring the growth of one organism to the exclusion of others (Encyclopedia Britannica, 2015).

2.2.2 Mixed Culture

Mixed-culture fermentations are those in which the inoculum always consists of two or more organisms. Mixed cultures can consist of known species to the exclusion of all others, or they may be composed of mixtures of unknown species.

The earliest studies of microorganisms were those made on mixed cultures by Van Leeuwenhoek in 1684. Micheli, working with fungi in 1718, reported his observations on the germination of mold spores on cut surfaces of melons and quinces. In 1875, Brefeld obtained pure-culture of fungi, and in 1878 Koch obtained pure cultures of pathogenic bacteria.

Mixed-culture fermentations offer a number of advantages over conventional single-culture fermentations. Firstly, product yield may be higher. Yogurt is made by the fermentation of milk with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Driessen *et. al.,* (1982) demonstrated that when these species were grown separately, 24 mmol and 20 mmol, respectively, of acid were produced; together, with the same amount of inoculum, a yield of 74 mmol was obtained. The number of *S. thermophilus* cells increased.

Next, the growth rate may be higher. In a mixed culture one microorganism may produce needed growth factors or essential growth compounds such as carbon or nitrogen sources beneficial to a second microorganism. It may alter the pH of the medium, thereby improving the activity of one or more enzymes. Even the temperature may be elevated and promote growth of a second microbe.

Furthermore, mixed cultures are able to bring about multistep transformations that would be impossible for a single microorganism. Examples are the miso and shoyu fermentations in which *Aspergillus oryzae* strains are used to make koji. Koji produces amylases and proteases, which break down the starch in rice and proteins in soybeans. In the miso and shoyu fermentations, these compounds are then acted on by lactic acid bacteria and yeast to produce flavor compounds and alcohol. In some mixed cultures a remarkably stable association of microorganisms may occur. Even when a mixture of cultures is prepared by untrained individuals working under unsanitary conditions, such as in ragi, mixtures of the same fungi, yeasts, and bacteria remain together even after years of subculture. Probably the steps in making

the starter were established by trial and error, and the process conditions were such that this mixture could compete against all contaminants.

In addition, compounds made by a mixture of microorganisms often complement each other and work to the exclusion of unwanted microorganisms. For example, in some food fermentations yeast will produce alcohol and lactic acid bacteria will produce lactic acid and other organic acids and change the environment from aerobic to anaerobic. Inhibiting compounds are thus formed, the pH is lowered, and anaerobic conditions are developed that exclude most undesirable molds and bacteria.

Mixed cultures permit better utilization of the substrate. The substrate for fermented food is always a complex mixture of carbohydrates, proteins, and fats. Mixed cultures possess a wider range of enzymes and are able to attack a greater variety of compounds. Likewise, with proper strain selection they are better able to change or destroy toxic or noxious compounds that may be in the fermentation substrate.

Mixed cultures also can be maintained indefinitely by unskilled people with a minimum of training. If the environmental conditions can be maintained (i.e., temperature, mass of fermenting substrate, length of fermentation, and kind of substrate), it is easy to maintain a mixed-culture inoculum indefinitely and to carry out repeated successful fermentations.

Besides that, mixed cultures offer more protection against contamination. In mixed-culture fermentations phage infections are reduced. In pure-culture commercial fermentations involving bacteria and actinomycetes, invariably an epidemic of phage infections occurs, and the infection can completely shut down production. Since mixed cultures have a wider genetic base of resistance to phage, failures do not occur, often because if one strain is wiped out, a second or third phage-resistant strain in the inoculum will take over and continue the fermentation. In such processes, especially with a heavy inoculum of selected strains, contamination does not occur even when the fermentations are carried out in open pans or tanks.

The other advantages of mixed culture fermentations are enable the utilization of cheap and impure substrates. In any practical fermentation the cheapest substrate is always used, and this will often be a mixture of several materials. For example, in

the processing of biomass, a mixed culture is desirable that attacks not only the cellulose but also starch and sugar. Cellulolytic fungi along with starch-and sugar-utilizing yeasts would give a more efficient process, producing more products in a shorter time.

Mixed cultures also can provide necessary nutrients for optimal performance. Many microorganisms, such as the cheese bacteria, which might be suitable for production of a fermentation product, require growth factors to achieve optimum growth rates. To add the proper vitamins to production adds complications and expense to the process. Thus, the addition of a symbiotic species that supplies the growth factors is a definite advantage (Hesseltine, 1992).

Mixed culture is used in this study because it is more effective in fermentation of lactic acid besides it is cheaper than pure culture. According to the study done by Abdel-Rahman *et al*., (2013), amount of lactic acid produced by mixed culture is almost equal with the amount produced by pure culture even though same substrate have been used in the fermentation. The mixed culture can be easily found in probiotic milk drink (PMD) such as Nutrigen, Vitagen and Solivite.

2.3 Substrate for Fermentation of Lactic Acid

Banana stem waste is one of the cheap raw materials that can be found easily in Malaysia. Cheap raw material is used because of the price for lactic acid is not too high. Moreover, the glucose content in banana stem is relatively high which makes it suitable to use in fermentation of lactic acid. The content of glucose in the pseudostem is higher than other parts of the banana plant. According to Bernstad *et al*., (2012), the contents of monosaccharides (glucose) and cellulose in banana pseudostem are high, while the content of lignin is commonly low. The content of glucose and cellulose was found to be in the ratio of 42.0-74.0 and 34.0-63.9% of disaccharide, respectively. The high glucose content in banana pseudostem makes it very suitable to use as substrate for lactic acid production.

Oh *et. al.,* (2005) has done lactic acid fermentations from agricultural resources such as barley, wheat, and corn on a laboratory-scale bioreactor without any supplementations

in order to investigate the possibility of those raw materials as a sole nutrient source. Table 2.1 shows the results of lactic acid production when the medium was composed of each saccharified liquor from 200 g of agricultural resources without any supplementations. The volumetric productivities of lactic acid and maximal dry cell weight reached to 0.51–0.88 g/l h and 1.67–2.25 g/l, respectively, where barley was found to be the most efficient nutrient among three resources tested. On the other hand, glucose yield after liquefaction and saccharification was highest at corn flour. Lactic acid yields based on consumed glucose were above 0.92 g/g in all the cases experimented. Javanainen and Linko (1995) previously reported that 36 g/l of lactic acid (0.75 g/l h) was produced from 180 g of barley flour (equivalent to 119 g of glucose) as a single nutrient source after 48 h of mixed culture of *Lb. amylovorus* and *Lb. casei* and 120 g/l of lactic acid (0.83 g/l h) was produced by simultaneous saccharification and fermentation through the addition of glucoamylase after 144 h of fermentation. We obtained 133 g of glucose from 200 g of barley flour through enzymatic hydrolysis, from which 38 g/l of lactic acid was produced and 2.3 g/l of maximal dry cell weight was obtained after 43 h of fermentation. This result is quite similar to those of Javanainen and Linko (1995).

Table 2.1 Lactic Acid Yield Using Agricultural Waste

Agricultural Resources (200 g)	Lactic Acid Yield (g/g)
Barley	0.94
Wheat	0.93
Corn	0.94

2.4 Optimization Using Central Composite Design (CCD)

Optimizing refers to improving the performance of a system, a process, or a product in order to obtain the maximum benefit from it. The term optimization has been commonly used in chemistry as a means of discovering conditions at which a procedure produces the best possible response (Kurbus et al., 2003). Optimization is done so that the optimum condition for the fermentation of lactic acid can be obtained. The optimum condition means that the process is effective and can produce high yield of lactic acid. Central Composite Design (CCD) was introduced by Box and Wilson. CCDs are

formed from two level factorials by addition of just enough points to estimate the curvature and interaction effects. The number of runs increasing exponentially with number of factors (Kennedy and Krouse, 1999). CCD can be combined with Response Surface Methodology (RSM), in which experiments were designed by CCD and thereafter optimized RSM (Chakravati and Sahai, 2002; Dahiya et. al., 2005).

Box and Wilson developed a comprehensive methodology employing factorial designs to optimize chemical production processes. The Box–Wilson methodology, now known as response surface methodology (RSM), employs several phases of optimization (Box and Draper, 1987). The two main optimization phases are following the path of steepest ascent; and locating a stationary point (using canonical analysis). The key to RSM is representing the yield as a surface. RSM includes factorial design and regression analysis, which can help in evaluating the effective factors and in building models to study interaction and select optimum conditions of variables for a desirable response. It can be used to evaluate the relative significance of several factors in the presence of complex interactions. It is a powerful technique for testing multiple-process variables because fewer experimental trials are needed as compared with the study of one variable at a time, thereby minimizing experimental cost. RSM is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes in which a response of interest is influenced by several variables and the objective is to optimize this response. RSM has important application in the design, development and formulation of new products, as well as in the improvement of existing product design. It defines the effect of the independent variables, alone or in combination, on the processes. In addition to analyzing the effects of the independent variables, this experimental methodology generates a mathematical model which describes the chemical or biochemical processes (Anjum *et al*., 1997 and Myers and Montgomery, 1995).

2.5 Factors Affecting Lactic Acid Production

Generally, there are few factors that affect the fermentation process such as fermentation mode, nitrogen source, microorganism, carbon source, temperature and fermentation time. In screening process of previous study done, there are five factors that have been chosen. The factors are agitation, sources of inoculums, ratio substrate to inoculums, fermentation time and temperature. In this study, there are two main factors

that have been chosen. The factors selected are fermentation time and temperature. Those factors were chosen because they gave positive effects to the yield of lactic acid and have high percent of contribution based on previous study.

