BIOETHANOL PRODUCTION USING OIL PALM TRUNK SAP (OPTS) BY *KLUYVEROMYCES MARXIANUS* - EFFECT OF TEMPERATURE AND PH

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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).

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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

To my parents, Mr. Abd Rahman Bin Abd Jalal and Mrs. Siti Samrah Bt Salleh who always support me in every minute of my life.

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In particular, my sincere appreciation also extends to my parent and family for their unbreakable love and continuous support to me either financially or morally. Many thanks also towards my fellow colleagues, friends and others who have provided assistance at various occasions. Their views, tips and opinions are useful indeed. Unfortunately, it is not possible to list all of them in this limited space. Last but not least, thank you to all who have been involved and helping during my study, I wish all will be blessed by Allah on the world and hereafter

ABSTRACT

This paper presents the kinetic study of bioehanol production using Oil Palm Trunk Sap (OPTS) by *Kluyveromyces marxianus.* Bioethanol have been discovered as the substitute of petrol that is biodegradable and less toxic than fossil fuels. Oil palm trunk can be used to produce bioethanol from its sap because it contains high glucose and other types of sugars. This study is focus on studying the kinetic parameters of bioethanol production from oil palm trunk sap (OPTS) using *Kluyveromyces marxianus.* By analyzing specific growth rate, sugar consumption rate and ethanol production rate the kinetic parameters can be determined. The analytical techniques for bioethanol production and substrate consumption were monitored by high-performance liquid chromatography (HPLC) and cell dry weight (CDW) for growth profile. In this study, pH and temperature were varied to study the effect of different pH (3, 4, 5, 6, and 7) and temperature (25ºC, 30ºC, 35ºC, 40ºC, and 45ºC). The highest ethanol concentration was produced at pH 5 which is 26.75 g/L and at temperature 35ºC ethanol concentration production was 45.06 g/L.

Keywords: K. marxianus, Bioethanol production, oil palm trunk sap (OPTS), Kinetic parameters

ABSTRAK

Kertas kerja ini membentangkan mengenai penghasilan bioethanol menggunakan cairan batang kelapa sawit oleh *Kluyveromyces marxianus*. Bioethanol telah ditemui sebagai pengganti kepada petrol yang mesra alam dan kurang toksik berbanding bahan bakar fosil. Cairan batang kelapa sawit boleh digunakan untuk menghasilkan bioethanol kerana ia mengandungi kandungan glukosa yang tinggi dan jenis gula yang lain. Kajian ini memfokuskan kepada kinetik parameter terhadap penghasilan bioethanol daripada cairan batang kelapa sawit oleh *Kluyveromyces marxianus.* Dengan menganalisis kadar pertumbuhan spesifik, kadar penggunaan gula dan kadar penghasilan ethanol. Teknik analisis untuk penghasilan bioethanol dan penggunaan gula telah dipantau menggunakan kromatografi cecair prestasi tinggi (HPLC) dan berat sel kering (CDW) untuk profil pertumbuhan. Dalam kajian ini, pH dan suhu telah dipelbagaikan untuk mengkaji kesan perbezaan pH (3, 4, 5, 6, dan 7) dan suhu (25ºC, 30ºC, 35ºC, 40ºC, dan 45ºC). penghasilan kepekatan ethanol yang tertinggi telah terhasil di pH 5 (26.75 g/L) dan di suhu 35ºC (45.06 g/L).

TABLE OF CONTENTS

SUPERVISOR'S DECLARATION IV
STUDENT'S DECLARATION V
Dedication VI
ACKNOWLEDGEMENT VII
ABSTRACT VIII
ABSTRAK IX
TABLE OF CONTENTS X
CHAPTER 1 1
INTRODUCTION 1
1.1 Background study 1
1.2 Motivation 2
1.3 Objective 2
1.4 Scope of study 2
CHAPTER 2 4
LITERATURE REVIEW 4
2.1 Overview 4
2.2 Bioethanol production 4
2.3 Oil palm trunk sap (OPTS) as the substrate 6
2.5 Factors affect the bioethanol production 9
2.5.1 Effect of pH 9
2.5.2 Effect of temperature 10
2.6 Kinetic study on effect of pH and temperature 11
CHAPTER 3 12
METHODOLOGY 12
3.1 Raw Materials and Chemical 12
3.2 Medium preparation 12
3.3 Microorganism preparation 13

