PRODUCTION OF LACTIC ACID FROM BANANA STEM WASTE USING MIXED CULTURE

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PRODUCTION OF LACTIC ACID FROM BANANA STEM WASTE USING MIXED CULTURE

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Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
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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 :

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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.

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Dedication

High appreciation gave to the people who helped me finished this thesis

To my supervisor, Dr. Norazwina bt. Zainol,
My parent, sibling, and family
My friends and colleagues
Lab assistants
Those who contributes to the labworks

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ABSTRACT

Lactic acid is a chemical compound that plays a role in various biochemical processes and also widely used in the food, cosmetic, pharmaceutical, and chemical industries. Lactic acid can be obtained by either microbial fermentation or chemical synthesis. Presently more than 95 % of industrial production of lactic acid is based on fermentation by using lactic acid bacteria. The research was done to produce lactic acid by using mixed culture of facultative anaerobic bacteria and banana stem waste. By using BSW as substrate and PMD as inoculums, the process was done according to selected factors. Source of inoculums (A), agitation (C) and ratio of substrate to inoculums (D) are categorical factors while temperature (B) and fermentation time (E) are numerical factors. Two factor A are used, Yakult and Nutrigen; three levels of factor B, 30, 35, 40 ^{o}C; agitate or non-agitate for factor C; while factor D used, 4:1 and 2:3; and also three levels of factor E, 20, 30, 40 hours. Design Expert 6.0 software is used to set the total number of experimental run, suggested validation experiment and also the best condition to produce lactic acid. The samples were analyzed using High Performance Liquid Chromatography while glucose content in the BSW is determined using Dinitrosalicylic Colorimetric (DNS) method and Ultra Violet-Visible (UV-Vis) Spectrometer. Factor A contributes the most in lactic acid fermentation with percentage of 1.07%. During preliminary experiments, Yakult and Nutrigen have respectively initial pH of 3.74 and 3.77 and decreases to 3.36 and 3.58 at the end of experiment. Previous study stated that when pH of the culture decreases, the concentration of lactic acid increases. Thus, it is showed that factor A could contribute the most in producing lactic acid. Factor's interaction between factor C and E contributes the most which is 35.55%. A total of five validation runs were suggested in order to confirm the experiment result. All runs have error between experimental and predicted lactic acid yield for about 8 – 35%. The best conditions to produce lactic acid for this research were at temperature 39^{o}C, no agitation, 20 hours of fermentation time and only 8% error with predicted lactic acid yield of 0.1009 g/g and experimental lactic acid yield of 0.0925 g/g. The production of lactic acid is affected by glucose content in the substrate used, temperature, agitation and fermentation time. Utilization of total sugar increased as the fermentation time increased, reducing the available sugar content in the media, thus increasing lactic acid production. Therefore, the result obtained in the study showed that BSW can be used as substrate in producing lactic acid and the parameter tested does affect the production of lactic acid.

ABSTRAK

Asid laktik adalah sebatian kimia yang memainkan peranan dalam pelbagai proses biokimia dan juga digunakan secara meluas dalam industri makanan , kosmetik , farmaseutikal, dan kimia. Asid laktik boleh didapati sama ada dengan penapaian mikrob atau sintesis kimia. Pada masa ini lebih daripada 95 % daripada pengeluaran perindustrian asid laktik adalah berdasarkan kepada penapaian dengan menggunakan bakteria asid laktik. Kajian ini dilakukan untuk menghasilkan asid laktik dengan menggunakan kultur campuran bakteria anaerobik fakultatif dan sisa batang pisang . Dengan menggunakan BSW sebagai substrat dan PMD sebagai inoculums , proses itu dilakukan mengikut faktor dipilih. Sumber inoculums (A) , putaran (C) dan nisbah substrat ke inoculums (D) adalah faktor mutlak manakala suhu (B) dan masa penapaian (E) adalah faktor berangka. Dua faktor A digunakan, Yakult dan Nutrigen ; tiga tahap faktor B, 30, 35 , 40 ^{o}C ; putaran atau tiada putaran untuk faktor C; sementara faktor D digunakan, 4:1 dan 2:3 ; dan juga tiga paras faktor E, 20, 30, 40 jam. Perisian 'Design Expert 6.0' digunakan untuk menetapkan jumlah jangka eksperimen , mencadangkan eksperimen pengesahan dan juga keadaan terbaik untuk menghasilkan asid laktik. Sampel dianalisis dengan menggunakan 'High Performance Liquid Chromatography' manakala kandungan glukosa dalam BSW ditentukan menggunakan kaedah 'Dinitrosalicylic Colorimetric' (DNS) dan 'Ultra Violet – Visible (UV -Vis) Spectrometer' . Faktor A menyumbang yang paling tinggi dalam penapaian asid laktik dengan peratusan sebanyak 1.07% . Semasa eksperimen awal, Yakult dan Nutrigen mempunyai pH masing-masing awal 3.74 dan 3.77 dan berkurangan kepada 3.36 dan 3.58 pada akhir eksperimen. Kajian terdahulu menunjukkan bahawa apabila pH kultur berkurangan, kepekatan asid laktik meningkat. Oleh itu, ia menunjukkan faktor yang A boleh menyumbang paling tinggi dalam menghasilkan asid laktik. Interaksi antara faktor C dan E menyumbang sebanyak 35.55 %. Sebanyak lima pengesahan eksperimen telah dicadangkan untuk mengesahkan hasil percubaan. Semua eksperimen mempunyai ralat di antara hasil asid laktik eksperimen dan diramalkan untuk kira-kira 8 - 35% . Faktor-faktor yang terbaik untuk menghasilkan asid laktik untuk kajian ini berada pada 39^{o}C suhu , tiada putaran, 20 jam masa penapaian dan ralat hanya 8 % dengan meramalkan hasil asid laktik daripada 0.1009 g / g dan eksperimen hasil asid laktik daripada 0. 0925 g / g. Pengeluaran asid laktik dipengaruhi oleh kandungan glukosa dalam substrat yang digunakan, suhu , putaran dan penapaian masa. Penggunaan daripada jumlah gula meningkat sebagai masa penapaian meningkat , mengurangkan kandungan gula yang terdapat di media, sekali gus meningkatkan pengeluaran asid laktik. Oleh itu, keputusan yang diperolehi dalam kajian ini menunjukkan bahawa BSW boleh digunakan sebagai substrat dalam menghasilkan asid laktik, dan faktor yang diuji boleh menjejaskan pengeluaran asid laktik.

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LIST OF ABBREVIATIONS

^{o}C	Degree Celcius
rpm	Revolution per minute
%	Percentage

ANOVA	Analysis of variance
BSW	Banana stem waste
C	Concentration
DNS	Dinitrosalicylic Colorimetric
FFD	Fractional Factorial Design
HPLC	High Performance Liquid Chromatography
PMD	Probiotic milk drinks
S:I	Substrate to inoculums
SSF	Solid state fermentation
UV-Vis	Ultra Violet-Visible
Y	Yield

1 INTRODUCTION

1.1 Motivation and statement of problem

Lactic acid is a chemical compound that plays a role in various biochemical processes enough to grabs all the attention as a chemical with many potential applications. Lactic acid is widely used in the food, cosmetic, pharmaceutical, and chemical industries and has received widely interest for use as a monomer for the biodegradable poly (lactic acid) production (Mudaliyar *et al*., 2011). Besides, lactic acid is also used commercially in the meat processes industry, together with hams, fish and poultry. It's also provide products with an increase in shelf life, better controls of food borne pathogens and enhanced flavour (Nagarjun *et al*., 2004). Presently the main growing application of lactic acid is in the production of biodegradable and renewable raw material based poly lactic acid (PLA) polymers (Sheeladevi *et al*., 2011)

Lactic acid can be obtained by either microbial fermentation or chemical synthesis and presently more than 95 % of industrial production of lactic acid is based on fermentation (Wee *et al*., 2006). Fermentation is known to be happened in anaerobic condition. Through fermentation, lactic acid bacteria also have the ability to produce lactic acid from sugar.

Lactic acid bacteria are generally considered facultative anaerobic obligate fermentative bacteria (Brooijmans, 2008). Facultative anaerobic bacteria are microorganisms which grow in the presence or absence of air (Condon, 1983). Holzapfel *et al.,* (1995) stated that lactic acid bacteria produce various metabolic products with antimicrobial properties such as organic acids like lactic acid and acetic acid. Lactic acid bacteria (LAB) are the most commonly used as probiotics. Probiotics are a live microbial food and feed supplement, which beneficially affects host by improving the intestinal microbial balance (Thongheam *et al*., 2008). According to Elmarzugi *et al*., (2010), most of commercially used probiotic products include different strains of *Lactobacilli* such as *L. acidophilus, L. casei*, *L. plantarum* and *Lactococcus lactis*. Nowadays, microorganisms of different groups or mixed culture are widely used in many probiotic applications as single strains or in combination. However, bacteria belong to *Lactobacilli* and *Bifidobacteria* groups are typical found in many probiotic products for human and animal use (Sanders, 1999). A mixed culture encompasses more than one species. Mixed culture of lactic acid bacteria are currently used in dairy industries for cheese and fermented probiotic milk manufacture. On industrial scale,

mixed culture has not yet being used. In this culture type, the existence of symbiotic relationship among various bacteria has been demonstrated (Moon and Reinbold, 1976). There were lot of research done to produce lactic acid by pure culture and also co-culture (Jawad *et al.,* 2012; Trontel *et al.*, 2011; Farooq *et al.*, 2012). Due to that, this study is focusing more on the used of mixed culture rather than pure culture.

Fermentative production of lactic acid offers the advantages in the utilization of renewable carbohydrates (Patil, *et al.*, 2006). Furthermore, lactic acid bacteria have the property of producing lactic acid from various fermentable carbohydrates. The main problem in producing lactic acid is to recognise the suitable and reliable raw materials. Most of the materials used for lactic acid production are sugar based feedstock such as glucose, molasses, food wastes and corn steep liquor. Jawad *et al.*, (2012) stated that application of agro-industrial wastes in bioprocesses provides an alternative way to replace the refined and costly raw materials. In addition, the bulk use of such materials helps to solve many environmental hazards. However, according to Bulut *et al.,* (2004), the application of microorganisms for the production of lactic acid using cost-effective raw materials is rare. Hence, research efforts are focused on looking for new and effective nutritional sources enabling the achievement of both high substrate conversion and high production.

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). According to Taskila and Ojamo (2013), chemical purity is mainly depending on the constituents in the fermentation medium especially when cheap materials are being used. The cheap substrate for lactic acid production may come from agricultural wastes containing cellulose and hemicelluloses, which can be converted into soluble sugars by chemical or enzymatic hydrolysis and then by microbial fermentation to lactic acid (Vodnar *et al.*, 2008). Vegetable and fruit processing wastes contain mainly starch, cellulose, soluble sugars and can be used for lactic acid production (Konings *et al.,* 2000). The study focus more on investigating the possibility of producing lactic acid by using banana stem waste instead of glucose, molasses, and other common substrate used. Therefore, consistent to the statement, by using banana stem waste in this study, it proved that production of lactic acid still can be obtained.

In this study, the parameters used for screening are fermentation time, agitation, source of inoculums, temperature and also ratio substrate to inoculums. Most of the screening

procedures generally involve the use of a differential medium occurrence containing a pH indicator (Choudhury *et al.*, 1990). According to the statement, at preliminary experiment, the culture was expected to lower the pH value as they grow and it showed that lactic acid is being produced. A two level factorial design was employed to determine the best condition to produce lactic acid according to the parameter selected. Two-level factorial is widely used in early stages of experiments to screen important factors from a large number of potential factors (Xu *et al.*, 2012). Two-level designs are commonly used to screen factors in the initial stage given a small number of runs.

1.2 Objectives

The following are the objectives of this research :

I. To produce lactic acid by using mixed culture of facultative anaerobic bacteria and banana stem waste.
II. To investigate on the effect of different source of inoculums, temperature, agitation, ratio of substrate to inoculums and fermentation time on the production of lactic acid using mixed culture of facultative anaerobic bacteria and banana stem waste.

1.3 Scope of this research

The following are the scope of this research :

I. Lactic acid fermentation by using banana stems waste and mixed culture of facultative anaerobic bacteria in form of commercially probiotic milk drink.
II. Determination of best condition to produce lactic acid by considering five parameters such as source of inoculums, temperature, agitation, ratio of substrate to inoculums and fermentation time.
III. Determination of glucose concentration in banana stems waste using Dinitrosalicylic Colorimetric (DNS) method and Ultra Violet-Visible (UV-Vis) Spectrometer.
IV. Determination of the lactic acid concentration using High Performance Liquid Chromatography (HPLC).
V. Data analysis of the yield of lactic acid concentration per glucose concentration by Two Level Factorial Design using Design Expert Software.