2.5.1 Temperature

The effect of temperature on the production of LA has only been studied in a few reports as shown in Table 2.2. Temperature is a crucial parameter affecting lactic acid fermentation. Temperature is one of the important factors that affect the growth of microorganism. The temperature giving the highest productivity was in some cases lower than the temperature resulting in highest lactic acid mass concentration and yield (Siebold *et al.,* 1995), whereas in others the same temperature gave the best results in all categories (Hujanen *et al.,* 1996). Most species have a characteristic range of temperature in which they can grow, but they do not grow at the same rate over the whole of temperature range. Microbial growth is governed by the rate of chemical reaction catalyzed by enzymes with the cell. Lactic acid bacteria can grow at temperatures from 5 to 45 °C. The yield of lactic acid increased with each increase at temperature between 30 to 40 °C. The lactic acid production decrease above temperature 45 °C due to at this temperature the growth not optima (Busairi, 2010). Table 2.2 shows the previous study done to investigate the effect of temperature in fermentation of lactic acid.

Table 2.2: Previous Studies on the Effect of Temperature in Lactic Acid Fermentation.

Temperature (°C)	Yield of Lactic Acid (g/L)	References
20-40	Optimum	Jawad *et al.,* (2013)
41	5.23	Djukuvic – Vukovic *et al.*,(2012)
42	10.00	Tango and Ghaly (1999)
37	28.73	Idris and Suzana (2006)
30	60	Akerberg *et al.* (1998)
37	70	Richter and Trager (1994)
30	80	Hujanen and Linko (1996)
30	4.9	Hofvendahl (1998)
34	65	Hofvendahl *et. al.*(1998)

In the table 2.1, the highest concentration of lactic acid produced is at the temperature of 37 °C (Richter and Trager, 1994). So, the optimum temperature is around that temperature.

2.5.2 Fermentation Time

Fermentation time is one of the critical environmental parameters affecting content, molecular mass, and sugar composition. Higher initial substrate concentration used causes the growth phase to be slower. According to Palaniraj *et al.*, (2012), the growth phase is observed from 0–40 hours with different initial substrate concentration. The death phase is found in above 40 hours. The exponential phase occurred in between 12 to 40 hour. Besides that, in study done by Bhatt and Srivastava (2012), it was found that after 48 hour, the lactic acid production rate is almost constant. The highest concentration of lactic acid was found at 48 hour. In addition, according to Vishnu *et. al.* (2002), by using low substrate concentration, the highest yield of lactic acid was obtained in 48 hours. Table 2.3 shows the previous studies on the effect of fermentation time in lactic acid fermentation.

Table 2.3: Previous Studies on the Effect of Fermentation Time in Lactic Acid Fermentation.

Fermentation Time	Concentration of Lactic Acid	References
40 hour	52 g/l	Palaniraj *et. al.*, (2012)
After 40 hour	Constant	
After 48 hour	Constant	Bhatt and Srivastava (2012)
48 hour	78±1.2 (g/l)	
48 hour	8.7-9.6 g/l	Vishnu *et. al.*, (2002)

In the Table 2.3, the highest concentration of lactic acid produced is at the fermentation time of below 48 hour (Bhatt and Srivastava, 2012). So, based on those previous studies, the optimum fermentation time is expected to be at below 48 hours.

2.5.3 Agitation

Different lactic acid bacterial strains differed in their requirement for growth conditions. According to Demirtas *et al.,* (2003), there was 8% increase in growth rate when agitation is increase to 200 rpm to 300 rpm. Meanwhile, some cases where the growth lag was longer due to the constant agitation increase rate, thus taking more time for microbes to adjust under such condition. The increase in agitation speed is expected to result in higher shear stress, causing the fungal to grow in smaller size but increasing the lactic acid production when 0 – 300 rpm is used (Bai *et al.,* 2003). This was supported by Tinocco-Valencia *et al.,* (2014), who stated that the shear forces from high agitation can create cell wall rupture, changes in physiological and morphology, biomass concentration, growth rates and also variation in product rate synthesis. However, increase of agitation rates from 50 to 500 rpm, under the experimental condition used, although increase the cell's glucose consumption, did not have significant effect on biomass production, lactic acid concentration and productivity (Gao and Ho, 2013). Table 2.4 shows the summary of agitation effect on lactic acid production.

Table 2.4: Previous Studies on the Effect of Agitation on Lactic Acid Production

Agitation (rpm)	Description	References
0-300	• Shear stress is higher. • Fungal size becomes smaller. • Lactic acid production is higher.	Bai *et. al.,* (2003)
50-500	• Cell's glucose consumption increase. • No significant effect on lactic acid production.	Gao, T., & Ho, K. P. (2013)

2.5.4 pH

The fermentation pH is either set at the beginning and then left to decrease due to acid production or it is controlled by base titration, or by extraction, adsorption, or electrodialysis of lactic acid. The optimal pH for lactic acid production varies between 5.0 and 7.0. A pH below 5.7 was optimal for *Lactobacillus* strains, which are known to tolerate lower pH than *lactococci*. Wee *et al.* (2006) investigated the influence of culture pH on lactic acid fermentation from molasses where lactic acid fermentations were performed on a jar fermenter at 38°C and pH 5.0 – 9.0 using 200 g/L of molasses. Although the optimum pH for cell growth of *Enterococcus faecalis RKY1* was seen to be 8.0, the lactic acid fermentation at pH 7.0 was completed faster than that at pH 8.0. The cell growth at pH 5.0 almost ceased even after 10 h of fermentation. The highest lactic acid mass concentration (4.0 g/L) was obtained at pH 7.0 with a comparable yield with pH 6.0. Huang *et al*. (2005) determined the impact of pH on the starch saccharification and fermentation of lactic acid by the *Rhizopus arrhizus* 3*6017* and *Rhizopus oryzae 2062*, the pH was controlled at 4.0, 5.0, 6.0 and 7.0 by adding 4 mol/L NaOH solution at t = 4 h intervals during the course of cultivation. The volumetric concentration of lactic acid and biomass in the *Rhizopus arrhizus 36017* cultures increased with the increase in pH. Senthuran *et al*. (1999) investigated the lactic acid production by Lactobacillus casei at different pH values showed that reactor productivity was highest at pH 6.0 with free cells and at pH 6.5 with immobilized cells. Productivity as well as free cell density decreased at both lower and higher pH values. The productivity was seen to at pH 5.5. Fu *et al*. (1999) produced lactic acid in batch fermentations with synthetic lactose media by using *Lactobacillus plantarum* at various pH values ranging from 4 to 7. The optimal pH range of 5–6 yielded the highest values of biomass (11.0 g/L) and lactic acid mass concentration (41 g/L). Ohkouchi and Inoue (2006) produced lactic acid economically by direct bioconversion from starchy substrates by using *Lactobacillus manihotivorans LMG18011*. The optimum initial pH was found to occur between 5.0 and 5.5. Above pH 6.0 or below pH 4.5, this strain could not convert all of the starch to lactic acid. Table 2.5 summarizes the previous studies on the effect of pH in production of lactic acid.

Table 2.5: Previous Studies on the Effect of pH on Lactic Acid Production

pH	Concentration of Lactic Acid	References
7.0	Highest yield (4 g/l)	Wee *et. al.,* (2006)
4.0 – 7.0	Increase with the increasing of pH.	Huang *et. al.,* (2005)
6.0 - 6.5	Highest yield of lactic acid obtained.	Senthuran *et. al.,* (1999)
5.0 – 6.0	Highest yield (41.0 g/l)	Fu *et. al.,* (1999)

2.5.5 Nitrogen Sources

The medium composition has been investigated from many aspects, including the addition of various mass concentrations of nutrients in the form of yeast extract, peptone or corn steep liquor. The addition of nutrients and higher nutrient mass concentrations generally had a positive effect on the lactic acid production. MRS medium, which contains yeast extract, peptone and meat extract, was superior to yeast extract, which in turn was better than malt extract. A variety of nitrogen sources have been tested for lactic acid production, but they did not give the product concentrations as high as those obtained with yeast extract (Nancib *et. al.,* 2001). Ohkouchi and Inoue (2007) reported that lactic acid productivities were improved by the supplementation of nitrogen sources with tryptone, beef extract, and MRS-complex. In particular, supplementation with tryptone or MRS-complex, the total nitrogen contents were improved to 0.58 % and 0.72 %, respectively, and both the acceleration of the bioconversion rate and a doubling of lactic acid production, from 35 g/L up to 70 g/L, was observed. There was a little improvement of the bioconversion only by nitrogen supplementation with beef extract (the total nitrogen content is 0.48 %). Nancib *et al.* (2005) reported the effects of supplementing date juice with different nitrogen sources such as yeast extract, ammonium sulfate, tryptic soy, urea, peptone and casein hydrolysate on the lactic acid production by *Lactobacillus casei subsp. rhamnosus*. None of the non-yeast-extract nitrogen sources gave lactic acid concentrations as high as that of yeast extract. Among the nitrogen sources tested, urea gave the lowest mass concentrations of lactic acid (14.1

g/L). The summary of previous studies on this effect in lactic acid production is shown in Table 2.6.

