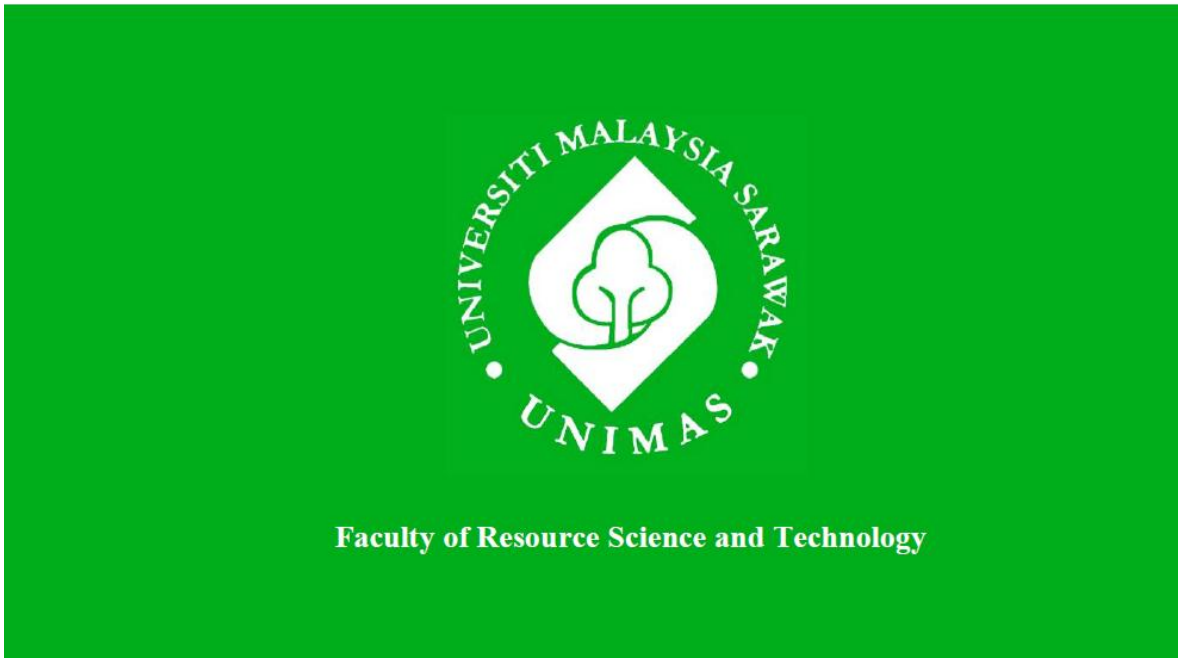

MATHEMATICAL MODELLING ON THE GROWTH OF THERMOPHILIC LACTIC ACID BACTERIA IN MOLASSES

Thian Sing Kit
(39152)

Bachelor of Science with Honours
(Resource Biotechnology)
2015

MATHEMATIC MODELING ON THE GROWTH OF THERMOPHILIC LACTIC ACID BACTERIA IN MOLASSES

Thian Sing Kit

(39152)

A final project report submitted in partial fulfilment of the requirement for the degree of Bachelor Science with Honours (Resource Biotechnology)

Supervisor: Associate Professor Dr. Cirilo Nolasco Hipolito

Resource Biotechnology
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2015

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to my supervisor, Associate Professor Dr. Cirilo Nolasco Hipolito for his dedicated guidance, supervisions and advices throughout this project. He constantly and convincingly conveyed a spirit of adventure in research and encouraged me to learn new things. Without his persistent guidance, this project would not have been possible.

I am grateful to my friends of the Biochemistry Laboratory, for companionship, friendship and assistance. I would like to acknowledge the support of Universiti Malaysia Sarawak for providing facilities and equipment for conducting the research study.

I thank my family, for always being my support, foundation and motivation to continue even in difficult times. Their encouragements have kept me going through tough times during the project.

I also take this opportunity to thank all who, in any way, have lent their helping hand in this venture.

DECLARATION OF ORIGINAL WORK

This declaration is made on the ……..day of……..2015.