2 LITERATURE REVIEW

2.1 Overview

The worldwide demand of lactic acid in 2007 was estimated to be 130 000 – 150 000 metric tons per year (John *et al.,* 2007). According to the forecast, the production should increase significantly over the coming years mainly to provide the polylactic acid manufacturing sites and its other applications (Mujtaba *et al.,* 2010). Numerous industries uses lactic acid to produce their desired product such as chemical, food, pharmaceutical, textile, cosmetic and others (Coelho *et al.,* 2010). This was supported by Mudaliyar *et al.,* (2011), who stated that lactic acid is widely used in every segment of food industry. Food, cosmetic, pharmaceutical and chemical industries is proved to be the four major categories for current uses and applications of lactic acid. Worldwide demand on the uses of lactic acid in food related industries almost reached about 85%. In the broad range of application, lactic acid play a vital roles in chemical industries as a precursor for ethyl lactate, propylene oxide, propylene glycol, acrylic acid, 2,3-pentadione and dilactide syntheses (Sheeladevi *et al.,* 2011)

2.2 Mixed Culture of Facultative Anaerobic Lactic Acid Bacteria

Mixed culture has been determined to be effective for certain lactic acid fermentation. Mixed culture of lactic acid bacteria is largely used in dairy industry for manufacturing fermented drinks (Lee, K., 2004). Fermented drinks contain probiotic bacteria that are good to body. National Centre for Complementary and Alternative Medicine (2013) indicated that probiotics are live microorganisms, mostly beneficial bacteria like *Lactobacilli* sp. Probiotics are available to consumers mainly in the form of dietary supplements and foods. In food industries, milk or dietary drink often contains probiotic bacteria such as *Lactobacillus acidophilus* and *Lactobacillus casei*. All the bacteria are gram-positive, facultatively anaerobic, non-motile, non-spore-forming, and rod-shaped lactic acid bacteria. It can be found in dairy and also plant products and in the digestive tract of humans and animals (Gunduz, M., 2005).

Martinez *et al.,* (2013) stated that mixed culture of *Lactobacilli sp.* were employed in lactic acid production to shows better result compared to pure cultures, which is one type of microorganism that usually used in the production of lactic acid. 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. Mixed cultures of lactic acid bacteria is more effective than single culturing for improving lactic acid production (Lee, K., 2005).

2.3 Banana Stem Waste

Major factor in the economic production of lactic acid is the raw material cost. From ages, in order to lower the fermentation cost and also producing a pure lactic acid product, pure sugars or edible crops always have been used as substrate (Abdel-Rahman *et al.,* 2013). By products of agriculture industries are one of the alternatives substrate and renewable resources for lactic acid fermentation. According to Litchfield (1998), nowadays, depending on the availability of the substrate in the producing country, variety of carbohydrates is used to produce lactic acid such as starchy and lignocellulosic biomasses.

Fermentative production routes offer advantages of utilization of cheap renewable substrates, low production temperatures and low energy consumption in producing desired products. According to Sheeladevi *et al.,* (2011), various fermentable carbohydrates can act as substrate to produce lactic acid from lactic acid bacteria. Food production utilized traditional feedstock for lactic acid production which is starch based substrates (Bilanović *et al.,* 2011). The profitability of the process could have improved if cheaper substrates such as ligno-cellulosic biomass or agro-industrial wastes are used. Cheap by-products and waste substrates are recommended generally for fermentative production of chemicals to avoid a competition with food industry (Ozalp and Hyman, 2009).

The common used substrates for lactic acid production are glucose, molasses, corn steep liquor and more. Lot of studies have been done using various agriculture resources such as cassava bagasse, apple pomace, date juice, food waste, mango peel and others, Banana stem waste is one of the new renewable carbohydrate sources that have been used as substrate in lactic acid fermentation. As we can see from Table 2.1, Mohapatra *et al.,* (2010) stated that glucose content in the banana pseudo stem is 74.0 %, higher comparing to the other waste. The statement also supported by Sinha *et al.,* (2012), informed that in the pressed juice from banana stem there were 0.41% of carbohydrates per 100 gram. Futhermore, banana stem sap contains 0.191% total sugar, 0.0141% protein and negligible amount of lipids (Feriotti and

Iguti). The carbohydrates existed in the banana stem waste proved that it can act as substrate to produce lactic acid.

Table 2.1 : Chemical composition of different morphologic regions of banana plant

	Pseudostem	**Petioles/mid rib**	**Leaf blade**	**Floral Stalk**	**Leaf Sheaths**	**Rachis**
Glucose	74.0 [a]	68.1 [a]	60.0 [a]	79.8 [a]	74.2 [a]	31.8 [a]
Xylose	13.1 [a]	23.6 [a]	17.5 [a]	9.3 [a]	13.8 [a]	14.0 [a]
Galactose	2.5 [a]	1.1 [a]	3.8 [a]	2.9 [a]	2.2 [a]	1.7 [a]
Arabinose	9.1 [a]	4.9 [a]	15.5 [a]	5.1 [a]	7.5 [a]	4.1 [a]
Mannose	1.3 [a]	1.5 [a]	2.3 [a]	2.2 [a]	1.5 [a]	2.9 [a]
Rhamnose	-	0.8 [a]	0.9 [a]	0.7 [a]	0.8 [a]	0.7 [a]
Lignin	12.0 [a]	18.0 [a]	24.3 [a]	10.7 [a]	13.3 [a]	10.5 [a]
Cellulose	34 - 40 [a]	31.0 [a]	20.4 [a]	15.7 [a]	37.3 [a]	31.0 [a]
Holocellulose	60 - 65 [a]	62.7 [a]	32.1 [a]	20.3 [a]	49.7 [a]	37.9 [a]
Ash	14.0[a]	11.6 [a]	19.4 [a]	26.1 [a]	19.0 [a]	26.8 [a]
Potassium	33.4*	9.4*	11.6 [*]	23.1 [a]	21.4 [*]	28.0*
Calcium	7.5*	32.3*	8.0*	0.6*	5.5 *	0.6*
Magnesium	4.3*	2.9*	1.1*	0.5*	1.9*	0.3*
Silicon	2.7*	7.0*	24.9*	7.8*	2.7*	1.2*
Phosphorous	2.2*	0.7*	0.7*	0.7*	0.9*	1.7*
Pentosans	-	16.2 [a]	12.1 [a]	8.0 [a]	12.3 [a]	8.3 [a]
Starch	-	0.4 [a]	1.1 [a]	26.3 [a]	8.4 [a]	1.4 [a]
Proteins	-	1.6 [a]	8.3 [a]	3.2 [a]	1.9 [a]	2.0 [a]
[a] Expressed in terms of % molar properties; * Expressed in % ash basis						

Table 2.2 : Composition of different parts of banana waste (per 100g)

Waste Product	Moisture (g)	Protein (g)	Fat (g)	Minerals (g)	Fibers (g)	Carbohydrates (g)
Banana peel	79.2	0.83	0.78	2.11	1.72	5.00
Banana stem-central core	95.1	0.30	0.03	1.04	0.68	1.20
Banana stem-outer hard fibrous sheath	91.9	0.12	0.06	0.98	1.81	2.44
Banana stem-pressed juice from stem	98.6	0.05	-	0.63	-	0.41
Sources : Salunkhe and Kadam (1995)						

2.4 Lactic Acid Fermentation

Recently, lactic acid has been produced from a variety of carbohydrates, including starchy and lignocellulosic biomasses, depending on the availability in the producing country (Abdel-Rahman *et al.*, 2013). Sheeladevi *et al.,* (2011) claimed that using various lactic acid bacteria yield different amount of lactic acid under optimized condition of temperature, pH, inoculums level and fermentation period. The statement is supported by Coelho *et al.*, (2010), the temperature and pH are the key of environmental parameters that affect the lactic acid fermentation process. Lactic acid bacteria can grow at temperatures from 5 to 45 °C and, not surprisingly, are tolerant of acidic conditions, with most strains capable of growing at pH 4.4. It is therefore important to determine the temperature and pH at which optimal microbial growth is achieved.

2.5 Factors Used in Lactic Acid Fermentation

In this study, factors that have been effected lactic acid fermentation are pH, fermentation time, temperature and agitation.

2.5.1 pH

The pH has a serious influence on enzyme activities and nutrient assimilations for microorganisms. Increased undissociated lactic acid in accordance with decreasing pH due to lactic acid production is considered to inhibit the fermentation of several lactic acid producers (Abdel-Rahman *et al.,* 2013). According to Busairi (2010), lower pH value indicated that higher lactic acid is being produced. The decrease in pH with time during the fermentation process may be attributed to the production of lactic acid from sugars (Jawad *et al.,* 2013). The fermentation pH is either set at the beginning or then left to decrease due to acid production, and also controlled by base titration, or by extraction, adsorption or electrodialysis of lactic acid (Hofvendahl *et al.,* 2000). Lactic acid production usually have pH varies between 5.0 and 7.0. Kashket (2006) claimed that for *Lactobacillus* strain which known by the abilities to tolerate lower pH have the optimal pH below 5.7. At initial pH 6.5, cell started to utilize glucose earlier and at a faster rate than at other initial pH. Maximum lactic acid concentration was attained at initial pH 6.5 (Boontawan, 2010). The statement is supported by Kohajdová *et al.,* (2005) who stated that a rapid pH reduction in early stages of fermentation is important to obtain a high-quality final product.

2.5.2 Fermentation Time

Fermentation time is one of the critical environmental parameters affecting content, molecular mass, and sugar composition. Utilization of total sugar increased as the fermentation time increased, thus reducing the available sugar content in the media (Farooq *et al.,* 2012). This indicates the utility of sugars during fermentation period. According to Palaniraj *et al.,* (2012), the growth phase is observed from 0 – 55 hours with different initial substrate concentration. Higher initial substrate concentration used causes the growth phase to be slower. Generally, the exponential phase where highest amount of lactic acid produced is between 4 – 40 hours depending on the substrate concentration.

2.5.3 Temperature

Temperature is one of the important factors that affect the growth of microorganism. The characteristics of the microorganism used were affecting the maximum temperature at which the growth rate is the highest. Meanwhile if the optimum temperature was above or over limit the temperature required for certain microbes, they will eventually die or lesser the microbial activity (Tango and Ghaly, 1999). The yield of lactic acid increased with each increase at temperature level of fermentation in between 30 to 40 ^{o}C. The lactic acid production decrease above temperature 45 ^{o}C due to at this temperature the growth not optima therefore the yield become smaller, and the highest yield of lactic acid at 79.8 %, was achieve at 40 ^{o}C. Busairi (2010). The statement is supported by Jawad *et al.,* (2013) where most lactic acid bacteria which are responsible for the conversion of sugar to lactic acid are classified as thermophilic or mesophilic bacteria and usually have an optimum growth between 20 ^{o}C and 40 ^{o}C. Final lactic acid concentration of 5.23 g/L was obtained at 41^{o}C after the fermentation process end indicating that certain amount of sugar is not utilized and hardly converted by the microorganisms (Djukic´-Vukovic *et al.,* 2012). Besides that, Tango and Ghaly (1999) also reported a total of 10 g/L lactic acid was produced at 42^{o}C. Meanwhile, the highest lactic acid production was obtained at 37 ^{o}C and the yield obtained were 28.73 g/L. L. delbrueckii growth seems to grow well at 37 ^{o}C promoting maximum cell concentration and this contributes to maximum lactic acid production (Idris and Suzana, 2006)

Table 2.3 : Previous study on temperature effect in lactic acid fermentation

Temperature (^{o}C)	**Yield of Lactic Acid**		**Reference**
	C (g/L)	Y(g/g)	
20 – 40	Optimum		Jawad *et al.*, (2013)
37	28.73	-	Idris and Suzana (2006)
40	-	0.798	Busairi (2010)
41	5.23	-	Djukic´-Vukovic *et al.*, (2012)
42	10.0		Tango and Ghaly (1999)

2.5.4 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, D. M. *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, T. *et al.,* 2013).

Table 2.4 : Previous study on agitation effect in lactic acid fermentation

Agitation (rpm)	Description	Reference
200 – 500	• Longer lag phase thus affecting microbes growth • Less growth rate increase	Demirtas *et al.,* (2003)
50 - 500	• Increase the cell's glucose consumption • No significant effect on biomass production, lactic acid concentration and productivity	Gao, T. *et al.,* (2013)
0 – 300	• Higher shear stress • Smaller fungal's size • Increase the lactic acid production	Bai, D. M. *et al.,* (2003)

2.6 Previous Study on Lactic Acid Production

Lactic acid production using *Kluyveromyces marxianus* (IFO 288), *Lactobacillus delbrueckii ssp. bulgaricus* (ATCC 11842) and *Lactobacillus helveticus* (ATCC 15009) individually or as mixed culture on cheese whey in stirred or static fermentation conditions was evaluated. The highest lactic acid concentrations were achieved when *K. marxianus* yeast was combined with *L. delbrueckii ssp. bulgaricus* (Plessas *et al.*, 2007). Lee (2005) observed that mixed cultures of lactic acid bacteria maybe more effective than single culturing for improving lactic acid production.