Table 2.6: Previous Studies on the Effect of Nitrogen Sources on Lactic Acid Production

Nitrogen source	Concentration of Lactic Acid	References
Nitrogen source supplementing with MRS complex	Highest yield (70 g/l)	Ohkouchi and Inoue (2007)
Yeast extract	Highest yield of lactic acid obtained.	Nancib *et al.* (2005)
Urea	Lowest yield of lactic acid obtained (14.1 g/l)	

2.5.6 Carbon Sources

A number of different substrates have been used for the fermentative production of lactic acid by lactic acid bacteria. The purest product is obtained when a pure sugar is fermented, resulting in lower purification costs. However, this is economically unfavorable, because pure sugars are expensive and lactic acid is a cheap product. Instead, waste products from agriculture and forestry were utilized. The study on lactic acid production by Senthuran *et al.*(1999) with free cell cultivations in a medium containing different sugars revealed that *Lactobacillus casei* preferred lactose as a carbon source for its growth and lactic acid production, followed by glucose and maltose, while sucrose was poorly utilized. The preliminary experiment of Yun and Ryu (2001) with vial cultivation in a medium containing the different sugars revealed that *Enterococcus faecalis RKY1* utilized glucose, fructose, and maltose as carbon sources for growth and lactic acid production, while galactose and sucrose were metabolized to formic acid and acetic acid as main products, and xylose, glycerol, whey, and starch were poorly utilized. When *Enterococcus faecalis RKY1* was cultivated on these three carbon sources, cell growth and lactic acid formation patterns were similar. The highest volumetric productivity was found to

be with cells grown in a medium containing fructose, which was completely utilized within t = 35 h. The average volumetric productivity and yield of lactic acid was 4.12 g/L and 0.96 g-lactic acid/g-fructose respectively. In many lactic acid bacteria (LAB), fructose metabolism generally differs from glucose metabolism in that fructose acts both as a growth substrate and electron acceptor. When *Enterococcus faecalis RKY1* was grown on a mixture of glucose (75 g/L) and fructose (75 g/L) the cell growth and volumetric productivity were higher than growth on each sugar alone. Furthermore, for the lactic acid fermentations with glucose/fructose, glucose/maltose, and fructose/maltose mixtures as carbon sources, *Enterococcus faecalis RKY1* grown on a mixture of glucose/fructose simultaneously consumed these sugars, and the cell growth and average volumetric productivity were higher than when grown on the individual sugars. Ohkouchi and Inoue (2006) selected glucose, soluble starch, and starch from rice or potato as carbon sources. At initial pH 6.5 the saccharification of starch was inhibited. Therefore, *Lactobacillus manihotivorans LMG18011* was unable to take up carbohydrate or produce lactic acid under this condition. The summary of previous studies on this effect in lactic acid production is shown in Table 2.7.

Table 2.7: Previous Studies on the Effect of Carbon Sources on Lactic Acid Production

Carbon source	Concentration of Lactic Acid	References
Fructose	Highest yield (4.12 g/l)	Yun and Ryu (2001)
Mixture of glucose and fructose.	Higher yield of lactic acid obtained compared to in single type of sugar.	

2.6 Previous Studies on Lactic Acid Production

There were various researches had been done using different source of substrate to produce lactic acid. Garde *et al.,* (2002) obtained lactic acid from wheat straw hemicellulose by using mixed culture of *Lactobacillus pentosus* and *Lactobacillus brevis.*

Pintado *et. al.,* (1999) has done a study about the production of lactic acid using mussel processing waste (MPW). The culture used is mixed culture consists of *Lactobacillus plantarum* A6 (LMG 18053), *Lactobacillus manihotivorans* (LMG 18010T), *Lactobacillus plantarum* R10101/2 and *Pediococcus* sp. VA403. The fermentation process was done at 30°C. The yield of lactic acid from the fermentation is 0.87 g/g.

Huang *et. al.* (2005) also had done a study about the production of lactic acid using potato starch waste by using *Rhizopus sp*. This study was done to investigate the effect of pH, temperature, starch source and concentration to the yield of lactic acid. At pH 6.0 and temperature of 30 °C was favourable for lactic acid fermentation, resulting in lactic acid yield of 0.87-0.97 g/g in 36-48 hour fermentation time.

Hovhendahl & Han-Hagerdal (1997) also has been studied the lactic acid production from whole wheat flour hydrolysate using strain of *Lactobacilli* and *Lactococci.* Whole wheat flour containing bran and gluten was hydrolyzed by a commercial mixed-amylase preparation and fermented to lactic acid by two *Lactococcus lactis* strains and two *Lactobacillus delbrueckii* strains. The yield of lactic acid from the experiment done was 0.93-0.95 g/g.

Besides that, Wee et. al., (2004), has studied the production of lactic acid using sugarcane molasses and corn steep liquor by using pure culture. The yield between using sugarcane molasses and corn steep liquor is almost the same which is 0.94 g/g and 0.95 g/g. Table 2.8 shows the summary of previous studies done on the production of lactic acid.

Table 2.8: Different Substrate for Lactic Acid Fermentation Using Pure and Mixed Culture

Fermentation Substrate	Type of culture	Lactic Acid Produced	Reference
Wheat straw hemicellulose hydrolysate	Mixed	0.61-0.90 g/g	Garde *et al., (*2002)
Mussel Processing Waste	Mixed	0.87 g/g	Pintado *et. al* (1999)
Potato Starch Waste	Mixed	0.87-0.97 g/g	Huang *et. al* (2005)
Whole Wheat	Mixed	0.93-0.95 g/g	Hovhendahl & Han-Hagerdal (1997)
Pineapple Waste	Pure	0.83 g/g	Busairi (2007)
Sugarcane Molasses	Pure	0.95 g/g	Wee *et al.* (2004)
Corn Steep Liquor	Pure	0.94 g/g	Wee *et. al* (2005)
Sugarcane Bagasse	Pure	0.97 g/g	Rojan *et. al* (2005)

3 MATERIALS AND METHODS

3.1 Overview

A schematic structure of the whole process flow has been constructed and is illustrated in Figure 3.1. As the starting point, the banana stem was collected and prepared for fermentation process. Then, inoculums were prepared in term of probiotic milk drink. The range for factors selected was decided beforehand using Response Surface Methodology (RSM) in Design-Expert® software. The experimental runs were done by Central Composite Design (CCD). The effects of fermentation time and temperature on lactic acid fermentation were investigated. After fermentation process, the samples were collected and were analyzed by High Performance Liquid Chromatography (HPLC). The last step was data analysis by using Design Expert.

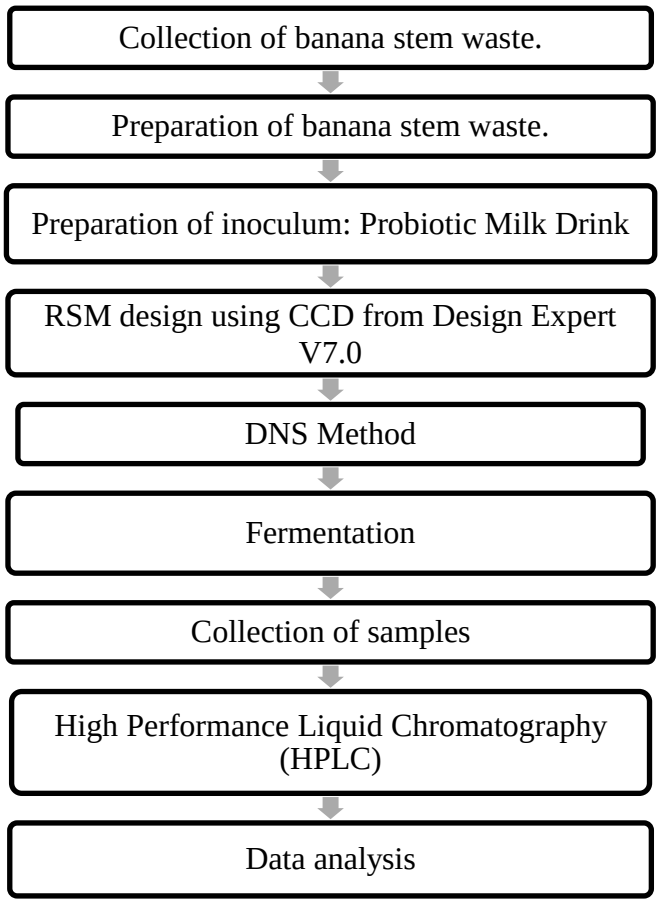


Figure 3.1: Flow chart process of experiment.