Student's Declaration:

I **Thian Sing Kit, 39152, Faculty of Resource Science and Technology** hereby declare that the work entitled "**Mathematical Modelling on the Growth of Thermophilic Lactic Acid Bacteria in Molasses"** is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

_____ **Thian Sing Kit (39152)**

Date submitted

TABLE OF CONTENTS

Page

LIST OF ABBREVIATIONS

LAB	lactic acid bacteria
LA	lactic acid
DCW	dry cell weight
DNS	dinitrosalicylic acid
OD	optical density
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})
X	biomass concentration (g/l)
α	growth associated constant in Leudeking-Piret model (g/U.O.D.)
β	non-growth associated constant in Leudeking-Piret model (g/U.O.D./h)
P	product formation (g/l)
t	time (h)
S	substrate concentration (g/l)
K_S	substrate saturation constant (g/l)

LIST OF TABLES

LIST OF FIGURES

MATHEMATICAL MODELING ON THE GROWTH OF THERMOPHILIC LACTIC ACID BACTERIA IN MOLASSES

Thian Sing Kit
(39152)

Resource Biotechnology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

A recently isolated lactic acid bacteria (LAB) from the Biochemistry Laboratory of UNIMAS that is capable to produce lactic acid (LA) from carbon source by fermentation under high temperature was studied. The strain was used for LA production from molasses at 20 g/l, 40 g/l and 60 g/l substrate concentration under controlled pH at 6.0 and temperature at 45 °C. Molasses is an abundant and low-cost bio-resources from the sugar mill industry and contain fermentable sugars. In this study, a mathematical model was generated to describe the batch fermentation of molasses to LA with the LAB strain, with the bacterial density, LA production and residual sugar as responses. The Monod equation was applied to describe the bacterial growth, while the Luedeking-Piret equation was employed to describe the production of LA. The fermentations produced a maximum specific growth rate, μ_{max} of 0.381 h^{-1} and substrate saturation constant of 13.81 g/l. The model depicted that the production of LA from molasses medium was related to the rate of bacterial growth and the quantity of bacteria present. The model could be used to study and optimize the growth and production of lactic acid using the LAB in molasses medium fermentation system.

Key words: Lactic acid bacteria (LAB); Batch fermentation; Molasses; Monod equation; Luedeking-Piret equation

ABSTRAK

Kajian telah dilakukan pada bakteria asid laktik (LAB) yang baru diperoleh dari Makmal Biokimia UNIMAS. LAB tersebut dapat menghasilkan asid laktik daripada sumber karbon dalam suhu yang tinggi dan telah digunakan untuk penghasilan asid laktik daripada molasses dengan kepekatan substrat 20 g/l, 40 g/l dan 60 g/l dalam keadaan pH pada 6.0 serta suhu pada 45 ° C. Molases mempunyai kos yang rendah dan banyak dihasilkan daripada industri penghasilan gula serta mengandungi gula beragi. Dalam kajian ini, model matematik telah dijana untuk menjelaskan penapaian molases kepada asid laktik oleh LAB tersebut, dengan pertumbuhan bakteria, pengeluaran asid laktik dan gula sebagai jawapan. Kinetika Monod telah digunakan untuk menghuraikan pertumbuhan bakteria, manakala kinetika Luedeking-Piret telah digunakan untuk menjelaskan pengeluaran asid laktik. Kadar pertumbuhan spesifik maksimum, iaitu μ_{max} bernilai 0.38 h^{-1} dan konstanta saturasi substrat 13.81 g/l telah dihasilkan dalam eksperimen fermentasi yang dijalankan. Kinetika ini menunjukkan penghasilan asid laktik daripada molases adalah berkait dengan faktor kadar pertumbuhan bakteria dan kuantiti bakteria. Model ini dapat digunakan untuk mengkaji dan mengoptimumkan pertumbuhan dan penghasilan asid laktik dengan menggunakan LAB dalam molasses melalui sistem fermentasi sederhana.

Kata kunci: bakteria asid laktik (LAB); fermentasi sederhana; molases; Kinetika Monod; Kinetika Luedeking-Piret

1 INTRODUCTION

Lactic acid ($CH_3CHOHCOOH$) (LA) is a carboxylic acid commonly used in the production of food, pharmaceutical, cosmetic and various chemical industries (Wee *et al.*, 2006). Biotechnological fermentation or chemical synthesis is applied in both food and non-food production including LA. Due to the concerns towards the environment and abrupt decrease in petrochemical supplies, biotechnological fermentation methods are proven to be more superior in producing LA with reduced cost using inexpensive alternative raw materials (Dumbrepatil *et al.*, 2008).