Oh *et al.,* (2005) has been studied on the comparison in lactic acid production by using three different substrates which are wheat, corn and barley. All the research took place about 48 hours with temperature of 38^{o}C and 200 rpm speed. The highest production (0.94 g/g) was obtained when barley and corn is used as substrate. However, the tiny margin between all lactic acid yields by using the three substrates showed that the content of carbohydrates in this case was glucose, is relatively have almost the same amount. The utilization of the sugar content is parallel throughout the fermentation process, thus producing different amount of lactic acid.

Meanwhile, Farooq *et al.,* (2012) reported that the concentration of lactic acid produced with single culture *Lactobacillus delbrueckii*, was 77.6 g/L. Significantly the highest lactic acid production took place after 7 days of fermentation with 42^{o}C temperature and no agitation used. During second and third days of fermentation, rapid increase in production of lactic acid was observed until day 7, meanwhile at the 8th, the yield started to show a non significant decrease, correspond to the utilization of sugar content in the sugarcane molasses.

Mixed cultures or co-cultures of lactic acid producing microorganisms may increase the conversion efficiency of substrate (Cui *et al.*, 2010). Lee (2005) stated that the biotechnological process, by a mixed cell culture, has the advantage of better growth of cells, higher lactic acid production and lower nutrient consumption.

On the other hand, Nancib *et al.,* (2009) investigated the production of lactic acid from date juice by the single and mixed culture system of *Lactobacillus casei* and *Lactococcus lactis*. Using the same parameters for both single and mixed culture with only 19 hours fermentation time, 150 rpm agitation and operating at 30 ^{o}C, there were distant differences in lactic acid production. The concentration of residual glucose and fructose for

single culture is higher compared to mixed cultures which are 17.8; 5.6 and 4.0; 0.0 respectively. Lactic acid concentration of 60.3 g/L and glucose efficiency of 96% were achieved with the mixed culture whereas 53 g/L of lactic acid concentration and 82.2% of glucose efficiency were obtained using single culture. The statement also similar with Taniguchi *et al.*, (2004), where a co-culture of *Entrococcus casseliflavus* and *L.casei* was reported to produce 95 g/L of lactic acid after fermentation completed.

Jawad *et al.*, (2013) evaluated the bio-fermentation process by producing lactic acid from mango peels. Natural mixed lactic acid bacteria act as microbes in the breakdown of peel polysaccharides to glucose and the eventual conversion of the glucose into lactic acid by microorganisms in the fermentation broth throughout the process. The operational variables were carefully observed and showed that the production of lactic acid depended significantly on those factors, which are initial pH, temperature and incubation time. The results for maximum production of lactic acid (17.4 g/L) achieved at initial medium pH of 10; incubation time of 6 days; and at a temperature of 35^{o}C.

Table 2.3 summarize all different substrate for lactic acid fermentation using pure and mixed culture. According to Garde *et al.*, (2002), by using mixed culture, the lactic acid yield is 0.95 g/g, almost similar with the result from using pure culture.

Table 2.5 : Different Substrate for Lactic Acid Fermentation Using Pure and Mixed Culture

Fermentation Substrates	Cultures	Glucose Efficiency	Lactic Acid Produced	Reference
Barley	Pure	65.0 ± 1.7 g/g	0.94 ± 0.02 g/g	Oh *et al.*, (2005)
Wheat	Pure	52.5 ± 1.4 g/g	0.93 ± 0.01 g/g	
Corn	Pure	67.5 ± 1.5 g/g	0.94 ± 0.01 g/g	
Sugarcane Molasses	Pure	-	77.6 g/L	Farooq *et al.*, (2012)
Dates Juice	Pure	82.2 g/L	53.0 g/L	Nancib *et al.*, (2009)
	Mixed	96.0 g/L	60.3 g/L	
Mango Peel	Mixed	17.4 g/L	-	Jawad *et al.*, (2013)
Cheese Whey	Mixed	-	0.35 g/g	Plessas *et al.*, (2008)
Wheat Straw	Mixed	-	0.95 g/g	Garde *et al.*, (2002)
Mango Peel	Mixed	-	17.4 g/L	Jawad *et al.*, (2013)

2.7 Screening

2.7.1 Fractional Factorial Design (FFD)

According to Kiew, P. L. *et al.,* (2013), two level fractional factorial design is a popular experimental design and mostly applied in engineering analysis. FFD allows possible consideration of multitudinous factors and determine the most relevant factors from all the outcomes. The statement is supported by Khalil, M. *et al.,* (2011) where FFD is said to investigate the effect of tested independents variables to the response within the investigation range. FFD also is a technique where not only the determination of the influence of several variables on the response but also estimating the overall main factor effects and interaction of different factors (Golshani, T. *et al.,* 2013). Jawad *et al.,* (2013) stated that the factorial design was employed to study the effect of independent variables and the level of selected factors can be chosen based on preliminary experiments.

In this study, based on preliminary experiments, five different factors was tested which are temperature, agitation, source of inoculums, ratio of substrate to

inoculums and fermentation time.. The levels of selected factors were stated according to preliminary experiments as below :

Table 2.6 : Process variables and levels for FFD

Process Variables	**Levels**		
Temperature (^{o}C)	30	35	40
Fermentation time	20 hour	30 hour	40 hour
Agitation (rpm)	No	Yes	
Sources of inoculums	Yakult	Nutrigen	
Ratio of Substrate to Culture	4:1	2 : 3	

The experiment design protocol was contrived with the aid of the software Design Expert.

3 METHODOLOGY

3.1 Overview

This chapter discussed the materials and methods adopted in the experimental work. This chapter explained the fermentation process and also the analysis of lactic acid produced. The subchapter covers in this chapter was substrate preparation, inoculums preparation, preliminary study, experimental design, analysis, summary of experimental design and also validation experiment. These methodologies were being used thoroughly in this study.

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 following by DNS Method. Then, fulfilling the first objective, inoculums was prepared in term of probiotic milk drink. Screening factors were decided beforehand and using Design Expert 6.0 software, the experimental runs was done by full factorial design (FFD). The effect of all five factors, agitation, source of inoculums, ratio of substrate to inoculums, fermentation time and temperature on lactic acid fermentation were investigated to achieved the second objective. After fermentation process, the samples were collected and ready to be analyzed by High Performance Liquid Chromatography (HPLC). Then, using the software again, the R^2 value was calculated to see whether the data collection was valid or not. The last step would be the experimental validation run. It is to confirm and to find the best condition to produce lactic acid from banana stem waste.

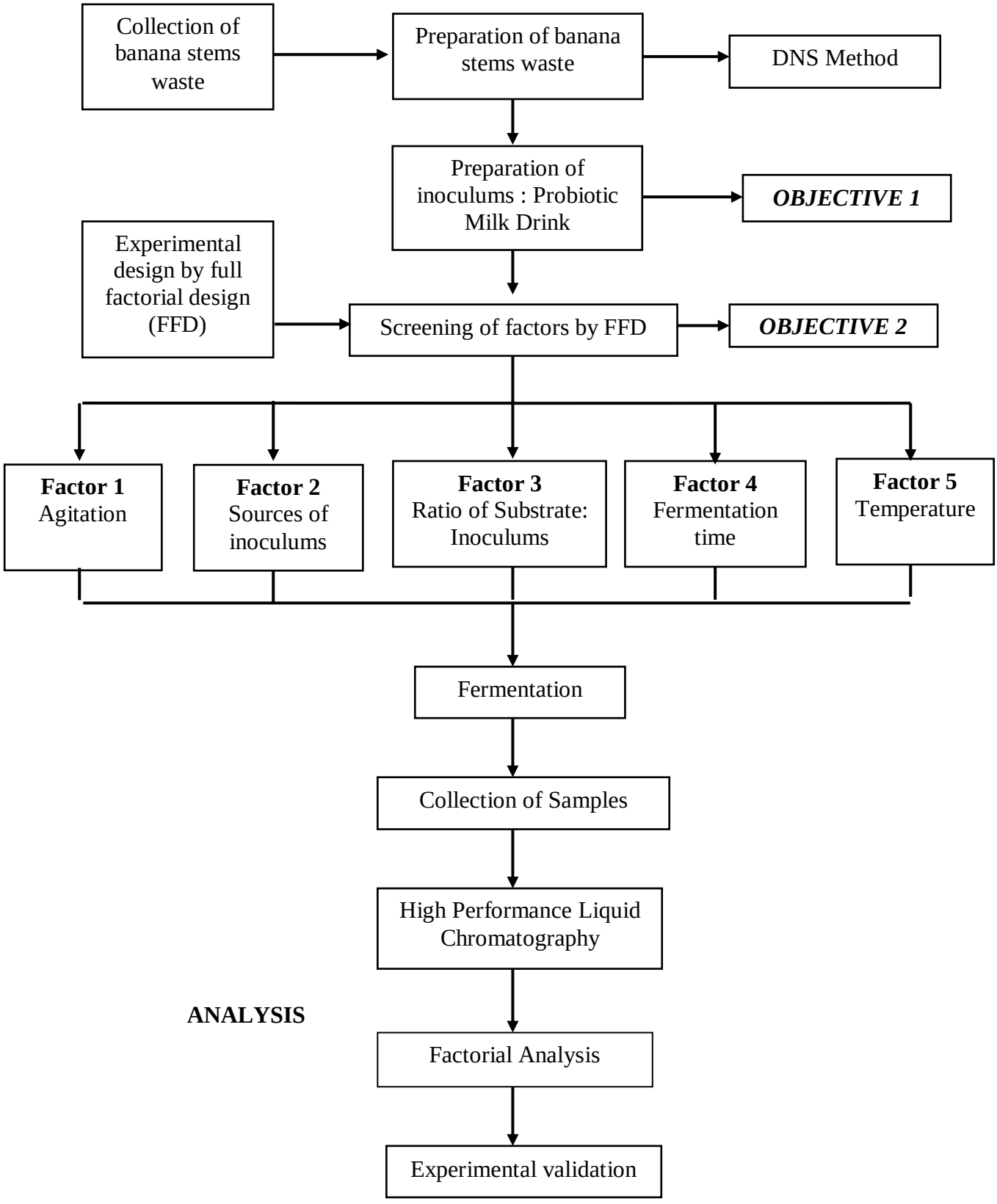


Figure 3.1 : Flow chart process of experiment

3.2 Substrate Preparation

Banana stem waste was collected from Gambang, Pahang, Malaysia. According to Alwi *et al.,* (2013), the pith of the pseudostem is separated. Figure 3.2 shows the pseudostem was crush, filter and the juice was kept. The fresh banana stem sap is stored in a refrigerator at 4°C to ensure its freshness. To avoid any fermentation, the experiment using this banana stem sap was carried out on the same day.