3.2 Substrate Preparation

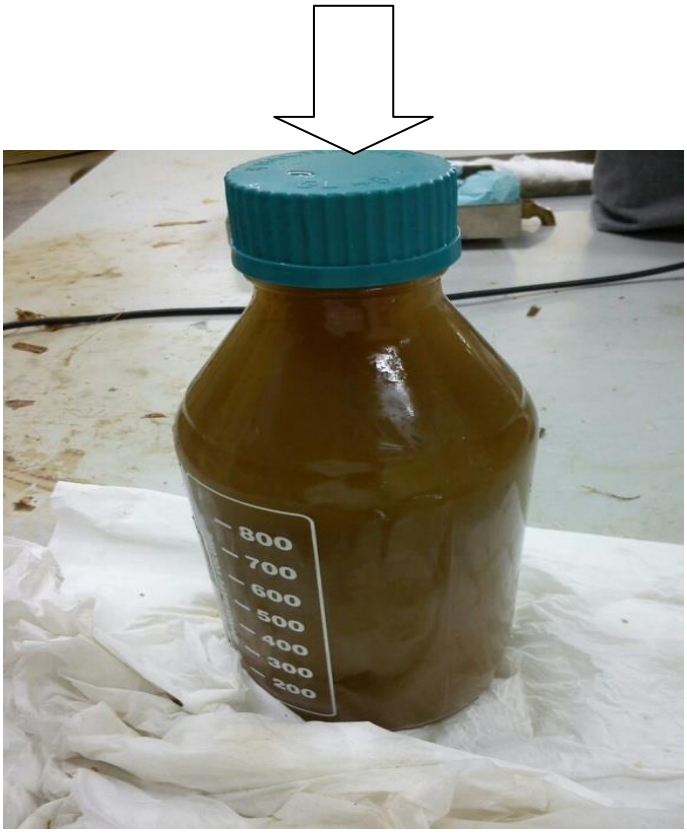
Banana plants were collected from Gambang, Pahang. Each banana stem was washed and cut into parts. The banana stems were pressed using sugarcane presser machine to get the juice. The fresh banana stem sap was stored in a refrigerator at 4°C to ensure its freshness (Hamid, 2013). To avoid any fermentation, the experiment using this banana stem sap was carried out on the same day. Figure 3.2 shows the step to get the juice from banana stem.

Banana stem was cut into shorter length.

The banana stem was cut into small pieces.

Sugarcane machine was used to press the banana stem.

The waste of banana stem after pressed with sugarcane presser.



Banana stem juice was kept in schott bottle.

Figure 3.2: Banana stem waste.

3.3 Inoculum Preparation

The inoculum was prepared using commercially probiotic milk drink (PMD). The PMD was purchased from nearer supermarket at Gambang. The experiment required the original flavour of PMD to ensure the accuracy of lactic acid production. The PMD were stored at 4°C to assure that bacteria are in inactive state. The PMD drinks must be at ambient temperature when the experiment was conducted. Figure 3.3 shows the probiotic milk drink that was used in this research.

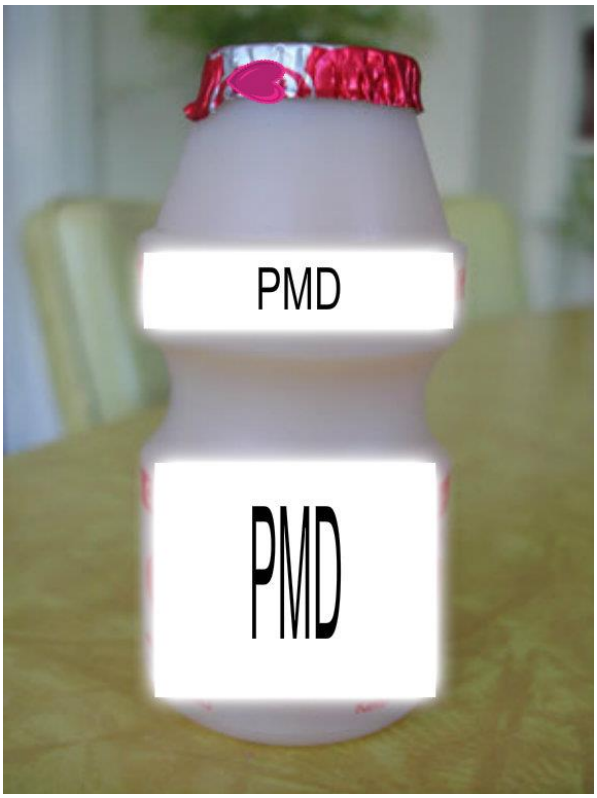


Figure 3.3: Probiotic Milk Drink (PMD)

3.4 Experimental Set Up

Fermentation was carried out in 250ml Erlenmeyer flasks in the ratio of substrate and culture 4:1. 40 ml of banana stem juice and 10 ml of inoculums were put in the Erlenmeyer flask. All the apparatus used were sterilized before using in the fermentation process. Table 3.1 shows the parameter used throughout the experiment. The cultures were incubated at different temperatures for different fermentation time. No agitation was provided during the fermentation time. The source of inoculums was from PMD. After fermentation was done, the samples were collected and centrifuged at 5000 rpm for 10 minutes. The samples were ready to be analysed by using HPLC to determine the yield of lactic acid. The samples were stored in -20 °C freezer to avoid contamination and error in result. Factors that have been selected are fermentation time and temperature. By using Design-Expert® software, a total of 13 runs were carried out. Using CCD, the range of value for every parameters selected are determined as in the Table 3.1. After data was collected, using Design-Expert® software, the data was analysed with Response Surface Methodology (RSM) by using Central Composite Design (CCD). Table 3.1 shows the range for factor 1 and 2 that is used in the experiment. The range for fermentation time is 18-22 hour, while the range for

temperature is 37-41 °C. The concentration of lactic acid was recorded at the end of every run.

Table 3.1: Range for Factors

Variables	-α	-1	Centre points	+1	+α
Fermentation time (hour)	18	19	20	21	22
Temperature (°C)	37	38	39	40	41

*α = 2

Table 3.2 shows the CCD table that had been constructed before the experiment was done.

Table 3.2: Parameters used.

Std	Run	Factor 1: Temperature (°C)	Factor 2: Fermentation Time (Hour)
1	3	38	19
2	7	40	19
3	10	38	21
4	5	40	21
5	1	37	20
6	13	41	20
7	12	39	18
8	8	39	22
9	6	39	20
10	2	39	20
11	4	39	20
12	11	39	20
13	9	39	20

3.5 Analysis of Lactic Acid

3.5.1 HPLC

Lactic acid was analyzed by using High Performance Liquid Performance (HPLC) equipped with UV -Vis detector set to 210nm. A Zorbax Eclipse Plus C18 column (250 x 4.6 mm) was used with 50mM Potassium Phosphate as mobile phase in ratio of 99 : 1 with acetonitrile and adjusted its pH of 2.5 by hydrochloric acid. The retention time was 5 minutes. Injection volume for samples and standard was set at 10 µl with rate of 1.0 ml/min. All standard and samples were analyzed in triplicate. The graph for standard lactic acid was drawn and concentration of lactic acid in each sample was calculated using Microsoft Excel software.

3.5.2 DNS Method

Three hundred grams of potassium sodium tartrate tetrahydrate was weighed into 1 L conical flask. 16 g of sodium hydroxide and 500 ml of water were added and dissolved by heating gently. When the solution was clear, 10 g of 3,5-dinitrosalicylic acid (DNS) was slowly added. The solution was cooled to room temperature and made up to 1 L with distilled water. The solution was kept covered to protect from light until the DNS was totally dissolved. DNS reagent was stored in a tightly stopped dark container to protect it from light and carbon dioxide. Glucose solution was prepared at different concentration of 0, 0.02, 0.04, 0.06, 0.08 and 0.10 g/L. DNS reagent was added to the glucose solution by ratio of 1:1 in the test tubes and mixed properly. The tubes was covered and placed in a boiling water bath for exactly 10 min and then cool down to room temperature. By using UV-Vis spectrophotometer, the absorbance for standard solution and banana stem sap was measured. The concentration of glucose in the banana stem sap was calculated from the standard given.

3.5.3 Validation Experiment

Validation is the process of repetition in order to get the confirmation of a result. The total of runs and all the parameters values were suggested by Design Expert 6.0 software. In this study, after the data was collected, the yield of lactic acid was compared from the experimental run before with error less than 20%. Therefore, in order to support the parameters that have been investigated, validation experiment will be run according to all the parameters. Following with the result from validation experiments, the best condition to produced lactic acid will be found.

4 RESULT AND DISCUSSION

4.1 Introduction

This chapter discusses the outcome of this study that related to the objectives and scopes. The topic covers in this chapter is main effect analysis, ANOVA analysis, optimization of lactic acid production using RSM and determination of optimum condition for lactic acid production. High Performance Liquid Chromatography (HPLC) was used to measure the concentration of lactic acid while the content of glucose in the banana stem waste was determined by using UV-VIS spectrophotometer. This study was conducted to investigate the optimum condition for the production of lactic acid using facultative anaerobic bacteria and banana stem waste as substrate. From the study, the optimum condition was obtained. The analysis of the interaction between the parameters was done by Design Expert software.

4.2 Effect of Temperature and Fermentation Time on Lactic Acid Production

Temperature and fermentation time are the factors that affect the production of lactic acid. Figure 4.1 shows the 3D surface for the effect of temperature and fermentation time on the production of lactic acid. The three-dimensional (3D) graphical represent the system behavior, called the response surface, was used to describe the individual and the cumulative effects of the variables as well as the mutual interactions between the independent variables and the dependent variables (Li et al., 2007). The units for the lactic acid concentration, fermentation time and temperature were mg/mL, hours and °C, respectively. From the figure, as the fermentation time increasing, the concentration of lactic acid is decrease. The concentration of lactic acid started to decrease when the fermentation time is below 20.5 hours. After 20.5 hours, the concentration of lactic acid is decreasing. It was found that the exponential phase for fermentation is between 12 – 40 hours meanwhile after 40 hours is the deceleration phase (Palaniraj et al., 2012). The statement has explained why the yield of lactic acid concentration per glucose concentration for both inoculums is higher at 20 hours of fermentation time. For the temperature as the temperature increase, the concentration of lactic acid increase. Above temperature of 39 °C, the concentration of lactic acid decrease. According to Sheeladevi

et al., (2011), the highest lactic acid productivity value is obtained at temperature 37 ^{o}C by using isolated lactic acid bacteria, *Lactobacillus casei* compared to the room temperature. The lactic acid production decrease when the temperature is above 45^{o}C. Lactic acid production using *Lactobacillus casei* strains is most efficient at 37 ^{o}C when compared to the room temperature (Hujanen et al., 1996). Busairi (2010) also stated that the lactic acid yield increased with each increase at temperature level of fermentation between 30 to 40 ^{o}C. The maximum lactic acid concentration is about 0.014 mg/ml at fermentation time is 20 hours and temperature of 39 °C. Figure 4.2 shows the interaction between the factors in perturbation plot and while Figure 4.3 shows the contour plot for production of lactic acid from Design Expert.