Currently, biodegradable polylactic acid (PLA) plastics, which is also known as "green plastics" has become the present interest of substantial commercial industries. PLA production utilizes renewable resources for instance starch, and could be a replacement for synthetic plastic (Gavrilescu & Chisti, 2005). The production of the LA involves natural feedstocks such as starch and cellulose and pure substrates like glucose and lactose. However, these substrates are sometimes less favourable as high expenses and complex pretreatment process are required (Dumbrepatil *et al.*, 2008). In order to minimize the manufacturing cost, waste products containing fermentable sugars are utilized to produce lactic acid. One of the methods is using molasses generated as a by-product in the production of sucrose from sugar cane. Microorganisms with fermentative ability can be employed to ferment the sucrose in molasses to yield lactic acid.

Lactic acid bacteria (LAB), like *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Pediococcus* are fermentative bacteria which efficiently use nutrients in the environment and produce LA as the main end-product and a variety of other compounds from fermentation. LABs are classified biochemically into two groups: 1) homofermenters that produce mainly

lactic acid, and 2) heterofermenters that are capable of producing other compounds from fermentation such as carbon dioxide, acetic acid, ethanol, and formic acid (Mozzi *et al.*, 2010).

In this study, a newly isolated LAB was employed for microbial fermentation. The LAB strain is found to be a thermophile that could grow and produce LA in high temperature. Due to its ability to produce lactic acid, the growth and fermentative ability of the LAB in molasses media have to be studied.

In order to describe the growth kinetics of microorganism in quantitative terms, the significant parameter has to be selected, for instance, the rate of product formation. The significant parameters involved include many environmental factors like pH, temperature, oxygen supply, and inhibitory by-products (El-Mansi, 1999). Hence, mathematical modelling is an expression for the representation of the growth kinetics and growth dynamics of the microorganism of interest in mathematical terms.

The main aim of this study is to develop a mathematical model that describes the growth of the LAB in molasses media. The objectives of this study are to:

I. Observe the growth pattern of the thermophilic LAB in molasses media.

II. Produce LA through fermentation utilising the thermophilic LAB in molasses media and determine the concentration of LA produced.

III. Perform mathematical modelling on the growth of the thermophilic LAB in molasses media.

2 LITERATURE REVIEW

2.1 Thermophilic Lactic Acid Bacteria

Lactic acid bacteria (LAB) are widely used as starter cultures in the manufacturing industries such as dairy products, meat processing, and alcoholic beverages production. Some examples of LAB are *Enterococcus, Lactococcus, Lactobacillus and Streptococcus.* LAB are generally characterized as Gram-positive rods or cocci, range from aerobic to facultative anaerobic (Mayo *et al*., 2010). They can be found in nutrient-rich environments, especially in food and dairy products.

The choice of LAB employed in fermentation depends largely on the objectives of the project and types of carbohydrates to be fermented, as LAB are classified into two groups related to the fermentative metabolism, namely homofermentative and heterofermentative. Homofermentative LAB mainly produce LA from glucose, whereas heterofermentative LAB produce LA and a considerable amount of acetic acid, ethanol and carbon dioxide apart from LA (Mayo *et al.*, 2010). LAB are also chosen based on their ability to ferment specific types of carbohydrates.

Thermotolerant LAB strains that produce LA and able to resist high temperature was used in this study. The isolated LAB can survive and produce lactic acid under the regulated temperature at 45 °C. According to Perez-Chabela *et al*. (2008), thermotolerant LAB are important in food production for their ability to prevent spoilage from other microorganisms or pathogens, extend shelf-life of food products. These thermotolerant strains can survive through heat processing, and become dominant during the cold storage of meat products. Thus, thermotolerant LAB can act like bio-preservatives.

2.2 Lactic acid

LA is commodity industrially important, especially in food industries such as the production of dairy milk, cured meat, and stearoyl lactylate which is used in the baking industry (Mozzi, 2010). It is a natural acidulant, preservative and antimicrobial agent in food. Although primarily used in the food industry, LA is also used as a feedstock for the polylactic acid (PLA) production, which is required to produce biodegradable plastics (Gavrilescu & Christi, 2005).

The LA molecule has an asymmetrical carbon, where the optically pure L- or D-lactic acids are the criteria needed for PLA polymerization process (Dumbrepatil *et al*., 2008). These isomers are identical in physical and chemical properties, but differ in optical characteristics. L(+)- lactic acid can be metabolized by the human body, thus are relatively important and high-demanding for biomaterial field (Adeva *et al*., 2013).