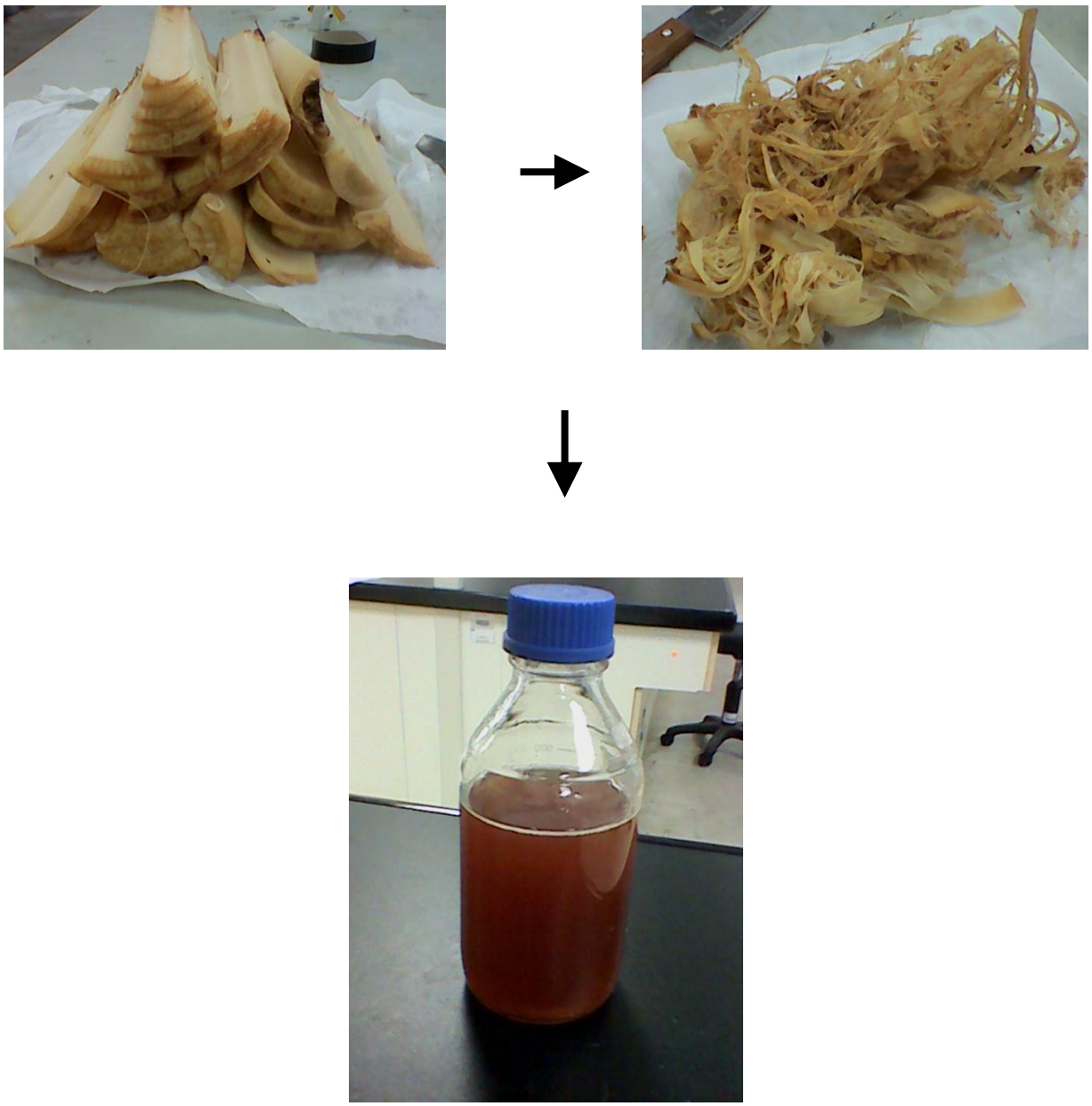


Figure 3.2 : Banana stem waste

3.3 Inoculums Preparation

Both commercially probiotic milk drinks, Yakult and Nutrigen were purchased from Giant Hypermarket at Kuantan, Pahang, Malaysia. Figure 3.3 shows the probiotic milk drinks that have been purchased as laboratory used. The experiment required the original flavour of both milk drinks to ensure the accuracy of the lactic acid production. Both milks drinks must be stored at 4^{o}C to assure that the bacteria are in inactive state. The milk drinks must be at ambient temperature when the experiment is conducted by leaving it at the laboratory locker.

Figure 3.3 : Commercially Probiotic Milk Drinks

3.4 Preliminary Study

The preliminary was conducted for determining the best condition between the factors that has been selected. It also done to investigate the experimental factors and to narrow the corresponding ranges before the application of statistical design. For preliminary experiment, temperature of 37°C and agitation of 100 rpm will be used as the parameters. Five different kinds of commercially probiotic milk culture such as Yakult, Nutrigen, Solivite, Vitagen and Farm Fresh Milk will be used as the inoculums for the fermentation. The pH of the fermented broth was measured using indicator to ensure the present of lactic acid bacteria in the solution. In this preliminary study, other purpose of doing it is to determine the fermentation time to conduct the experiment with other variables. The growth profile from the fermentation will be plotted and from the data that will be obtained, fermentation time will be identified to be carried out for the real run. The ratio used for banana stem sap to inoculums is 3:2 where 75 ml of substrate in the Erlenmeyer flask were added with 50 ml of inoculums. According to Goksungur *et al.*, (1997), parameter to incubate culture in incubator shaker is :

- Temperature : 37^{o}C
- Fermentation time : 30 hour
- Agitation : 100 rpm
- Source of Inoculums : Five commercially probiotic milk drink
- Ratio of Banana Stem Waste to Inoculums : (3:2)

3.5 Experimental Design

Fermentation was carried out in 250ml Erlenmeyer flasks containing different ratio of substrate and culture. Table 3.1 shows the parameter used throughout the experiment. The cultures were incubated at 3 controlled temperatures (30, 35 and 40 ^{o}C) for different fermentation time (20, 30, 40 hours). After fermentation was done, the samples were all collected and ready to be analysed by using High Performance Liquid Chromatography. The sample can be stored in -20^{o}C freezer to avoid contamination and error in result. By using Design-Expert® software, a total of 24 runs will be carried out. After data is collected, using Design-Expert® software, the data will be analysing with two-level factorial designs. Two-level factorial designs will analyse multiple parameter interaction.

Factors that have been selected were source of inoculums, fermentation time, temperature, ratio of substrate to inoculums and agitation. From preliminary experiment, according to the data collected, all the parameter was chosen based on the effects on the fermentation. A range of value for every parameter have been selected and listed in the Table 3.1. The parameters can be used in the experimental run in order to fulfil the objectives of the study as to find the best condition to produce lactic acid by using mixed culture of facultative anaerobic bacteria and banana stem sap

Table 3.1 : Parameter used

Process Variables	**Levels**		
Temperature (^{o}C)	30	35	40
Fermentation time	20 hour	30 hour	40 hour
Agitation (rpm)	No	Yes	
Sources of inoculums	Yakult	Nutrigen	
Ratio of Substrate to Inoculums	4:1	2 : 3	

3.6 Analysis

3.6.1 Lactic Acid Analysis

Lactic acid was analyzed by 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. A flow rate of 1.5 ml/min was used for column while the column temperature was maintained at 30 ^{o}C. Injection volume for samples and standard was set at 10 ml with rate of 1.0 mol/min. All standard and samples were analyzed in triplicate. The graph for standard lactic acid was drawn and concentration of lactic acid in each samples were calculated using Microsoft Excel software.

3.6.2 DNS method

Three hundred grams of potassium sodium tartrate tetrahydrate is weighed into 1 L conical flask. Sixteen grams of sodium hydroxide and 500 ml of water were added and dissolve by heating gently. When the solution is clear, 10 g of 3,5-dinitrosalicylic acid (DNS) was slowly added. The solution was cool 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.

3.7 Validation

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. According to Table 3.2, the experiment was carried out using the following parameters listed.

Table 3.2 : Validation experiment

Culture	**Temperature(^{o}C)**	**Agitation**	**Ratio Substrate : Culture**	**Fermentation Time (hour)**	**Yield LA Conc/ Glucose Conc (mg/mg)**
Nutrigen	39.7028125	No	4:1	22.42634375	0.09949549
Nutrigen	37.9635	No	4:1	20.6355	0.099606854
Nutrigen	38.557	No	4:1	20.442	0.100885105
Nutrigen	39.46684375	No	4:1	21.2314375	0.101067927
Nutrigen	30.55525	No	2:3	20.17675	0.101837127

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 ANOVA and regression analysis, main effect analysis, and interaction between factors. High Performance Liquid Chromatography (HPLC) is used to measure the concentration of lactic acid while the content of glucose in the banana stem waste is determined by using UV-VIS spectrophotometer. The recent study was conducted to investigate the production of lactic acid using facultative anaerobic bacteria and banana stem waste as substrate. From the study, it is show that the parameters gave different effect to the lactic acid production. The analysis of the interaction between all the parameters is done by Design Expert software.

4.2 Preliminary

Preliminary was done to identify the fermentation time of lactic acid fermentation using banana stem waste and also mixed culture in probiotic milk drinks. Beside, the best condition to conduct the experiment was also determined. A total of 3 run is done with 5 samples of banana stem and different probiotic milk drinks such as Yakult, Vitagen, Solivite, Nutrigen, and Farm Fresh Milk. From the experiment, only three probiotic milk drinks is chose as their acidity is decreasing as the time flows. The table 4.2 and graph 4.1 showed the data collected for every sample taken.

Yakult, Solivite and Nutrigen are chose as the best three out of five samples of milk drinks. The experiment is repeated to get the exact fermentation time where the acidity of the samples is measured. The temperature used in the study is 37^{o}C with agitation of 100 rpm. pH of the sample is measured every 6 hours for 2 days (48 hours). 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 statement also supported by Busairi (2010) which stated that the lactic acid yield increased with each increase at temperature level of fermentation between 30 to 40 ^{o}C. 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).

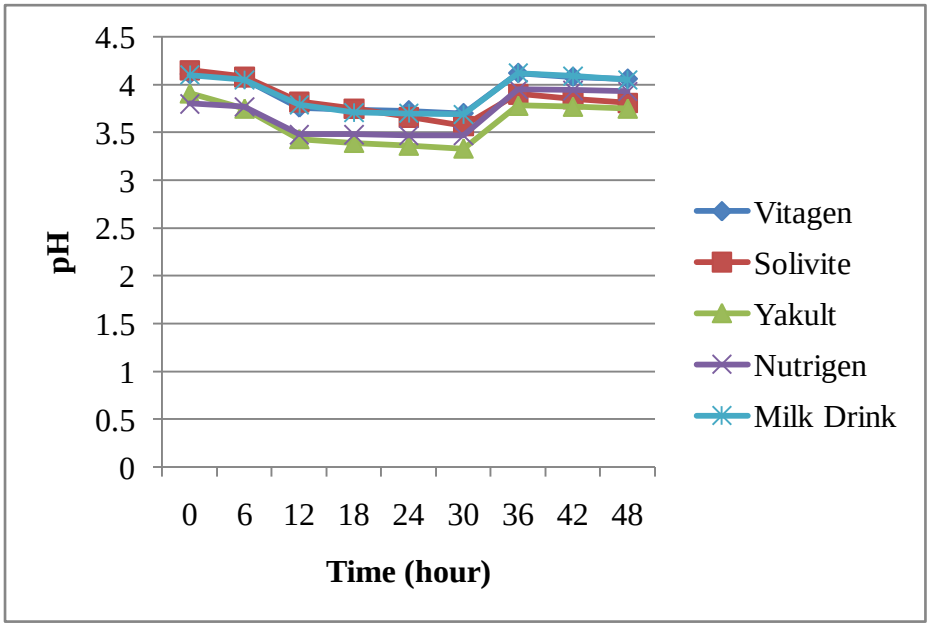
The initial pH for banana stem waste and every mixed cultures used is as tabulated in table 4.2. The experiment is carried out in the incubator shaker for 48 hours. The data of pH measured for every 6 hours is listed as in Table 4.3 and Graph 4.2. Based on the graph 4.2, fermentation time is decided to be stopped at 30 hours as the pH value for each sample keep decreasing until at 30 hours. The lactic acid cultures reduced the pH to 3.0 or below and increase the acidity. Yoon *et al.,* (2004) stated that as the pH reduces, the viable cell counts (CFU) increases rapidly after 72 hours thus enhance the production of lactic acid. Carlson *et al.,* (2002) also claimed that as the pH of the culture decreases, the concentration of lactic acid increases.