The response function for RSM data was assessed graphically by using perturbation plot as in Figure 4.2. The perturbation plot helps to compare the effect of all factors at particular point in RSM design space. In this plot, the effect of changing one factor from the reference point while holding the other factor constant was displayed. Based on Figure 4.2, both factors affected the lactic acid concentration in an almost similar trend of curvature. This indicates that both factors which are temperature (A) and fermentation time (B) showed significant quadratic effects that contributed the lactic acid concentration.

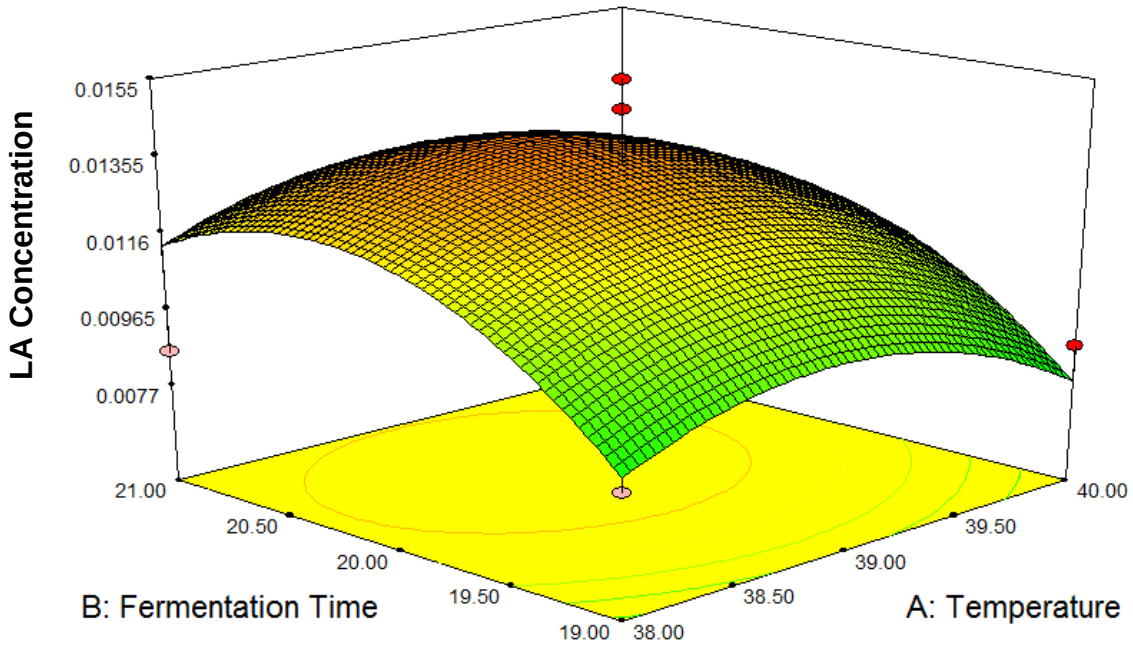


Figure 4.1: 3D Surface for The Effect of Temperature and Fermentation Time on Lactic Acid Production.

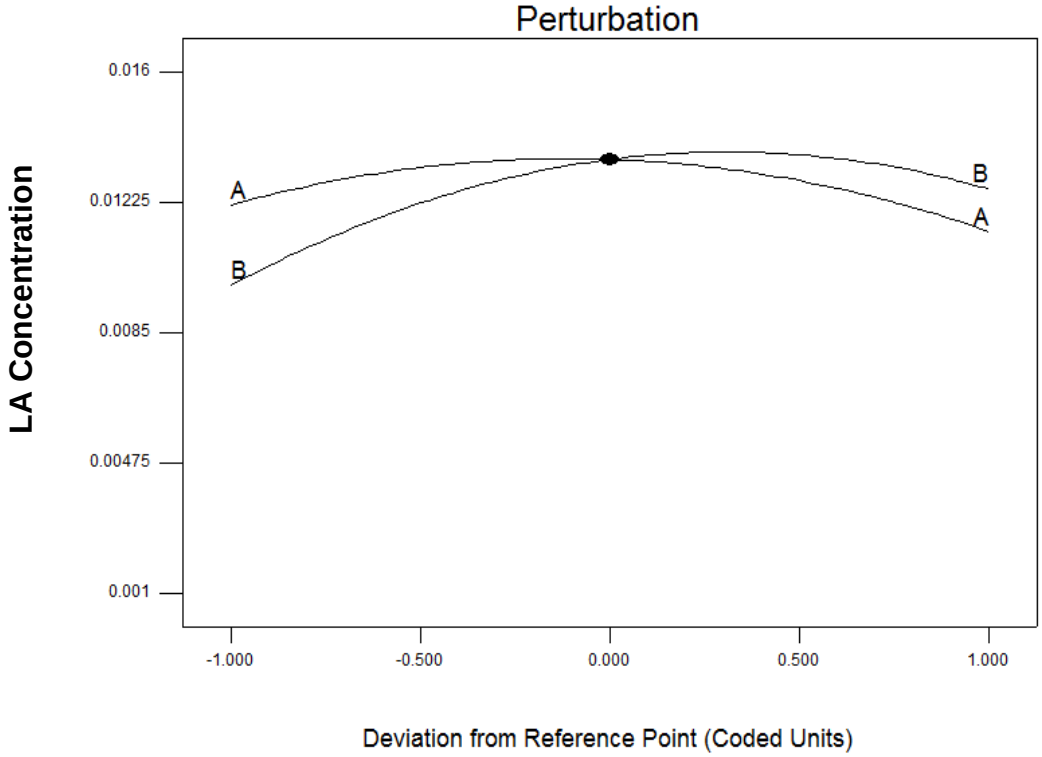


Figure 4.2: Perturbation Plot for the Interaction Between Fermentation Time and Temperature

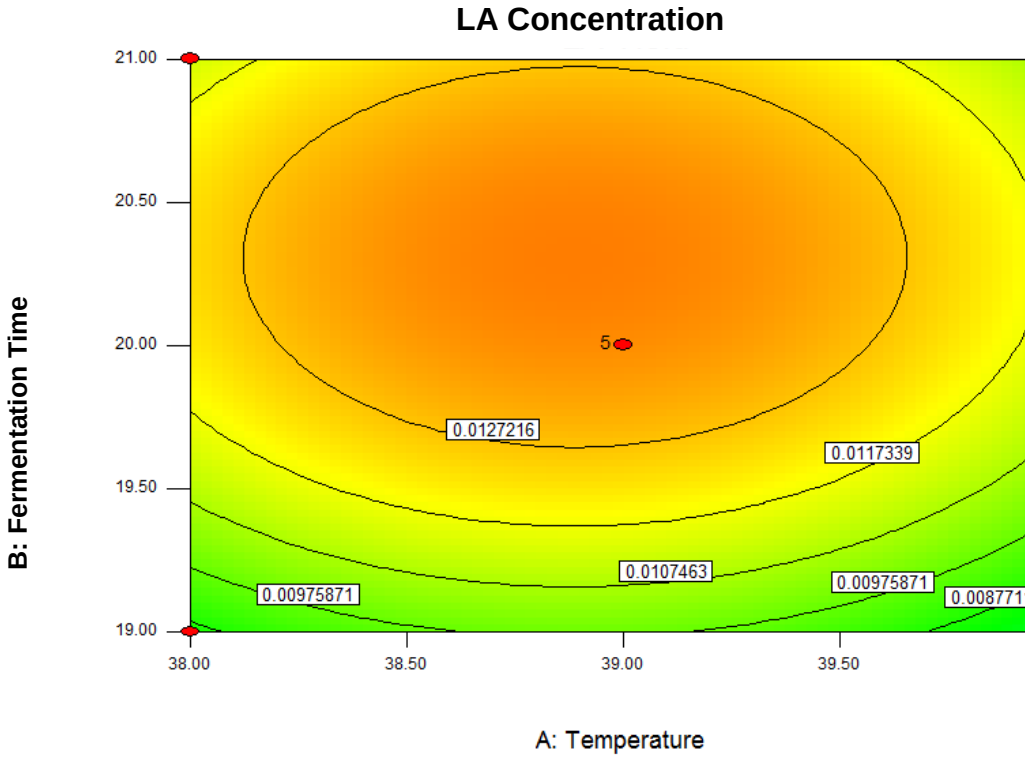


Figure 4.3: Contour Plot

4.3 ANOVA Analysis

Two factors were investigated in this research which is temperature and fermentation time. The results of 13 runs of the experiment were analyzed by analysis of variance (ANOVA) and a regression model was developed to describe the relationship between the selected factors. The regression model for lactic acid production is given in the Table 4.1.