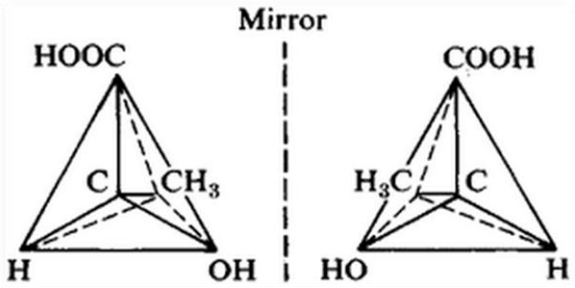

Figure 2-1. Mirror images of LA isomers (*isomerism*, n.d.)

Due to a racemic mixture of L- and D- lactic acids that are frequently resulted from chemical synthesis, microbial fermentation is applied in the manufacturing of almost 90% of the global production of LA (Dumbrepatil *et al.*, 2008). Several advantageous have attracted the global market for LA manufacturing via fermentation other than chemical synthesis, specifically for its use of renewable and low cost raw materials and low energy

consumption due to milk operation conditions (Komesu *et al.*, 2014). Different types of LA, namely L-, D-, and DL- lactic acids can be produced depending on the strain employed for fermentation. Microbial LA production from several renewable substrates such as cheese whey and wheat starch had been studied by Li and Cui (2010).

2.3 Molasses

The agro-industry contributes to considerable amount of waste, which causes a significant loss of valuable materials every day; this includes materials that can be reused in another production system. Similarly, molasses is a viscous by-product generated from the sugar manufacturing process and contains about 40% to 60% sucrose that is fermentable into LA by microorganisms (Dumbrepatil *et al.*, 2008). From an economic point of view, molasses is an inexpensive raw material to reduce the cost of LA production. Some common types of molasses include cane molasses and sugar beet molasses.

Cultivation of lactic acid bacteria requires complex nutrients, for instance, amino acids and vitamins are necessary for cell growth. Hence, yeast extract and vitamin B complex are commonly added to the culture media as nitrogen source and supplement respectively (Dumbrepatil *et al.*, 2008). In a study concerning molasses media, a small quantity of yeast extract is required, as there is sufficient nitrogen source present in molasses necessary for growth. Molasses also contain various non-sugar substances such as inorganic salts, raffinose, kestose, organic acids and nitrogen containing compounds (Elena *et al.*, 2009).

According to Dumbrepatil *et al.* (2008), research had demonstrated that fermentation of cane sugar molasses by *Lactobacillus delbrueckii* gives high productivity of lactic acid at 4.15 g/l/h.

2.4 Fermentation Process

In the study of microbial physiology, synchronous growth was induced by the operations of microbial fermentations (Waites *et al.*, 2001). This is done to prevent asynchronous growth, whereby cells do not divide at exactly at the same rate or react independently according to environmental conditions, which if happens, may influence the study of microbial population and biomass concentration.

Microbial fermentations can be conducted as a batch, fed-batch, and continuous operations (Waites *et al.*, 2001). Batch culture is a closed culture system provided with initial, restricted starting ingredients, which are given in a fixed volume at the beginning of fermentation. For regulation, acids or bases for pH control, or gases may be added. On the contrary, fed-batch fermentations involve additions of fresh medium or medium components continuously or intermittently, and the volume of the batch rises with time (Waites *et al.*, 2001). In continuous fermentations, the volume of the medium remains constant even though fresh medium is continuously fed as the remaining consumed medium and cells are removed at the same rate.

Batch fermentation is preferred in most large-scale fermentation as it is flexible in product volume, yet able to meet fluctuating market demands and minimizes overall production cost. In researches, batch fermentation is carried out in bioreactor, as the experimental set-up is relatively simple (Fleming & Patching, 2008). Although the dynamic conditions vary from time to time, well-instrumented bioreactors manage to keep some important variables to be constant throughout the experiment.

2.5 Model development

Conventionally, trial and error methods that require an extensive amount of work and high expenses are applied in most of the process designs and industrial processes. With mathematical modelling, process efficiency can be predicted because the formulated models provide insight into the behaviors of fermentation variables and act as the basis for optimization works.

Mathematical models represent the pattern and behavior of objects or processes using mathematical terms, by means of well-established theory (Dym, 2004). In practicing modelling, all the relevant parameters and their influences on the process are defined in quantitative terms. In addition, process inputs such as medium, feeds, environmental conditions, and outputs, which include biomass, and product formation will be considered (Rehm *et al.,* 1993). Once a mathematical model is formulated, it can be validated by comparing with experimental data and any deviations in performance may be further refined to achieve good agreement.