Table 4.1 : Initial pH

	pH
Banana Stem Sap	6.08
Yakult	3.57
Nutrigen	3.59
Solivite	3.96

Table 4.2 : pH value for 5 different samples of fermentation

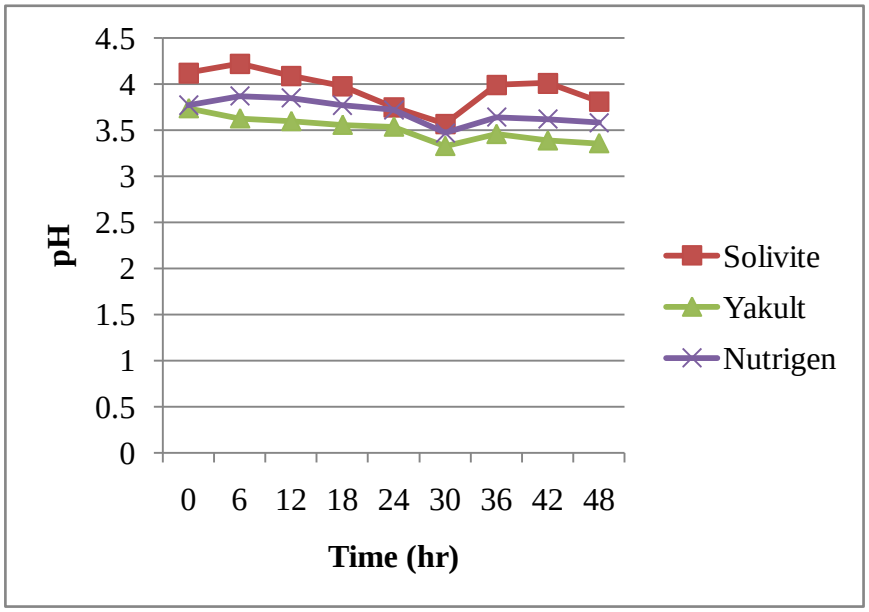
Time	pH				
	Vitagen	**Solivite**	**Yakult**	**Nutrigen**	**Milk Drink**
0	4.11	4.15	3.91	3.80	4.10
6	4.06	4.08	3.75	3.77	4.05
12	3.76	3.82	3.43	3.48	3.79
18	3.74	3.75	3.39	3.48	3.71
24	3.73	3.66	3.36	3.47	3.70
30	3.70	3.57	3.33	3.47	3.69
36	4.12	3.90	3.78	3.95	4.12
42	4.08	3.85	3.77	3.94	4.09
48	4.06	3.81	3.75	3.93	4.05



Graph 4.1 : Graph of time versus pH value for five different samples

Table 4.3 : pH value for 3 different samples of fermentation

Time (hr)	pH					
	Solivite		Yakult		Nutrigen	
0	4.12	4.12	3.74	3.74	3.77	3.77
	4.13		3.75		3.38	
6	4.22	4.22	3.63	3.63	3.87	3.87
	4.23		3.64		3.87	
12	4.09	4.09	3.61	3.60	3.85	3.85
	4.12		3.60		3.87	
18	3.98	3.98	3.56	3.56	3.78	3.77
	4.00		3.56		3.77	
24	3.75	3.75	3.54	3.54	3.72	3.72
	3.76		3.55		3.73	
30	3.57	3.57	3.33	3.33	3.47	3.47
	3.58		3.34		3.48	
36	3.99	3.99	3.46	3.46	3.64	4.64
	4.01		3.47		3.64	
42	4.01	4.01	3.39	3.39	3.63	3.62
	4.03		3.39		3.62	
48	3.9	3.9	3.36	3.36	3.58	3.58
	3.9		3.39		3.58	



Graph 4.2 : Graph of time versus pH value for three different sample

4.3 ANOVA and Regression Analysis

Five factors were thought to be influential on lactic acid production by facultative anaerobic bacteria during fermentation is used as response. The five factors are source of inoculums, fermentation time, temperature, ratio of substrate to inoculums and agitation. The results of 24 runs factorial design of lactic acid production were analyzed by analysis of variance (ANOVA) and a regression model was developed to describe the relationship between the selected factors. ANOVA results showed that source of inoculums (A), temperature (B), agitation (C), ratio of substrate to inoculums (D) and fermentation time (E) have influence lactic acid production. The regression model for lactic acid production is given in the following table :

Table 4.4 : Regression model for lactic acid production

Final Equation in Terms of Coded Factors:

Yield LA Conc/Glucose Conc	=
0.068777482	
-0.002117595	* A
-0.002291704	* B
-0.001512952	* C
-0.001893598	* D
0.001853217	* E
-0.00340051	* A * B
-0.002391489	* A * C
0.006123369	* A * D
0.008053385	* A * E
0.006993105	* B * D
-0.008071539	* C * D
0.014978219	* C * E
0.00481898	* D * E

The R^2 value for lactic acid production is 0.8881. 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.

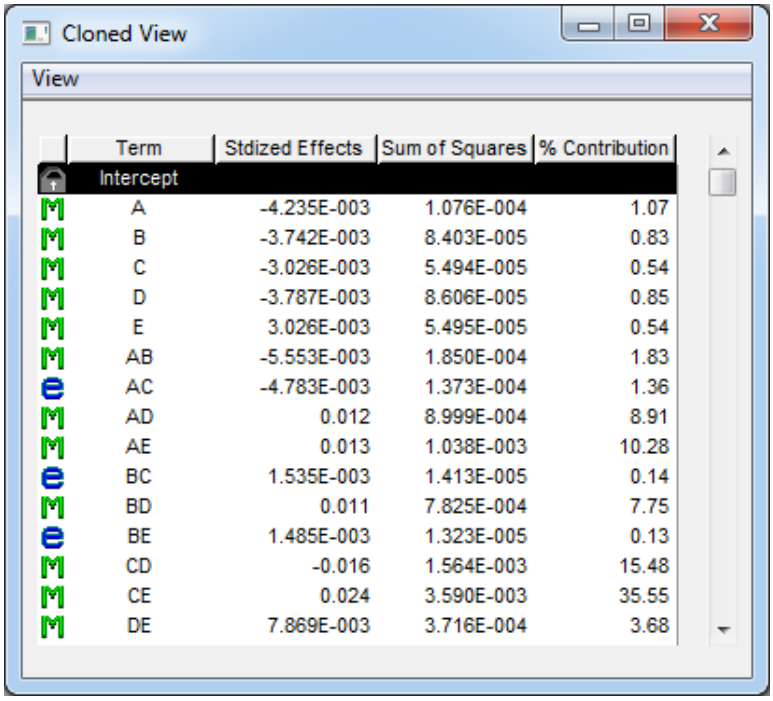
4.4 Main Effect Analysis

From Figure 4.3, it is shown that parameter A, source of inoculums, contributes the most in lactic acid fermentation with a percentage contribution of 1.07%. According to the preliminary experiments, both inoculums, Yakult and Nutrigen have respectively initial pH of 3.74 and 3.77. Fermentation took time for about 48 hours and the final pH of Yakult and Nutrigen decreases to 3.36 and 3.58. Carlson *et al.*, (2002) claimed that as the pH of the culture decreases, the concentration of lactic acid increases. Both mixed culture have affected the production of lactic acid and yield different amount of lactic acid.

The second factor that contributes to the production of lactic acid is the ratio of substrate to inoculums with the contribution of 0.85%. The amount of bacteria present in the fermentation medium seems to affect the production of lactic acid. Ratio of 2:3 and 4:1 is used during fermentation. The ratio of the substrate : media in the reaction mixture was varied from 0.25 to 4, so its suitable to use the chosen ratio (Bishai *et al.*, 2013). However, the higher present of bacteria does not yield higher production of lactic acid and higher amount of substrate might become too much for the bacteria to handle.

Temperature contributed of 0.83% to lactic acid production. 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). The temperature range used between 30^{o}C to 40^{o}C in the experiment seems to show its contribution to the process as Busairi (2010) also stated that the lactic acid yield increased with each increase at temperature level of fermentation between 30 to 40^{o}C.

Fermentation time and agitation have least contributes of 0.54%. From the result, both parameters might not be one of most affecting factors. For single effect parameter, although fermentation process is usually a time consuming and agitate process, fermentation time and agitation for this process have shown to be less important compared to the other factors. However, fermentation time and agitation contribute the most when they interact.



Term	Stdized Effects	Sum of Squares	% Contribution
Intercept			
A	-4.235E-003	1.076E-004	1.07
B	-3.742E-003	8.403E-005	0.83
C	-3.026E-003	5.494E-005	0.54
D	-3.787E-003	8.606E-005	0.85
E	3.026E-003	5.495E-005	0.54
AB	-5.553E-003	1.850E-004	1.83
AC	-4.783E-003	1.373E-004	1.36
AD	0.012	8.999E-004	8.91
AE	0.013	1.038E-003	10.28
BC	1.535E-003	1.413E-005	0.14
BD	0.011	7.825E-004	7.75
BE	1.485E-003	1.323E-005	0.13
CD	-0.016	1.564E-003	15.48
CE	0.024	3.590E-003	35.55
DE	7.869E-003	3.716E-004	3.68

Figure 4.3 : The percentage distribution of each main factor and their interaction. Terms : A, Source of inoculums; B, Temperature; C, Agitation; D, Ratio of Substrate to Inoculums; E, Fermentation Time

4.5 Interaction between Factors

The interaction between the factors will likely improve the production of lactic acid. Some factor interaction may contribute more than the main effects alone. There were four interactions discovered in this study.

In Figure 4.4, the first interaction is between factor A, source of inoculums and factor E, fermentation time. Figure shows that there exist an interaction between source of inoculums and also fermentation time. This interaction is possibly due to the highest contributes of factor A compared to the other factors. As we can see in Figure 4.4, the yield of lactic acid concentration per glucose concentration for Nutrigen is higher at 20 hours fermentation time compared to 40 hours. This also can be applied to Yakult as the yield of lactic acid concentration per glucose concentration at 20 hours is bigger than in 40 hours. For different initial substrate concentration, 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. According to Nancib *et al.*, (2009), lactic acid production from date juice

by mixed culture of lactic acid bacteria yield the highest when at 20 hours fermentation time compared to single culture. Utilization of total sugars increased as the time of fermentation was increased decreasing the available sugar content in the media. The statement is supported by Farooq *et al.*, (2012), who proved that during the first day of lactic acid fermentation, the utilization of glucose is significantly large thus assured that the yield of lactic acid is bigger during 20 hours and above.

The second interaction can be found in Figure 4.5, which shows the interaction between factor C, agitation and factor D, ratio of substrate to inoculums. The high effects or contribution of agitation has been explained in most of the past studies such as Abdel-Rahman *et al.*, (2013), but its interaction with another factor, substrate to inoculums ratio is something new and fresh to discuss. Ratio of substrate to inoculums that have been used in the experiment is 2:3 and 4:1. According to Figure 4.5, the lactic acid yield higher amount when 2:3 ratio and agitation was used for both inoculums. Bishai *et al.*, (2013) supported by stated that when ratio was varied from 0.25 to 4, the conversion increase for about 75.76% at ratio of 1.5 and beyond it the conversion got reduced gradually.

The third interaction is portrayed in Figure 4.6 which shows the interaction between factor C, agitation and factor E, fermentation time. At 20 hours of fermentation time, there were only slight differences in the yield of lactic acid concentration per glucose concentration. Naranong and Poochareon (2001) claimed that the values of specific growth rate increased with increasing the agitation rate demonstrating that growth was dependent on oxygen supply. In this study, the mixed culture that have been used is a facultative anaerobic bacteria, so the culture can grow in a medium that have less oxygen and thus make agitation less important.

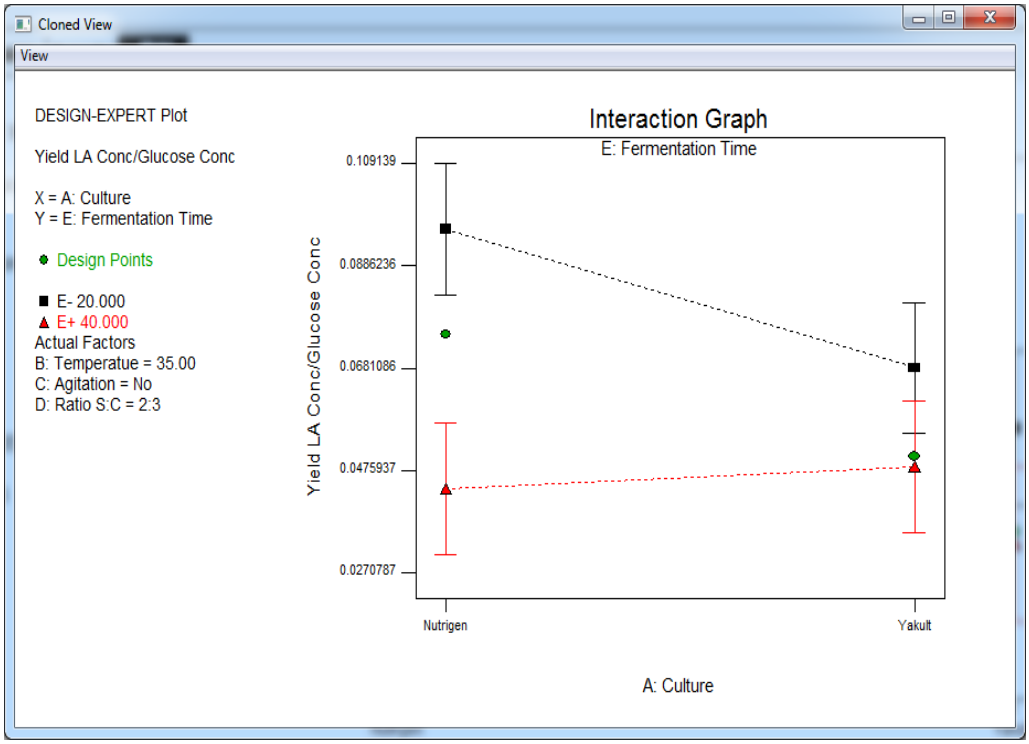


Figure 4.4 : The interaction graph between factor A, Source of Inoculums and factor E, Fermentation Time