Table 4.1: Regression Model for Lactic Acid Production

Final Equation in Terms of Coded Factors:

Lactic Acid Concentration	+0.013
*A	-3.800E-004
*B	+1.384E-003
*A*B	+7.201E-006
$*A^2$	-1.683E-003
$*B^2$	-2.237E-003

Final Equation in Terms of Actual Factors:

Lactic Acid Concentration	-3.44833
*Temperature	+0.13074
*Fermentation Time	+0.090598
*Temperature*Fermentation Time	+7.201E-006
$*Temperature^2$	-1.683E-003
$*Fermentation\ Time^2$	-2.237E-003

The quadratic equation of regression model for the lactic acid production:

Lactic acid = +0.013 - 3.800E-004 *Temperature + 1.384E-003 *Fermentation Time + 7.201E-006 *Temperature*Fermentation Time - 1.683E-003*$Temperature^2$ - 2.237E-003 *$Fermentation\ Time^2$.

The R^2 value for lactic acid production is 0.8852. According to Annuar *et al.*, (2008), the R^2 value is frequently used to judge whether the model is correctly represent the data, implying that if R^2 is close to one, then the regression model is correct. The results of the second order response surface model in the form of analysis of variance (ANOVA) are given in the Table 4.2.

Table 4.2: Analysis of Variance (ANOVA) for Response Surface Quadratic Model of Lactic Acid Production.

Source	Sum of Squares	df	Mean Square	F value	p-value Prob > F	
Model	1.668E-004	5	3.335E-005	10.80	0.0035	significant
A- Temperature	1.733E-006	1	1.733E-006	0.56	0.4782	
B- Fermentation Time	2.297E-005	1	2.297E-005	7.44	0.0295	
AB	2.074E-010	1	2.074E-010	6.716E-005	0.9937	
A^2	6.489E-005	1	6.489E-005	21.01	0.0025	
B^2	1.147E-004	1	1.147E-004	37.13	0.0005	
Residual	2.162E-005	7	3.089E-006			
Lack of Fit	1.588E-005	3	5.294E-006	3.69	0.1197	Not significant
Pure Error	5.739E-006	4	1.435E-006			
Cor Total	1.884E-004	12				

Values of Prob>F less than 0.05 indicate model terms are significant.

4.4 Interaction of Factors

The interaction between factors was plotted as in Figure 4.4. The plot shows that the lines are not parallel which indicates that there was an interaction effect between temperature (A) and fermentation time (B) on lactic acid production. The less parallel line shows that there is a significant interaction (Bakeman, 2002). Based on Figure 4.4, the lactic acid concentration response grows curvilinear when the temperature increasing at affixed level of fermentation time factor.

At lower coded fermentation time factor (B-) which is 19 hours, temperature had a significant effect on lactic acid production. This was because during limited fermentation time period, temperature became the important factor in lactic acid production. In such short fermentation time period, the capability of lactic acid production from banana stem waste was relatively lower compared to longer fermentation time. Higher temperature at 39 °C is the suitable temperature for the microorganism to grow. However, too high temperature (over 39 °C) will cause the microorganism die.

At higher coded fermentation time (B+) which is 21 hours, temperature also had a significant effect on lactic acid production. Lactic acid concentration was slightly increased because the microorganisms were given longer time duration to consume the substrate. In this longer fermentation time at 39 °C definitely gives higher lactic acid concentration but too long fermentation time will causes the cost to produce lactic acid increase.

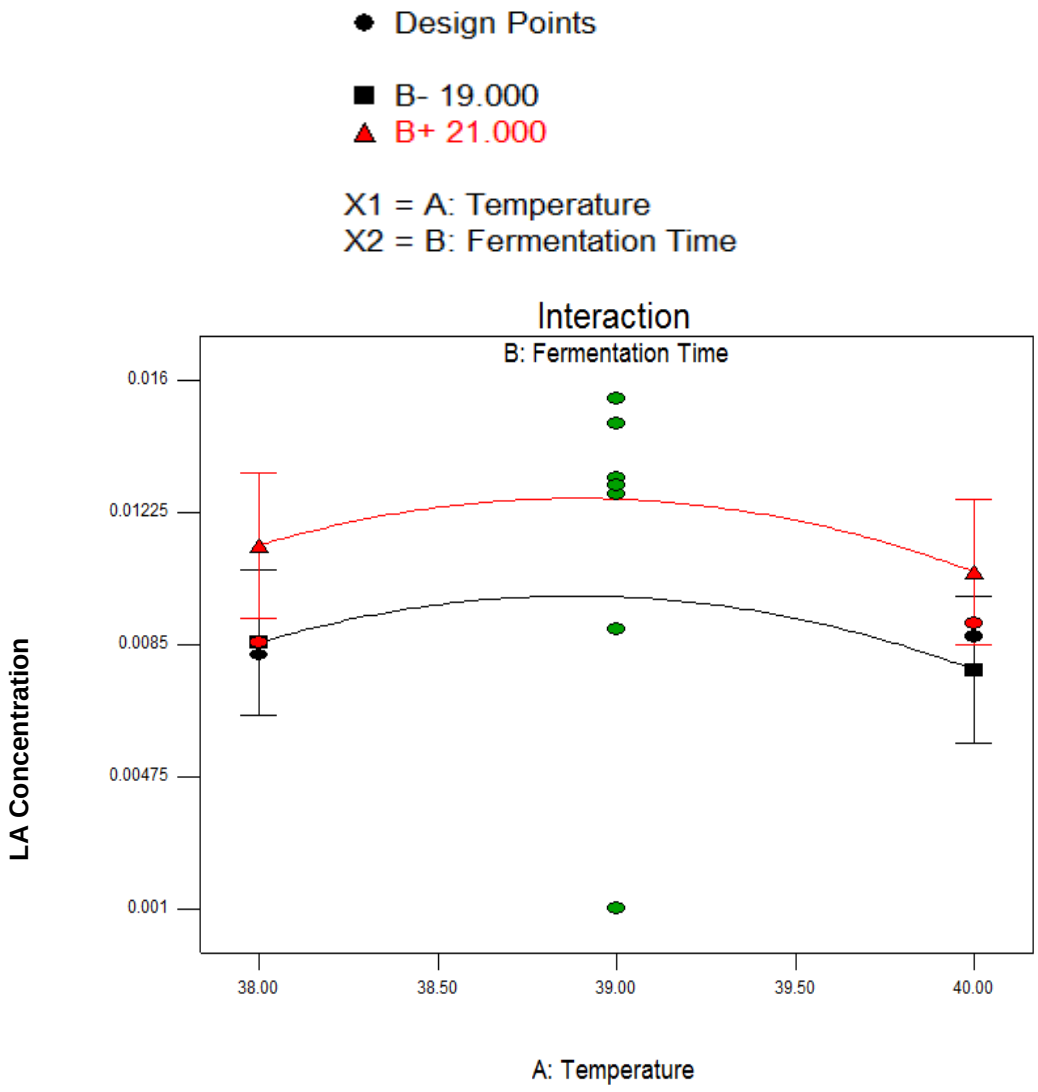


Figure 4.4: Interaction Plot of Temperature and Fermentation Time on Lactic Acid Concentration

4.5 Residual Analysis and Diagnostic Plots

Residuals are the difference between the observed values of the independent variable (y) and the predicted value ($\hat{y}$). From the least squares fit, the residual (e) play a crucial role in judging the adequacy of the model and are defined by equation (4.1).

$$e = y - \hat{y} \quad (4.1)$$

A residual plot is a graph that shows the residuals on the vertical axis and the independent variable on the horizontal axis. If the points in a residual plot are randomly

dispersed around the horizontal axis, a linear regression model is appropriate for the data; otherwise, a non-linear model is more appropriate. The residual plot shows a fairly random pattern which can be positive and negative. This random pattern indicates that a linear model provides a decent fit to the data.

Normal probability plot of residuals can be used to check the normality assumption. According to Kraber *et. al.,* (2002), a good normal probability plot must have a linear straight line whereas an S-shape indicating a bad normal plot. A review on the normal probability plot for lactic acid as illustrated in Figure 4.5 revealed that the residuals fall on a straight line implying that the errors are distributed normally. Figure 4.6 shows the residuals versus predicted response. It shows that they are randomly scattered without obvious pattern and unusual structure. This means that the model proposed was adequate and there was no reason to suspect any violation of the independence or constant variance assumption.