3.4 Inoculums preparation 13
3.5 Fermentation Preparation 13
3.6 Analytical Method 14
CHAPTER 4 16
RESULT & DISCUSSION 16
4.1 Effect of pH and temperature on growth 16
4.2 Effect of pH and temperature on bioethanol production 20
4.3 Effect of pH and temperature on sugars consumption 24
CHAPTER 5 28
CONCLUSION & RECOMMENDATION 28
5.1 Conclusion 28
5.2 Recommendation 28
REFERENCES 29
Appendix A 31
Raw Data 31
Appendix B 34
Sugar concentration and ethanol concentration 34

LIST OF FIGURES

Figure 2.1: The growth of different type renewable energy resources from 2011 to 2030. 6
Figure 4.1: Growth profile of K. marxianus in nutrient broth 17
Figure 4.2: Cell dry weight of K. marxianus at different pH..... 18
Figure 4.3: Cell dry weight of K. marxianus vs time at different temperature..... 18
Figure 4.4: Effect of pH on the specific growth rate (□) of K. marxianus..... 19
Figure 4.5: Effect of temperatures on the specific growth rate 20
Figure 4.6: Ethanol concentration vs time at pH 3, 4, 5, 6, and 7 21
Figure 4.7: Ethanol concentration vs time at temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC..... 21
Figure 4.8: Ethanol formation rate at pH 3, 4, 5, 6 and 7 22
Figure 4.9: Ethanol formation rate at temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC ... 23
Figure 4.10: Sugar concentration vs time at 35ºC 25
Figure 4.11: Substrate consumption rate, qs (g/L.h) at different pH 3, 4, 5, 6 and 7(a) and temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC (b)..... 26
Figure A.1: Calibration curve of (a)Sucrose (b)fructose (c)glucose (d)ethanol 32
Figure B. 1: Concentration of sugar and ethanol (g/L) vs time (h) at pH 3..... 34
Figure B.2: Concentration of sugar and ethanol (g/L) vs time (h) at pH 4..... 34
Figure B.3: Concentration of sugar and ethanol (g/L) vs time (h) at pH 5..... 35
Figure B.4: Concentration of sugar and ethanol (g/L) vs time (h) at pH 6..... 35
Figure B.5: Concentration of sugar and ethanol (g/L) vs time (h) at pH 7..... 36
Figure B.6: Concentration of sugar and ethanol (g/L) vs time (h) at 25ºC..... 36
Figure B.7: Concentration of sugar and ethanol (g/L) vs time (h) at 30ºC..... 37
Figure B.8: Concentration of sugar and ethanol (g/L) vs time (h) at 35ºC..... 37
Figure B.9: Concentration of sugar and ethanol (g/L) vs time (h) at 40ºC..... 38
Figure B.10: Concentration of sugar and ethanol (g/L) vs time (h) at 45ºC..... 38

LIST OF TABLE

Table 3.1: Chemical used in this study 12
Table A.1: cell dry weight (g) of K.marxianus at temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC and pH 3, 4, 5, 6 and 7. 31
Table A.2: Effect of specific growth rate at different temperature and pH 32
Table A.3: Ethanol formation rate, qp (g/L.h) at different pH and temperature 33
Table A.4: Sugar consumption rate, qs (g/L.h) at different pH and temperature 33

LIST OF ABBREVIATIONS

µ	– Specific growth rate
h	– Hour
no.	– Number
HPLC	– High Performance Liquid Chromatography
OD	– Optical Density
CDW	– Cell dry weight
OPTS	– Oil palm trunk sap
rpm	– rotation or revolution per minutes
rs	– sugar consumption
rp	– bioethanol production
v/v	– volume per volume
w/v	– weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background study

Bioethanol is the biotechnology-based production as the substitute of fuels due to the exhaustion of fossil fuels and the increase in their price. It is the alternative to replace the fossil fuels that could reduce vehicles carbon dioxide (CO_2) by 90 %. Bioethanol is mainly produced by the sugar fermentation process, although it can also be manufactured by the chemical process of reacting ethylene with steam. The main sources of sugar required to produce ethanol come from primary feedstock and agricultural crops. These sources are grown specifically for energy use which includes corn, maize and wheat crops, waste straw, willow and popular trees. Biomass wastes contain a complex mixture of carbohydrate polymers from the plant cell walls known as cellulose, hemicellulose and lignin. These carbon sources can be converted to bioethanol by various microorganisms.