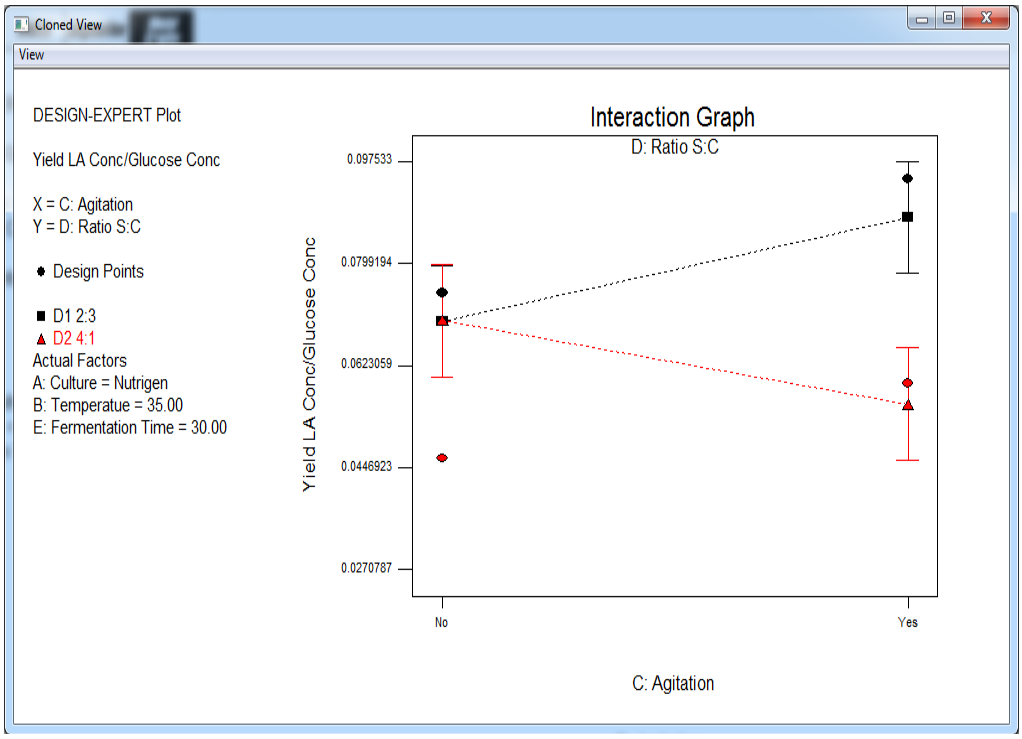


Figure 4.5 : The interaction graph between factor C, Agitation and factor D, Ratio of Substrate to Inoculums

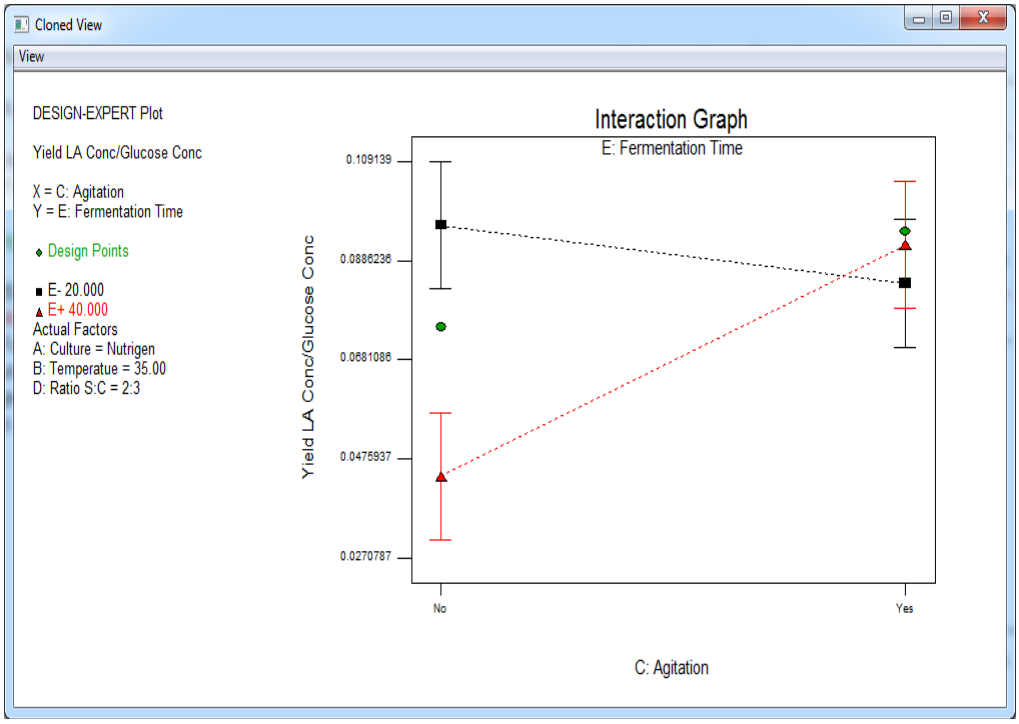


Figure 4.6 : The interaction graph between factor C, Agitation and factor E, Fermentation Time

4.6 Validation Experiment

Design Expert 6.0 software suggested a total of five validation runs in order to confirm the experiment result. According to Table 4.6, all five runs were predicted to produce different yield of lactic acid with their respective parameters. The lowest error was 8.3% where sample 3 yield 0.09 g/g of lactic acid whiles the predicted value was 0.10 g/g lactic acid. As a result, the best condition to produce lactic acid from banana stem waste was by using Nutrigen as inoculums, temperature of 39^{o}C, no agitation, the 4:1; ratio of substrate to inoculums and 20 hours of fermentation time. `

Table 4.5 : Validation Experiments

Culture	Temperature (^{o}C)	Agitation	Ratio S: C	Fermentation Time (hour)	Predicted LA Yield (mg/mg)	Experimental LA Yield (mg/mg)	Error (%)
Nutrigen	39.70	No	4:1	22.43	0.0995	0.0793	20.3
Nutrigen	37.96	No	4:1	20.64	0.0996	0.0644	35.3
Nutrigen	38.56	No	4:1	20.44	0.1009	0.0925	8.3
Nutrigen	39.47	No	4:1	21.23	0.1011	0.0882	12.8
Nutrigen	30.56	No	2:3	20.18	0.1018	0.0916	10.0

4.7 Comparison of Lactic Acid Production with other Researchers

Recently, 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 applied in bioprocess as an alternative way to replaced costly raw materials (Jawad *et al.,* 2013). In addition, agro-industrial waste such as barley, wheat, corn, sugarcane molasses, dates and more have been extensively studied for lactic acid production (Ohkouchi and Inoue, 2006; Tanaka *et al.,* 2006; Sreenath *et al.,* 2001; Chan-Blanco *et al.,* 2003).

Lactic acid fermentation is effected by several parameters such as temperature, agitation and fermentation time. The differences in lactic acid recovery during this study with that of some other researchers might be due to the variation in temperature, substrate levels, types of substrates and the selection of suitable culture for fermentation.

Ohkouchi and Inoue (2006) has been studied on several factors for direct and effective lactic acid production from food wastes by *Lactobacillus manihotivorans* LMG18011 and also optimum conditions to used food wastes as substrate. Different initial pH condition is used to identify the effect on lactic acid productivity. At the end of research, it is found that the residual sugar for pH5.0 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 is detected when using the temperature of 30^{o}C and at 48 hours fermentation time.

Tanaka *et al.,* (2006) reported that by using defatted rice bran, 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. Significantly, the highest lactic acid production took place after 36 hours of fermentation time with 37^{o}C temperature with agitation on. Throughout the research, solid state fermentation (SSF) process is used as the method to produced lactic acid and it has been proved that SSF method at low pH is applicable for microbial productions of other useful materials from agricultural by-products.

On the other hand, Sreenath *et al.,* (2001) investigated the production of lactic acid from alfalfa fibers by using pure culture of *Lactobacillus delbrueckii* strain. 0.35 g of lactic acid per gram of dry matter of alfalfa fibers was produced when the temperature used is 37^{o}C with agitation of 150 rpm at 47 hours of fermentation time. It is found that alfalfa fiber is an excellent substrate because it does not require any additional nutrient supplement along the

process to increase lactic acid production. *Lactobacillus delbrueckii* also suitable to used in microbial lactic acid fermentation due to their high yield and rate of production.

Chan-Blanco *et al.,* (2003) evaluated the usefulness of waste banana for generating lactic acid through batch fermentation, using *Lactobacillus casei* under three parameters. A Regular diluted banana puree is used as substrate and showed a 10% yield with maximum productivity of 0.10 g/g. Sugar concentration also showed a slow decreased over time, indicating that fermentation was indeed very slow and so, therefore, was the production of lactic acid. The result for the maximum yield of lactic acid is achieved at temperature of 37^{o}C and 70 hours of fermentation time.

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 in stirred or static fermentation conditions was evaluated (Plessas *et al.,* 2008). Mixed culture of lactic acid bacteria can be successfully used to enhance lactic acid production from cheese whey, thus minimize the cost for microorganism's growth and therefore lactic acid production. Highest yield (0.35 g/g) obtained when the temperature used was 37^{o}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^{o}C, no agitation used and 20 hours of fermentation time. The yield of lactic acid produced was 0.09 g/g, almost similar to Chan-Blanco *et al.,* (2003) and Ohkouchi and Inoue (2006) where the percentage error in lactic acid yield was only 0.1%. However, both of the study using pure culture to produce lactic acid while this research was using mixed culture in term of commercially probiotic milk drink. As we can see from Table 4.5, 0.35 g/g of lactic acid was obtained by using mixed culture and cheese whey (Plessas *et al.,* 2008). The difference in lactic acid yield between this research and Plessas *et al.,* (2008) were the substrate and also type of mixed culture used in the process.

Table 4.6 : Comparison for lactic acid production from agricultural source

Substrate	**Cultures**	**Temperature (^{o}C)**	**Agitation (rpm)**	**Fermentation Time (hours)**	**Lactic Acid** Y (g/g)	**Reference**
Food Wastes	Pure	30	-	48	0.10	Ohkouchi and Inoue (2006)
Defatted Rice Bran	Pure	37	Yes	36	0.28	Tanaka *et al.,* (2006)
Alfalfa fibers	Pure	37	150	47	0.35	Sreenath *et al.,* (2001)
Banana wastes	Pure	37	-	70	0.10	Chan-Blanco *et al.,* (2003)
Cheese Whey	Mixed	37	-	24	0.35	Plessas *et al.,* (2008)
This work	Mixed	39	-	20	0.09	-

5 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In this study, banana stem waste and probiotic milk drinks were investigated for its potential in producing lactic acid. Experimental runs showed that lactic acid was produced from banana stem waste by probiotic milk drinks over a period of 40 hours. The effect of temperature, source of inoculums, agitation, ratio substrate to inoculums and fermentation time were studied using the factorial design and the result obtained showed that all parameters do give a significant influence on the production process. Still, the source of inoculums affected the production of lactic acid the most by 1.07% of percentage contributions while the interaction of agitation and fermentation time gives an increase in lactic acid production.

In order to support the parameters that have been investigated, validation experiment will be run according to all the parameters. The total of runs and all the parameter 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%. Validation experiment showed and confirmed that the best conditions to produce lactic acid for this research were at temperature 39^{o}C, no agitation, 20 hours of fermentation time and only 8% error with predicted lactic acid yield of 0.1009 g/g and experimental lactic acid yield of 0.0925 g/g.

Previous study have mostly focused on producing lactic acid using pure culture, however this study clearly indicates that facultative anaerobic bacteria (mixed culture) can be used as the inoculums in fermentation process with selected parameters involved. 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.

As a conclusion, it is found that by using probiotic milk drinks as inoculums and banana stem waste as substrate, lactic acid can be produced. This finding 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. Further studies are needed to be done for extra evidence on the usage of probiotic milk drinks and banana stem waste in lactic acid fermentation process.

5.2 Recommendations

In conjuction with the result and conclusion obtained, the following recommendations were identified to further improve the future research in this field of study :

1. The optimization study will be done with the best factors in order to obtain the highest production of lactic acid.
2. Study on the optimization will be done to determine the best or optimize condition using banana stem waste and probiotic milks to get the highest amount of lactic acid.

REFERENCES

Abdel-Rahman, M. A., Tashiro, Y., & Sonomoto, K. (2013), "Recent Advances in Lactic Acid Production by Microbial Fermentation Processes", *Biotechnology Advances*, pp. 877-902.

Alwi, H., Idris, J., Musa, M., & Hamid, K. H. K. (2013), "A Preliminary Study of Banana Stem Juice as a Plant-Based Coagulant for Treatment of Spent Coolant Wastewater", *Hindawi Publishing Corporation*, Vol. 2013, pp. 7.

Annuar, M. S. M., Tan, I. K. P., & Ramachandran, K. B. (2008), "A Kinetic Model for Growth and Biosynthesis of Medium-Chain-Length Poly-(3-Hydroxyalkanoates) in Pseudomonas putida*," Brazilian Journal of Chemical Engineering,* Vol. 25, pp. 217 - 228.