The typical pattern of growth cycle of microorganisms in batch system is due to different phases. The phases are namely: lag, acceleration, exponential, deceleration, stationary, accelerated death, exponential death, and survival phase (El-Mansi, 1999). In doing mathematical modelling, the exponential or logarithm phase is taken into consideration, in which the bacterial cells start to multiply rapidly and increase in biomass and cell quantity exponentially with time. The cell growth rate in the logarithm phase can be expressed as:

$$\frac{dX}{dt} = \mu X \qquad (2.1)$$

where X is the concentration of cells, μ is the specific growth rate, and t is the time in hours.

In bioprocess engineering, cell growth models are grouped into structured and unstructured models (Chen *et al*., 2006). Structured models describe cellular function at individual processes and reactions, thus provide a realistic representation of the cell. On the other hand, unstructured models relate cell growth and environmental conditions of the culture, without involving intracellular state variables. In general, unstructured models are suitable for practical applications in designing cultivation processes as they provide predictions for the installation of appropriate control devices and process conditions in the industrial application.

Among the kinetic equations developed, Monod equation is popular as it emphasizes on the concept relating the microbial growth rate (μ) with growth-limiting substrate concentration (*s*) (Panikov, 1995). By cultivating a stationary culture on a filtrate, Monod proved that the deceleration of bacterial growth was caused by the depletion of nutrients, which was previously thought to be the accumulation of toxic metabolites. Monod modelled an equation concerning the dependence of growth rate on limiting substrate concentration.

$$\mu = \mu_m \frac{s}{K_S + s} \tag{2.2}$$

Equation 2.1 involves two parameters, whereby μ_m is the maximum specific growth rate and K_s stands for the saturation constant. Due to its simplicity, Monod equation has been used and derived into different forms to describe the rates of biomass, product formation and substrate utilization (Panikov, 1995). In addition, models with terms representing maintenance energy, inhibition effects of products and substrates had been developed by modifying the Monod's model (Zerajic & Savkovic-Stevanovic, 2007). Usually, the Monod model is integrated with the Luedeking-Piret model derived based on analysis of LA fermentation (Nielsen, 1999).

Optical density (OD) was used in this study to measure the bacterial growth. As mentioned in the paper by Monod (1949), OD measurements allow a convenient method of predicting the bacterial density. OD at the same time is generally found to be proportional to bacterial density during the positive growth phases of the cultures.

A model proposed by Luedeking and Piret (1959) is often used for describing the rate of product formation. It was reported that the production of LA was dependent on the biomass production during fermentation. Luedeking and Piret (1959) stated that the rate of product synthesis is correlated to the rate of growth and amount of bacteria present, with the expression as follows:

$$\frac{dP}{dt} = \alpha \frac{dx}{dt} + \beta X \qquad (2.3)$$

where $\frac{dP}{dt}$ = the rate of product formation

α = growth associated product formation

β = non-growth associated product formation

Several kinetic models are also studied to describe other factors related to growth and product synthesis, mainly by modifying the Monod model and Luedeking-Piret equation. The degree of complexity of each model varies in each of the models, and the applications of the model depend on the objectives of the modelling studies (Nielsen, 2004). These models are important, as they form the basis for identifying the key events for improvement in the fermentation process.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

LA producing culture thermophilic Lactic Acid Bacteria with optimum growth temperature at 45 ℃ isolated from the Biochemistry Laboratory of UNIMAS was used.

3.1.2 Substrates

Sugarcane molasses was obtained and stored at room temperature for application in fermentation.

3.1.3 Media

Bacterial culture and inoculum media contained 20 g/l glucose and 5 g/l yeast extract. Tryptic soy agar was used for plate-cell count.

3.2 Experimental methods

3.2.1 Preparation of media

3.2.1.1 Glucose yeast extract broth

Glucose yeast extract broth for culturing thermophilic Lactic Acid Bacteria from previous seniors was prepared. The dehydrated ingredients were weighed using electronic balance. 20 g of glucose and 5 g of yeast extract were added to 1 l of distilled water and then mixed well. The solution was dispensed as required into universal bottles prior to sterilization at 121 ℃ for 20 minutes. The color was amber and slightly opalescent.

3.2.1.2 Tryptic Soy Agar culture plate

Tryptic Soy Agar (Merck KGaA, Germany) was prepared to measure the colony-forming unit (CFU) of bacteria during growth cycle throughout the experiment. The weight of Tryptic Soy Agar in dehydrated form was measured using electronic balance. 40 g/l of dehydrated Tryptic Soy Agar was suspended in distilled water and then heated to completely dissolve the medium. The solution was then sterilized by autoclave at 121 °C for 20 minutes. After sterilization, the molten agar solution was poured onto sterile petri dish and allowed to solidify. The pouring of agar solution was carried out in the laminar flow hood and the agar plates were sealed with parafilm to avoid contamination. The colour of the prepared medium was pale yellow.