The economics of ethanol production by fermentation is influenced by cost of the raw materials, which accounts for more than half of the production cost. In recent years bioethanol production has been produced from oil palm trunk sap. Oil palm trunk has become one of the highest production crops in the world. There is no available method to utilize felled oil palm trunks except in plywood factories. As stated by Murata *et al.*, (2012), there are only small percentages of the felled trunks use for plywood production, but nearly all of the felled trunks are discarded. Oil palm trunk is the agricultural waste that contain large amount of sugars in its sap such as glucose and sucrose. These sugars can be converted easily to ethanol and lactic acid, thus the trunk was reported to be a potential significant resource for the production of fuel ethanol, biochemical and bioplastics (Murata *et al.*, 2012). Oil palm sap was reported to contain approximately 11% sugars with sucrose as a major component accounting for approximately 90% of total sugar (Yamada *et al*., 2010).

The production of bioethanol from OPTS usually using *Saccharomyces cerevisiae* as the microorganism. This is because this yeast strain can produce a high concentration of ethanol and it is preferred for most ethanol fermentations (Ngoh *et al.,* 2009). In this study *Kluyveromyces marxianus* is use as the microorganism to produce bioethanol from OPTS. *Kluyveromyces marxianus* is a thermotolerant yeast that shows considerable growth in the temperature range between 25ºC and 45ºC, while *Saccharomyces cerevisiae* did not grow at 45ºC (Matsuzaki *et al.,* 2012). Since there is no data or study have been done on bioethanol production from OPTS using *K. marxianus*, this study will use OPTS as the substrate and *K. marxianus* as the microorganism. The kinetics of ethanol production using *K. marxianus* was also studied.

1.2 Motivation

Production of bioethanol from oil palm trunk sap (OPTS) has developed from time to time. The development of bioethanol production using OPTS as substrate is due to contain of high glucose content in the sap. There is no previous research has been done for bioethanol production from substrate OPTS using *Kluyveromyces marxianus (K. marxianus).* Therefore, it is motivated to investigate the effect of several factors in influencing bioethanol production.

1.3 Objective

The objective of this study is to determine the effect of pH and temperature in the production of bioethanol from oil palm trunk sap (OPTS) using wild strain *K. marxianus*.

1.4 Scope of study

This study investigated the effect of temperature from 25°C to 45°C and pH factor from 3 to 7 during fermentation that affecting the bioethanol production using *K.*

marxianus and OPTS. The time series of growth and bioethanol production were monitored by cell OD and cell dry weight and high performance liquid chromatography (HPLC) respectively. HPLC was also need to monitor the sugars consumption. The specific growth rate (μ) and the kinetic parameters such as glucose consumption rate (r_s) and bioethanol production rate (r_p) were determined.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This study is to investigate the kinetic parameters towards the bioethanol production using oil palm trunk sap (OPTS) by *Kluyveromyces marxianus.* Previous studies have shown that most bioethanol production was using agricultural waste and primary feedstock such as corn, sugarcane, and wheat. Using primary feedstock as the main substrate in the industrial production will compete with the source to produce food stock. Currently OPTS have been found to be useful in bioethanol production. Most of the studies using *Saccharomyces cerevisiae* as the microorganism since yeast can produce more bioethanol in sugar fermentation. There is lack of information study on *K. marxianus* as the microorganism for bioethanol production using OPTS. Other than that, *K. marxianus* has been found as the thermotolerent yeast that can produce high production of bioethanol at the higher temperature until 47ºC compared to *Saccharomyces cerevisiae.*

2.2 Bioethanol production

Nowadays bioethanol production has been developed to be the substitute fuels. Dodic´ *et al*., (2012) stated that bioethanol is a modern energy source, which represents a significant replacement of liquid fossil fuels. It is necessary to consider and identify which process or combination of processes for bioethanol production gives the best results from the technological, economic and ecological aspect.