Bai, D. M., Jia, M. Z., Zhao, X. M., Ban, R., Shen, F., Li, X. G. & Xu, S. M., (2003), " L(+)-Lactic Acid Production by Pellet-form *Rhizopus oryzae* R1021 in a Stirred Tank Fermentor", *Chemical Engineering Sciences*, Vol. 58, pp. 785-791.

Brooijmans, R. J. W. (2008), "Electron Transport Chains of Lactic Acid Bacteria", *Applied and Environmental Microbiology*.

Bilanović D., Chang F. H., Isobaev P., & Welle P. (2011),"Lactic Acid And Xanthan Fermentations on an Alternative Potato Residues Media – Carbon Source Costs, *Biomass and Bioenergy*, Vol. 35, pp. 2683-2689.

Bishai, M., De, S., Adhikari, B. & Banerjee, R., (2013), "Zizyphus oenophlia : A potent for lactic acid production, *Bioresource Technology*, Vol. 133, pp. 627-629.

Boontawan, P., (2010), "Development of lactic acid production from cassava by using lactic acid bacteria", Suranaree University of Technology, Thailand.

Bulut, S., Elibol, M., & Ozer, D. (2004), "Effect of different carbon sources on L(+)-lactic acid production by Rhizopus oryzae", *Biochem. Eng. J.*, Vol. 21, 33–37.

Busairi, A. M. (2004), "Recycling of pineapple waste using *Lactobacillus Delbroeckii* to lactic acid", *Gema Teknologi*, Vol. 16, No. 1.

Carlson, T. L., & Peter, E. M. (2002)., "Low pH lactic acid fermentation", *Cargill, Inc*., Wayzata, MN.

Chan-Blanco, Y., Bonilla-Leiva, A. R., & Velazquez, A. C. (2003), "Using banana to generate lactic acid through batch process fermentation", *Appl Microbiol Biotechnol*, Vol. 63, pp. 147–52,

Choudhury, N., Hansen, W., Engesser, D., Hammes, W.P.and Holzapfel, W.H. (1990) Formation of histamine and tyramine by lactic acid bacteria in decarboxylase assay medium. *Lett Appl Microbiol*, Vol. 11,pp. 278–281.

Coelho, L. F., Christian, J. B., & Contiero, J. (2010), "The use of response surface methodology in optimize of lactic acid production : Focus on medium supplement, temperature and pH control", *Food Technol. Biotechnol,* Vol. 48, No. 2, pp. 175-181.

Cui F., Li Y., & Wan C. (2011), "Lactic acid production from corn stover using mixed cultures of Lactobacillus rhamnosus and Lactobacillus brevis", *Bioresour Technology*.

Demirtas, M. U., Kolhatkar, A. & Kilbane II, J. J. (2003), "Effect of aeration and agitation on growth rate of Thermus thermophilus in Batch Mode", *Journal of Bioscience and Bioengineering*, Vol. 95, pp. 113-117.

Djukic-Vukovic, A. P., Mojovic, L. V., Vukasinovic-Sekulic, M. S., Rakin, M. B., Nikolic, S. B., Pejin, J. D. & Bulatovic, M. L., (2012), "Effect of different fermentation parameters on L-lactic acid production from liquid distillery stillage", Food Chemistry, Vol. 134, pp. 1038-1043.

Elmarzugi, N., El Enshasy, H., Malek, R. A., Othman. R., Sarmidi, M. R., & Aziz, R. A. (2010), "Optimization of cell mass production of the probiotic strain *Lactococcus lactis* in batch and fed-bach culture in pilot scale levels", *Technology and education topic in applied microbiology and microbial biotech*.

Gao, T. & Ho, K., (2013), "L-lactic acid production by *Bacillus subtilis* MUR1 in continuous culture", *Journal of Biotechnology*, Vol. 168, pp. 646-651.

Hofvendahl, K. & Hahn-Hagerdal, B., (2000), "Factors affecting the fermentative lactic acid production from renewable resources", *Enzyme and Microbial Technology*, Vol. 26, pp. 87-107.

Holzapfel, W. H., Geisen, R. & Schillinger, U. (1995), "Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzyme", *International Journal of Food Microbiology, Vol 24*, pp. 343-362,.

Farooq, U., Anjum, F. M., Zahoor, T., Sajjad-Ur-Rahman, Randhawa, M. A., Ahmed, A. & Akram, K. (2012), " Optimization of lactic acid production from cheap raw material : sugarcane molasses", *Pak. J. Bot*, Vol. 44(1), pp. 333-338.

Feriotti, D. G. & Iguti, A.M. (2011), "Proposal for Use of Pseudostem from Banana Tree", *Maua Institute of Technology*, pp 1-5.

Goksungur, Y. & Guvenc, U. (1997), "Batch and Continuous Production of Lactic Acid from Beet Molasses by *Lactobacillus delbrtueckii*.", *J. Chem. Tech. Biotechnology*, Vol. 69, pp.399-404.

Golshani, T, Jorjani, E., Chelgani, S. C., Shafaei, S. Z., & Nafechi, Y. H. (2013), "Modelling and process optimization for microbial desulfurization of coal by using a two-level full factorial design, *International Journal of Mining Science and Technology*, Vol. 23, pp. 261-265.

Gunduz, M. (2005), "Lactic Acid Production By *Lactobacillus Casei NRRL* B-441 Immobilized In Chitosan Stabilized Ca-Alginate Beads", *Izmir Institute of Technology,* pp. 26.

Hujanen, H. & Y.Y, Linko, Y.Y., (1996), " Effect of Temperature and Various Nitrogen Sources on Lactic Acid Production by Lactobacillus caseii", *Microbiol Biotechnol.*,45, pp 307-313.

Idris, A. & Suzana, W., (2006), "Effect of sodium alginate concentration, bead diameter, initial pH and temperature on lactic acid production from pineapple waste using immobolized *Lactobacillus delbrueckii*", *Process Biochemistry,* Vol. 41, pp. 1117-1123.

Jawad, A. H., Alkarkhi, A. F. M., Jason, O. C., Easa, A. M. & Norulaini, N. A. N. (2013), "Production of lactic acid from mango peel waste – Factorial experiment", *Journal of King Saud University – Science,* Vol. 25, pp. 39-45.

John, R.P., Nampoothiri, K. M. & Pandey, A. (2007), "Production of L-(+) lactic acid from cassava starch hydrolyzate by immobilized *Lactobacillus delbrueckii*.", *J Basic Microbiol*, Vol. 47, pp. 25–30.

Kashket, E. R., (2006), "Bioenergetics of lactic acid bacteria : cytoplasmic pH and osmotolerance", *FEMS Microbiology Letters*, Vol. 46, pp. 233-344.

Khalil, M., Darusman, L. K., & Syafitri, U. D. (2011), "Application of fractional factorial design to optimize extraction method of artemisinin from *Artemisia annua*", *Science Asia*, Vol. 37, pp. 219-224.

Kiew, P. L., & Mat, D. M. (2013), "Screening of significant factors in collagen extraction from hybrid *Clarias* sp. using a statistical tool", *International Food Research Journal*, Vol. 20(4), pp. 1913-1920,.

Kohajdova, Z. & Karovicova, J., (2005), "Sensory and chemical evaluation of lactic acid-fermented cabbage-onion juice", *Chem. Pap,* Vol. 59, pp. 55-61.

Konings, W. N. (2000), "Lactic acid bacteria: The bugs of new millennium. Current Opinions inMicrobiology" Vol. 3, pp. 276-282.

Lee, K. (2004), "Comparison of fermentative capacities of *Lactobacilli* in single and mixed culture in industrial media", *Process Biochem.,* Vol. 40, pp. 1559–1564.

Litchfield, J. H. (1996), "Microbiological production of lactic acid. In: Neidelman SL and Laskin AI (Eds.) Advances in Applied Microbiology", Vol. 42, pp. 45–95.

Martinez, F. A. C., Balciunas, E. M., Salgado, J. M., González, J. M. D., Converti, A. &7 Oliveira, R. P. (2013), "Lactic acid properties, applications and production: a review. Trends *Food Sci Technol*, Vol. 30, pp. 70–83.

Mercier, P. & Yerushalmi, L. (1992), "Kinetics of Lactic Acid Fermentation on Glucose and Corn by L. amylophillus", *J. Chem. Tech. Biotechnology,* Vol. 55, pp. 111-121.

Mohapatra, D., Mishra, S., & Sutar, N. (2010), "Banana and its by-product utilization : an overview", *Journal of Scientific & Industrial Research,* Vol. 69, pp. 323-329.

Moon, N. J. & Reinbold, G. W. (1976), "Commensalism and competition in mixed cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*", *J. Milk Food Technol.,* Vol. 39, pp. 337-341.

Mudaliyar, P. & Kulkarni, L. S. C. (2011), "Food waste management-lactic acid production by *Lactobacillus* species", *International Journal of Pharmaceutical & Biological Achieves*, Vol. 1, No. 1, pp. 52-56.

Mujtaba, I. M., Edreder, E. A. & Emtir, M. (2010), "Optimization of batch reactive distillation process: production of lactic acid", *Computer Aided Chemical Engineering*, Vol. 27, pp. 1793-1798.

Nagarjun, P. A., Rao, R. S., Rajesham, S., & Rao, L. V. (2005), "Optimization of lactic acid production in SSF by *Lactobacillus amylovorus* NRRL B-4542 using Taguchi methodology", *The Journal of Microbiology*, pp 38-43.

Naranong, N. & Poochareon, D. (2001), "Production of L-Lactic Acid from Raw Cassava Starch by Rhizopus oryzae NRRL 395", *Kasetsart J. (Nat. Sci.),* Vol. 35, pp. 164 – 170.

Nancib, A., Nancib, N., & Boudrant, J. (2009), "Production of lactic acid from date juice extract with free cells of single and mixed cultures of Lactobacillus casei and Lactococcus lactis", *World J. Microbiol. Biotechnol*.

Oh, H., Wee, Y. J., Yun, J. S., Ho Han, S., Jung, S., & Ryu, H. W. (2005), "Lactic acid production from agricultural resources as cheap raw materials", *Bioresource Technology*, Vol. 96(13), pp. 1492-1498.

Ohkouchi Y, & Inoue Y. (2006), "Direct production of L-(+)-lactic acid from starch and food wastes using *Lactobacillus manihotivorans* LMG18011", *Bioresource Technology,* Vol. 97, pp. 1554–1562.

Ozalp N. & Hyman B. (2009),"Energy Allocation And Energy Intensity Estimates For The U.S. Organic Chemicals Industry", *Chemical Engineering Transactions,* Vol. 18, pp. 531-536.

Patil, S. S., Kadam, S. R., Bastawde, K. B., Khire, J. M. & Gokhale, D. V. (2006), "Production of lactic acid and fructose from media with cane sugar using mutant of *Lactobacillus delbrueckii* NCIM 2365", Letters in Applied Microbiology.

Palaniraj, R. & Nagarajan, P., (2012), " Kinetic studies in production of lactic acid from waste potato starch using Lactobacillus casei", *International Journal of ChemTech Research*, Vol. 4, pp. 1601-1614.

Plessas, S., Bosnea, L., Psarianos, C., Koutinas, A. A., Marchant, R., & Banat, M. (2008), "Lactic acid production by mixed cultures of *Kluyveromyces marxianus*, *Lactobacillus delbrueckii ssp. bulgaricus* and *Lactobacillus helveticus", Bioresource Technology*, Vol. 99, pp. 5951–5955.

Ryu, H.W., Yun, J.S., & Wee, Y.J. (2003), " Lactic acid", *Concise Encyclopedia of Bioresource Technology*. (Ed), pp. 635.

Salunkhe & Kadam, S. S., (1995), "Minor fruits-Tropical, In : Hankbook of fruit Science and Technology. Production, composition, storage and processing", *Marcel Dekker, Inc*., pp.563-591.

Sanders, M. E. (1999), "Probiotics", *Food Technology*, Vol. 53, pp. 67-75.

Sheeladevi, A. & Ramanathan, N. (2011), "Lactic acid production using lactic acid bacteria under optimized condition", *International Journal of Pharmaceutical & Biological Achieves*, *Vol. 2*, No. 6, pp. 1686-1691.

Sinha, N., Sidhu, J., Barta, J., Wu, J., & Cano, M. P. (2012), "Handbook of Fruits and Fruits Processing", *Technology & Engineering*, pp. 748.

Sreenath, H. K., Moldes, A. B., Koegel, R. G., & Straub, R. J. (2001), "Lactic acid production by simultaneous saccharification and fermentation of alfalfa fiber. *J Biosci Bioeng*, Vol. 92, pp. 518–23.