3.2.2 Inoculum preparation

Thermophilic LAB was subcultured in glucose yeast extract broth at 36 °C for 18 h in the incubator. The Glucose Yeast Extract broth for inoculum preparation was prepared by adding 20 g/l of glucose into distilled water and mixed well. The solution was dispensed as required into Duran bottles prior to sterilization by autoclave at 121 °C for 20 minutes.

3.2.3 Fermentation medium preparation

The molasses in the concentrated form (50-55% fermentable sugars) was diluted with distilled water to concentrations of 20 g/l, 40 g/l, and 60 g/l sugars prior to fermentation tests. The pH of the molasses medium was adjusted to 6.0 with 1 M NaOH prior to sterilization at 121 °C for 20 minutes. The medium was enriched with 5 g/l of sterilized yeast extract to provide the nutritional requirements for the bacterium. The yeast extract was prepared separately to avoid darkening of the fermentation medium after sterilization. 7.5 g of yeast extract was suspended in 0.2 l of distilled water and then heated to completely dissolve the medium prior to sterilization at 121 °C for 20 minutes.

3.2.4 Fermentation

Batch fermentation experiments were performed in a benchtop fermentor (Biostat® B, B. Braun Biotech International, Germany) with 1.5 l working volume. The batch fermentor was equipped with control instrumentation (pH and temperature) and rotor with controllable speed. The fermentation runs were performed at constant temperature at 45 °C, pH at 6.0 by the addition of 10 M NaOH, and agitation speed at 100 rpm. Samples for analyses were withdrawn every 3-hour intervals until stationary growth phase was achieved.

Table 1. Experiment runs with different initial substrate concentrations.

Experiment runs	**Initial substrate concentration (g/l)**
1	20
2	20
3	40
4	40
5	60
6	60

3.2.5 Analytical procedure

3.2.5.1 Determination of sugar content in molasses

In the sugar content determination of untreated molasses, the Lane and Eynon copper reduction method was used (Corn Refiners Association, 1996). 5 g of molasses was weighed and transferred to a round bottom flask with 325 ml of distilled water, the solution was added with 75 ml of 4N H_2SO_4. The solution was heated to the boiling point in about 25 minutes, and then continued to boil for 150 minutes. The flask was then removed from the heat and the hydrolysate was left to cool to room temperature in an ice water bath.

The hydrolysate was neutralized to pH 4 with the addition of 50% NaOH and then to pH 5 with 0.1 N NaOH. The neutralized hydrolysate was transferred to a 500 ml volumetric flask, diluted to volume and then filtered through Whatman No.1 filter paper.

Standardized mixed Fehling's solution that was prepared beforehand was pipetted into a 200 ml Erlenmeyer flask added with some glass beads. The glass beads were added to avoid overheating of the solution. Titration was carried out with the filtered hydrolysate solution by means of the burette to the anticipated endpoint (standardized). The flask was placed on the wire gauze and heated by a burner to reach the boiling point within 2 minutes. Three drops of methylene blue indicator ware added to indicate the completion of titration.

3.2.5.2 Determination of residual glucose

Residual glucose was determined by Dinitrosalicylic Acid Method (DNS Method). A standard curve of reducing sugar was prepared using glucose solution (1 g/l) in distilled water. 3 ml of each concentration of glucose solution was filled into a test tube and added with 3 ml of DNS solution, boiled for 15 min. The boiled samples were immediately cooled and 1 ml of Roselle salt was added. The reaction mixture was mixed thoroughly and the absorbance at 575 nm was measured. A standard curve of relation between glucose concentration and its absorbance was plotted to determine the reducing sugar in the sample solution.

Each sample obtained from the fermentation was treated with acid hydrolysis (1 ml of 20% H_2SO_4 for every 100 ml of sample) to breakdown the sucrose, a non-reducing sugar, into glucose and fructose. The samples were then filtered through cotton wool and activated carbon to remove the dark color of molasses, leaving only transparent liquid for the DNS analysis. The samples were diluted with distilled water to achieve readings of OD values of 0.1 to 0.5 in Shimadzu UV-Vis spectrophotometer at wavelength 575 nm. The reducing sugar content in each filtered sample during the fermentations was calculated by comparing to the standard curve (Appendix A).