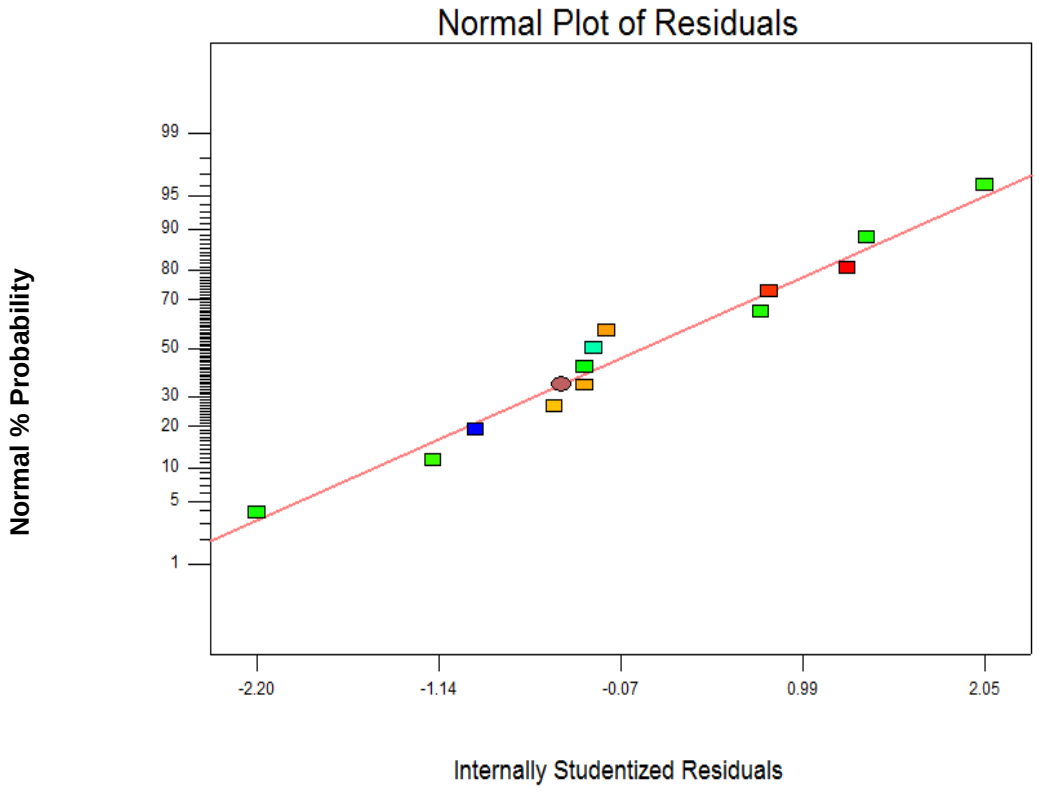


Figure 4.5: Normal Probability Plot of Residuals for Lactic Acid Concentration Data

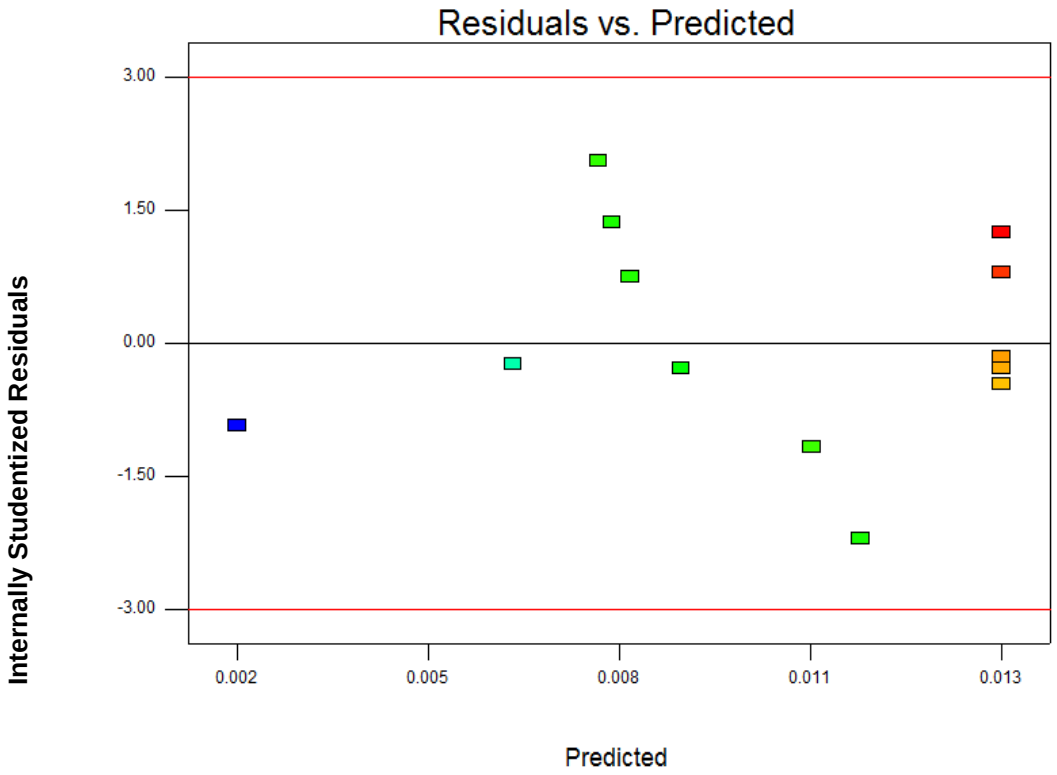


Figure 4.6: Residuals Versus Predicted Response Plot for Lactic Acid Concentration Data

4.6 Optimization of Lactic Acid Production using CCD

CCD was used in this study to determine the optimum condition for production of lactic acid. There are two factors involved in this study which are fermentation time and temperature. By using CCD, a total of 13 runs were generated with different conditions of fermentation time and temperature. The response of lactic acid concentration from the experiment was tabulated in Table 4.3. These data were the input for further analysis in Design Expert 7.0. Result from the optimization step using Response Surface Methodology (RSM) showed that the optimum condition for maximum lactic acid production were 39 °C of temperature and the fermentation time is 20 hours. The optimum lactic acid production predicted by the model was 0.01371 mg/mL. An experiment was done in order to validate this optimum condition.

Table 4.3: Combination Data Arranged by Response Surface Methodology (RSM)

Std	Run	Factor 1 A: Temperature (°C)	Factor 2 B: Fermentation Time (hour)	Response 1 LA Concentration (mg/mL)
1	3	38.00	19.00	0.0082
2	7	40.00	19.00	0.0087
3	10	38.00	21.00	0.0086
4	5	40.00	21.00	0.0091
5	1	37.00	20.00	0.0086
6	13	41.00	20.00	0.0058
7	12	39.00	18.00	0.0010
8	8	39.00	22.00	0.0089
9	2	39.00	20.00	0.0127
10	6	39.00	20.00	0.0132
11	4	39.00	20.00	0.0147
12	11	39.00	20.00	0.0130
13	9	39.00	20.0	0.0155

4.7 Validation Experiment

Design Expert 6.0 software suggested a total of two validation runs in order to confirm the experiment result. According to Table 4.4, all two runs were predicted to produce different yield of lactic acid with their respective parameters. The fermentation time was maintained at 20 hours. The only parameter that was changed in this validation experiment is the temperature.

Table 4.4: Validation Experiment

Temperature (°C)	Fermentation Time (hour)	Predicted LA Concentration (mg/mL)	Experimental LA Concentration (mg/mL)	Error (%)
39	20	0.01371	0.01370	0.06
38	20	0.0122	0.0120	1.38

Based on the table above, when the temperature was at 38 °C, the error of the result was higher than in temperature at 39 °C. So, the optimum condition for production of lactic acid is at temperature of 39 °C and fermentation time is 20 hours.

4.8 Comparison of Lactic Acid Production with Other Researchers

Nowadays, variety of carbohydrates is used to produce lactic acid, including agro-industrial waste, starchy, and lignocellulosic biomasses (Abdel-Rahman *et al.,* 2013). Depending on the availability of the substrate, a lot of agro-industrial waste is used to replace raw materials which are highly cost (Jawad *et al.,* 2013).

Previously, many researchers had studied about the production of lactic acid using different substrate and parameters of fermentation were varied. Tanaka *et al.,* (2006) reported that the yield of lactic acid produced based on total weight of the rice bran and the amount of utilized sugars is 0.28 g/g using solid state fermentation (SSF). Significantly, the highest lactic acid production took place after 36 hours of fermentation time with 37 °C temperature with agitation on.

Ohkouchi and Inoue (2006) had studied lactic acid production from food wastes by *Lactobacillus manihotivorans* LMG18011. Different temperature for fermentation and different initial pH condition were used to identify the effect on lactic acid productivity. At the end of the research, it was found that the residual sugar for pH 5.0 is the lowest and it showed that sample with initial pH 5.0 yields the highest amount of lactic acid (0.10 g/g) compared to the other two. The highest yield was at temperature of 30 °C and 48 hours fermentation time.

On the other hand, Chan-Blanco *et al.,* (2003) used waste of banana for generating lactic acid through batch fermentation, using *Lactobacillus casei*.. Sugar concentration showed a slow decreased over time, indicating that fermentation was indeed very slow. The result for the maximum yield of lactic acid is achieved at temperature of 37 °C and 70 hours of fermentation time.

Besides that, lactic acid production using *Kluyveromyces marxianus* (IFO 288), *Lactobacillus delbrueckii ssp. bulgaricus* (ATCC 11842) and *Lactobacillus helveticus* (ATCC 15009) as mixed culture on cheese whey was evaluated by Plessas *et al.,* (2008). Highest yield (0.35 g/g) was obtained when the temperature used was 37 °C for 24 hours fermentation time.

For this research, the best condition to produce lactic acid from banana stem waste by using facultative anaerobic bacteria was at temperature of 39 °C, no agitation used and 20 hours of fermentation time. The yield of lactic acid produced was 0.16 g/g, almost similar to Chan-Blanco *et. al.,* (2003) and Ohkouchi and Inoue (2006). From Table 4.5, 0.10 g/g of lactic acid was obtained by using pure culture while in this research was using mixed culture which is PMD. Mixed culture and cheese whey was used by Plessas *et al.,* (2008). The difference in lactic acid yield between this research and Plessas *et al.,* (2008) because of the different substrate used and also type of mixed culture used in the process.