Bioethanol is one of the renewable sources for the fuels nowadays. It has been widely produced due to the high demand in population and industrialization. As stated by Sarkar *et al.*, (2012), the world's present economy is highly dependent on various fossil energy sources such as oil, coal, natural gas, etc. These are being used for the production of fuel, electricity and other goods. In this scenario, renewable sources might

serve as an alternative. Bioethanol has been receiving widespread interest at the international, national and regional levels. The global market for bioethanol has entered a phase of rapid, transitional growth. Many countries around the world are shifting their focus toward renewable sources for power production because of depleting crude oil reserves. The trend is extending to transport fuel as well. Recently, the focus on renewable biofuels in Malaysia is restricted to biodiesel and bioethanol only. Most liquid fuel in Malaysia are utilized in transportation sector, that is why Malaysia need renewable energy to substitute fuel. The ethanol derived from biomass, or second-generation bioethanol (SGB), offers greater promise in replacing fossil fuels than bioethanol that was derived from edible sources, or first-generation bioethanol (FGB), because SGB does not compete with the human food supply (Tye *et al.*, 2011).

Besides solar energy, the other renewable energies are solid waste, mini hydro, biogas and biomass. The growth of different types of renewable energy resources from 2011 to 2030 is shown in Figure 2.1. From the figure, we can see that besides solar energy, the growth of biomass also increases every year. Malaysia has a significant amount of agriculture activities; thus, biomass can be a very promising alternative source of renewable energy (Tye *et al.*, 2011). Bioethanol can be produced from the fermentation of raw materials that has sugar in it to be metabolized to bioethanol. Yamada *et al.* (2010), investigated the possible ethanol yield from sap of old trunk and calculated it to be approximately 9000 L/m^2, which exceeds that of sugar cane juice. Other than that Kosugi *et al*. (2010) determined the amount of ethanol produced from OPTS is correspond to 94.2% of the theoretical yield calculated based on consumption of glucose, sucrose, fructose, and galactose. By this finding, unlike sugar cane, bioethanol production using felled OPT will not conflict with food usage and has a great potential as a feedstock for bioethanol.

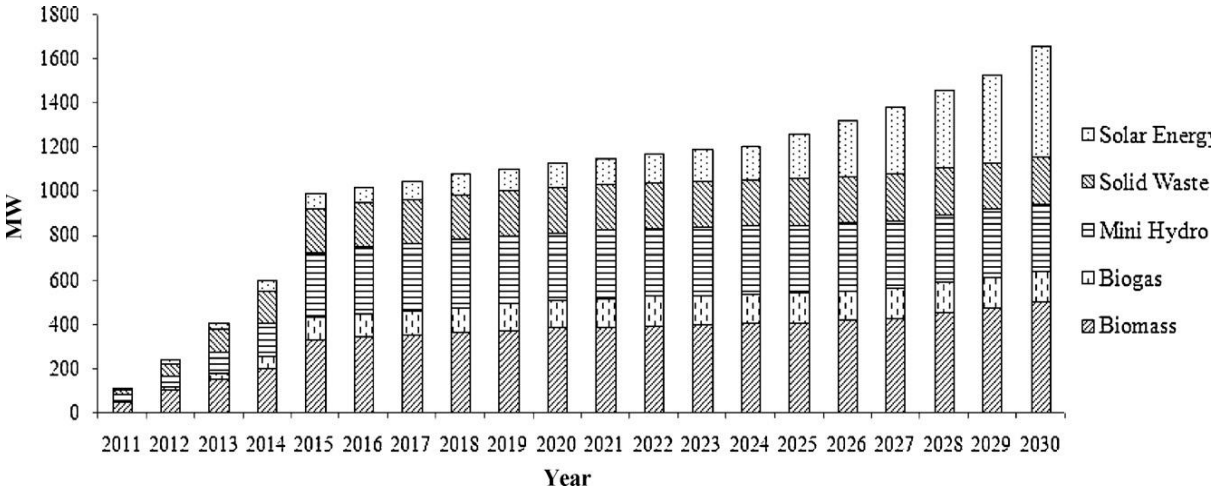


Figure 2.1: The growth of different type renewable energy resources from 2011 to 2030.