Tanaka, T., Hoshina, M., Tanabe, S., Sakai, K., Ohtsubo, S., & Taniguchi, M. (2006), "Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation", *Bioresource Technology*, Vol. 97, pp. 211–217.

Tango, M. S. A. & Ghaly, A. E., (1999), "Effect of temperature on lactic acid production from cheese whey using Lactobacillus helveticus under batch conditions", *Biomass and Bioenergy*, Vol. 16, pp. 61-78.

Taniguchi, M., Tokugana, T., Horiuchi, K., Hoshino, K., & Tanaka, T. (2004), "Production of L-lactic acid from a mixture of xylose and glucose by co-cultivation of lactic acid bacteria, *Appl. Microbial. Biotechnology*, Vol. 66, pp. 160-165.

Taskila, S. & Ojamo, H., (2013), "The Current Status and Future Expectations in Industrial Production of Lactic Acid by Lactic Acid Bacteria", University of Oulu, Finland.

Thongheam, R., Thongjub, A., Malaphan, W., & Chim–anage, P. (2008), "Probiotic Production by Mixed Culture of Lactic Acid Bacteria and Yeast", *Kasetsart J. (Nat. Sci.)*, Vol. 42,pp. 277 - 284.

Tinoco-Valencia, R., Gomez-Cruz, C., Galindo, E. & Serrano-Carreon, L., (2014), "Toward an understanding of the effect of agitation and aeration on growth and laccases production by *Pleurotus ostreatus*, *Journal of Biotechnology*, Vol. 177, pp. 67-73.

Trontel, A., Batusic, A., Gusic, I., Slavica, A., Santek, B., & Novak, S. (2011)," Production of D- and L-Lactic acid by Mono- and Mixed culture of Lactobacillus sp.", *Food. Technol. Biotechnol.,*Vol. 49(1), pp. 75-82.

Vodnar, D. & Carmen, S. (2008), "Comparative Analysis Of Lactic Acid Produced By Apple Substrate Fermentation, Using Hplc And Tectronik Senzytec Biosensor", *Bulletin UASVM, Agriculture*, Vol. 65(2).

Wee, Y. J., Kim, J. N., & Ryu, H. W. (2006), " Biotechnology Production of Lactic Acid and its Recent Applications", *Food Technol. Biotechnol.* Vol. 44 (2), pp. 163–172.

Xu, H., Jaynes, J. & Ding, X. (2012), "Combining Two-Level and Three-Level Orthogonal Arrays for Factor Screening and Response Surface Exploration", *Statistica Sinica*.

Yoon, K. Y., Woodams, E. E., & Hang, Y. D. (2004), "Probiotication of Tomato Juice by Lactic Acid Bacteria", *The Journal of Microbiology*, Vol. 42, pp. 315-318.

APPENDICES

1) Analysis for Lactic Acid Content

Table 6.1 : HPLC Reading for Standard Curve Lactic Acid

No.	Concentration [mg/ml]	Retention Time [min]	Area [mAU*s]	Height [mAU]	Equation
0	0	0	0	0	
1	0.01	3.526	3638.7745	375.4350	
2	0.05	3.586	1.13E+04	768.4805	
3	0.1	3.617	1.76E+04	1018.7617	
4	0.15	3.630	2.23E+04	1347.6160	y = 5860.x
5	0.2	3.630	6.00E+04	1653.6540	
6	0.3	3.626	7.49E+04	2186.3164	
7	0.4	3.621	8.16E+04	2415.0530	
8	0.45	3.614	7.73E+04	2642.7183	
9	0.5	3.611	7.76E+04	2759.0051	
10	0.6	3.598	7.49E+04	2998.9448	

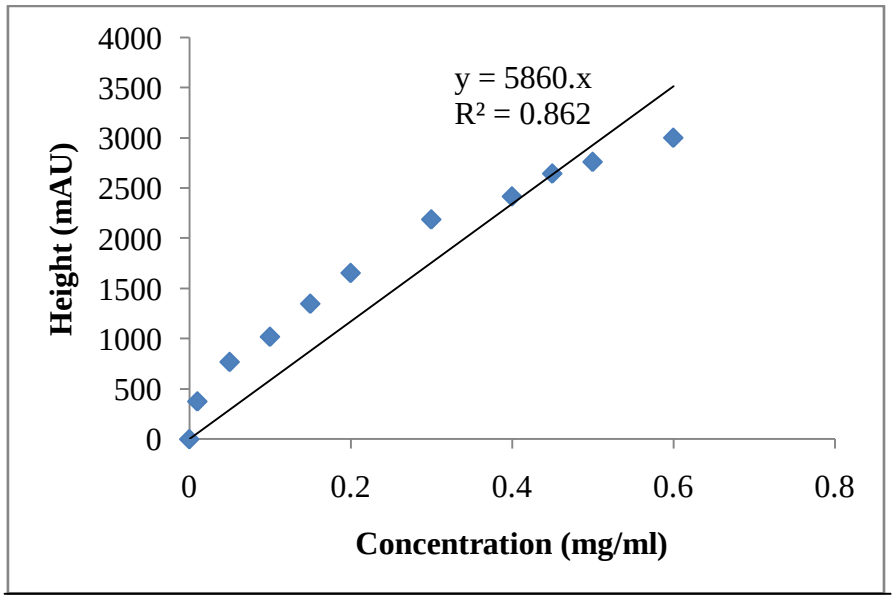


Figure 6.1: Standard curve for Lactic Acid

2) Analysis for 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 OD Reading
STANDARD				
0	0	0	0	0
0.05	0.038	0.037	0.036	0.0370
0.10	0.099	0.100	0.105	0.1013
0.15	0.156	0.156	0.157	0.1563
0.20	0.178	0.179	0.180	0.1790
0.25	0.381	0.383	0.383	0.3823
SAMPLE				
Banana Stem Waste (A)	0.962	0.814	0.817	0.8643
CONCENTRATION A (mg/ml)	**0.7126**			

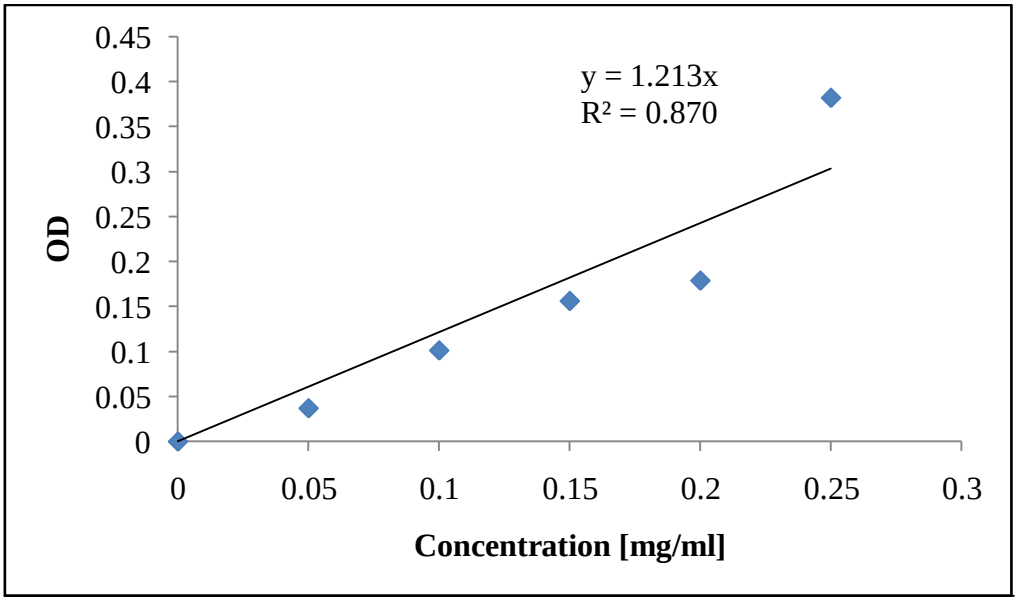


Figure 6.2 : Standard curve for glucose

$$Yield = \frac{\text{Lactic Acid Concentration } (\frac{\text{mg}}{\text{ml}})}{\text{Glucose Concentration } (\frac{\text{mg}}{\text{ml}})}$$

Table 6.3 : Experimental Runs

No.	Symbol	Inoculums	Temperature (^{o}C)	Agitation	Ratio Substrate to Inoculums	Fermentation time (hours)
1	B1	Nutrigen	35	Yes	4 : 1	30
2	B2	Nutrigen			2 : 3	
3	B3	Yakult			4 : 1	
4	B4	Yakult			2 : 3	
5	C1	Nutrigen	35	No	4 : 1	30
6	C2	Nutrigen			2 : 3	
7	C3	Yakult			4 : 1	
8	C4	Yakult			2 : 3	
9	D1	Yakult	30	Yes	4 : 1	20
10	D2	Nutrigen			2 : 3	
11	D3	Nutrigen			4 : 1	40
12	D4	Yakult			2 : 3	
13	E1	Nutrigen	30	No	4 : 1	20
14	E2	Yakult			2 : 3	
15	E3	Nutrigen			2 : 3	40
16	E4	Yakult			4: 1	
17	G1	Yakult	40	Yes	2 : 3	20
18	G2	Nutrigen			4: 1	
19	G3	Nutrigen			2 : 3	40
20	G4	Yakult			4: 1	
21	H1	Nutrigen	40	No	2 : 3	20
22	H2	Yakult			4: 1	
23	H3	Nutrigen			4 : 1	40
24	H4	Yakult			2 : 3	

Table 6.4 : HPLC reading for samples

No.	Samples	Retention Time [min]	Area [mAU*s]	Height [mAU]	Lactic Acid Concentration [mg/ml]	Yield LA Conc/Glucose Conc (mg LA/mg Glucose)
1	B1	3.520	2699.9627	312.6120	0.0533	0.0749
2	B2	3.511	1842.1658	247.6794	0.0423	0.0593
3	B3	3.532	3840.5110	395.1809	0.0674	0.0946
4	B4	3.529	3709.0593	379.9193	0.0648	0.0910
5	C1	3.527	3504.2707	367.2063	0.0627	0.0879
6	C2	3.507	2002.8046	249.2499	0.0425	0.0597
7	C3	3.520	3013.2912	339.5533	0.0579	0.0813
8	C4	3.520	3334.8599	357.0967	0.0609	0.0855
9	D1	3.500	1450.5134	207.0873	0.0353	0.0496
10	D2	3.499	1.53E+03	210.5646	0.0359	0.0504
11	D3	3.482	808.2874	129.1165	0.0220	0.0309
12	D4	3.519	3270.1760	351.9718	0.0600	0.0843
13	E1	3.484	697.1729	113.1444	0.0193	0.0271
14	E2	3.515	3203.2788	344.5645	0.0588	0.0825
15	E3	3.490	1395.9870	193.4455	0.0330	0.0463
16	E4	3.503	2459.2878	291.4814	0.0497	0.0698
17	G1	3.511	3209.8567	345.5598	0.0590	0.0827
18	G2	3.473	1040.6790	149.1752	0.0254	0.0357
19	G3	3.478	2079.2251	211.2080	0.0360	0.0506
20	G4	3.509	3293.4314	336.3355	0.0574	0.0805
21	H1	3.485	2012.7500	220.3866	0.0376	0.0528
22	H2	3.503	2920.0308	321.1537	0.0548	0.0769
23	H3	3.505	4035.7071	368.2902	0.0628	0.0882
24	H4	3.510	4257.8193	394.7785	0.0674	0.0945

Table 6.5 : Suggested Validation Experiments

No.	Culture	Temperature (^{o}C)	Agitation	Ratio Substrate : Culture	Fermentation Time (hour)	Yield LA Conc/ Glucose Conc (mg/mg)
1	Nutrigen	39.70	No	4:1	22.43	0.0995
2	Nutrigen	37.96	No	4:1	20.64	0.0996
3	Nutrigen	38.56	No	4:1	20.44	0.1009
4	Nutrigen	39.47	No	4:1	21.23	0.1011
5	Nutrigen	30.55	No	2:3	20.18	0.1018

Table 6.6 : HPLC Reading for Samples with Error between Predicted and Experimental Lactic Acid Yield

Samples	Retention Time [min]	Area [mAU*s]	Height [mAU]	Lactic Acid Concentration [mg/ml]	Yield LA Conc/Glucose Conc (mg LA/mg Glucose)	Expected Yield	Error (%)
1	3.434	1008.7243	88.4965	0.0538	0.0793	0.0995	20.25
2	3.374	819.2346	78.4542	0.0437	0.0644	0.0996	35.33
3	3.418	1176.9862	111.5159	0.0627	0.0925	0.1009	8.26
4	3.441	1121.3721	94.2364	0.0598	0.0882	0.1011	12.78
5	3.451	1164.4634	111.9930	0.0621	0.0916	0.1018	10.04