Table 4.5: Comparison of Lactic Acid Production with Other Researchers

Substrate	Cultures	Temperature (°C)	Fermentation Time (hours)	Lactic Acid Yield (g/g)	Reference
Defatted rice bran	Pure	37	36	0.28	Tanaka *et. al.,* (2006)
Food waste	Pure	30	48	0.10	Ohkouchi and Inoue (2006)
Banana stem	Pure	37	70	0.10	Chan- Blanco *et. al.,* (2003)
Cheese whey	Mixed	37	24	0.35	Plessas *et. al.,* (2008)
This research	Mixed	39	20	0.16	

5 CONCLUSION AND RECOMMENDATIONSTIONS

5.1 Conclusion

In this study, the effect of temperature and fermentation time on lactic acid production was studied. The RSM with CCD was used to determine the optimum condition for the production of lactic acid from banana stem waste using facultative anaerobic bacteria. From the ANOVA analysis, the effect of temperature and fermentation time for lactic acid production was significant.

There was a fairly strong correlation between the interactions of those two factors to the lactic acid production. The maximum lactic acid concentration was found at temperature 39 °C and fermentation time at 20 hours. Beyond this temperature and fermentation time, lactic acid production was decrease.

Validation experiment was carried out to validate the result obtained in this study. The optimal condition was at temperature 39 °C and fermentation time of 20 hours. The concentration of lactic acid obtained from this condition is 0.01370 mg/ml (0.16 g/g). The error from the predicted models was only 0.6%. Therefore, it is suggested that the models obtained from CCD can be used to optimize lactic acid production.

From current study, it can be concluded that banana stem waste can be potentially promising source of lactic acid production. These findings are important to show that banana stem waste and probiotic milk drinks can be used as a cheap substrate and inoculums respectively in order to reduce the cost in production of lactic acid.

5.2 Future Research Recommendation

This research was done to produce lactic acid from cheap raw material which is banana stem waste. It is recommended to construct a pilot study about optimization of lactic acid production under optimum conditions obtained from this study.

The use of cheap raw material is very important in order to cut the cost in production of lactic acid. Furthermore, the usage of banana stem waste in this study also showed that a new effective substrate has been found and can be used in the process to produce lactic acid.

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7 APPENDICES

1) Analysis for Lactic Acid Content

Table 6.1: HPLC Reading for Standard Curve Lactic Acid

Concentration (mg/mL)	Retention Time (min)	Area (mAU*s)	Equation
0	0	0	y = 19063x
0.05	2.084	15239.00	
0.08	2.095	20883.63	
0.10	2.101	17406.57	
0.15	2.088	44851.77	
0.20	2.083	26063.20	
0.25	1.818	64338.07	
0.30	1.789	65760.47	
0.35	1.727	73239.10	
0.40	1.666	79829.27	
0.45	1.649	81085.00	
0.50	1.639	77454.03	

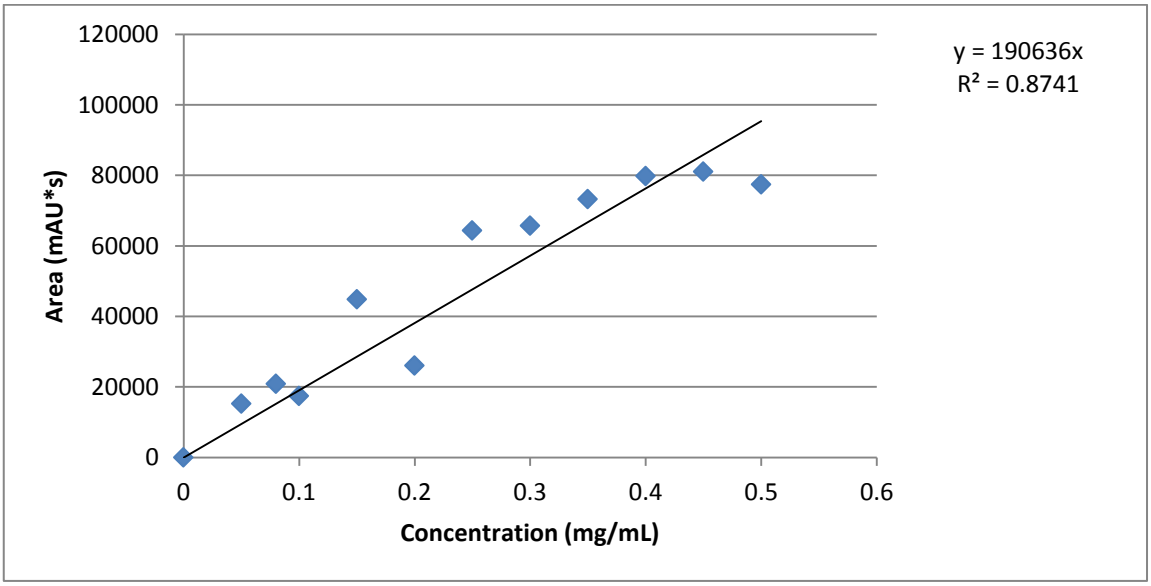

Figure 6.1: Standard curve for Lactic Acid

2) Analysis Glucose Concentration in Banana Stem Waste

Table 6.2: OD Reading for Standard Curve Glucose and Banana Stem Waste

Concentration (mg/ml)	OD Reading 1	OD Reading 2	OD Reading 3	Average
0	0	0	0	0
0.05	0.041	0.047	0.045	0.044
0.1	0.082	0.091	0.099	0.091
0.15	0.112	0.158	0.174	0.148
0.2	0.201	0.245	0.237	0.228
0.25	0.251	0.287	0.243	0.260
Banana stem waste	0.076	0.099	0.082	0.086
Concentration of banana stem waste (mg/ml)	0.086			
Yield (g/g)	0.160			
LA concentration (mg/ml)	0.014			

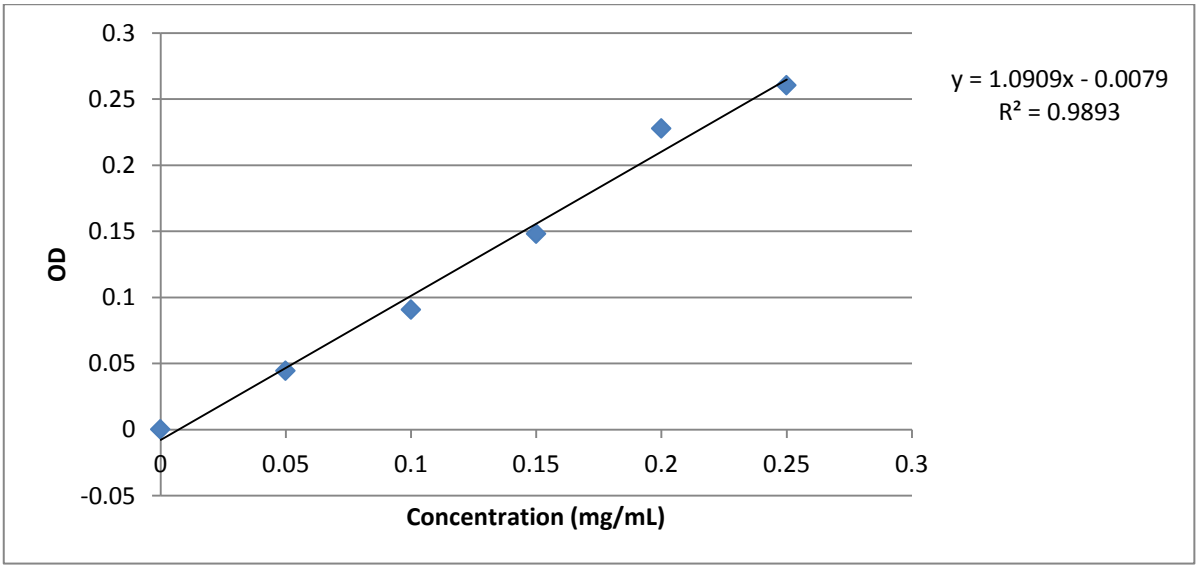

Figure 6.2: Standard curve for glucose

3) Analysis for Samples.

Table 6.3: HPLC reading for samples

Samples	Area (mAU*s)	Lactic Acid Concentration (mg/mL)
38c 19h	1563.688	0.008202
40c 19h	1661.213	0.008714
38c 21 h	1631.941	0.008561
40c 21h	1734.957	0.009101
37c 20h	1639.686	0.008601
41c 20h	1104.714	0.005795
39c 18h	1678.394	0.008804
39c 22h	1705.101	0.008944
39c 20h 1	2407.313	0.012628
39c 20h 2	2521.008	0.013224
39h 20h 3	2810.320	0.014742
39c 20h 4	2432.857	0.012762
39c 20h 5	2949.121	0.015470

Table 6.4: HPLC Reading for Samples in Validation Experiment with Error between Predicted and Experimental Lactic Acid Yield

Samples	Area (mAU*s)	Lactic Acid Concentration (mg/mL)
38c 20h 1	2918.998	0.015
38c 20h 2	1658.438	0.009
39c 20h 1	2485.639	0.013
39c 20h 2	2937.177	0.015
39c 20h 3	2412.753	0.013

Table 6.5: Errors between Predicted and Experimental Lactic Acid Yield in Validation Experiment

Fermentation Time (hours)	Lactic Acid Concentration (mg/mL)	Expected Yield	Error (%)
38c 20h	0.012005697	0.012	1.383
39c 20h	0.013700751	0.014	0.064