From the previous research it has been shown that bioethanol is the most important renewable source in the future. The increasing demand of the fuel will increase the demand of the bioethanol as it is the renewable energy that can obtain from the biomass. The most economical way to produce bioethanol by fermentation.

2.3 Oil palm trunk sap (OPTS) as the substrate

Malaysia is the tropical country that widely planted oil palm tree for its oil. As the oil palm is a tropical palm tree, it can be cultivated easily in Malaysia. The oil palm tree in Malaysia originates from West Africa, where it grows wild. It was later developed into an agricultural crop. Because oil palm is a high yielding crop, it can produce, on average, about 4–5 ton of oil/year. It has been forecasted that, in years to come, the demand will be higher with increasing world demand in oils and fats. It is already very profitable to invest in the oil palm industry in Malaysia, even just using existing technology (Tye *et al.,* 2011). As stated by Yamada *et al.* (2010), the oil is mainly used for food, raw material for various products such as detergents and cosmetic.

Oil palm trees are replanted at an interval of approximately 25 years because of decreased oil productivity, so the felled trunks are the enormous amount of biomass resources in the palm oil produces. The oil palm trunks usually utilized for plywood manufacturing and the inner part is discarded because of its weak properties for

manufacturing of the plywood. When replanting the oil palm trees, the old trunks are cut and most of them are discarded or burnt. This will produce pollution and abundant of biomass will produce. Therefore, the most efficient ways needed for utilizing oil palm trunks for the ideal oil palm plantation and sustainable palm oil industry (Yamada *et al.,* 2010).

From previous study, in order to utilize the old palm trunks felled for replanting, especially the inner part, attempt has been done to produce bioethanol and the material for bio-plastic from felled trunks (Kosugi *et al.,* 2010). The study focused on sugars in the sap of the felled trunk and observed a large quantity of high glucose content sap in the trunk. The water content of the trunk is surprisingly high at a mass fraction of 70-80%, which is much higher than that of freshly harvested wood species around 50-60% (Murata *et al.* 2012). Their research found that the major sugar in the sap from oil palm is glucose and the minor sugar components in the sap medium such as sucrose, fructose and galactose initially found at 4.2 g/L, 2.6 g/L and 0.6 g/L respectively. Because of the large amount of glucose in the sap, it can be a good feedstock for the bioethanol production.

Yamada *et al.* (2010), has investigated that the free sugar content in the sap is at the maximum in 30 to 60 days of storage after logging. Therefore the sap should be squeezed during this period to obtain the highest sugar concentration for the production of bioethanol. Studies have shown that oil palm will absorb carbon dioxide (CO_2) and return oxygen (O_2) to the atmosphere more than others plant, thus the conversion of oil palm biomass into second-generation bioethanol to be used as transport fuel can further reduce the emission of CO2 and conservation of environment can be achieved (Tye *et al.,* 2011).

Other research also state that sugar cane has sufficient organic nutrients and minerals that make it more suitable for the microorganism especially *Saccharomyces cerevisiae* for ethanol production (Limtong *et al.,* 2007).

From the research that has been done, felled oil palm trunk was the most abundant crops in Malaysia. It also have been proved to contain highest sugar concentrations, such as glucose, sucrose, fructose and galactose, which can be used for production of bioethanol. So, oil palm trunk sap was the most suitable substrate for the

production of bioethanol rather than sugar cane as it prevents the conflict with food usage and has a great potential as a feedstock for bioethanol.

2.4 *Kluyveromyces marxianus* as the microorganism

Ethanol fermentation is a biological process in which organic chemical is converted by microorganism to simpler compounds, such as sugars. These fermentable compounds are then fermented by microorganisms to produce ethanol and CO_2. During the whole process of ethanol fermentation, there are mainly two parts for microorganisms. One is for the microorganisms which convert fermentable substrates into ethanol, and the other is to produce the enzyme to catalyze chemical reactions that hydrolyzed the complicated substrates into simpler compounds (Lin and Tanaka, 2006). Furthermore, the lack of industrially suitable microorganisms for converting biomass into fuel ethanol has traditionally been cited as a major technical roadblock to developing a bioethanol industry.

Matsuzaki et al. (2012), stated that *K. marxianus* is a thermotolerent yeast that shows a considerable growth in the temperature range between 25ºC and 45ºC. Thermotolerent microorganism is the efficient way for the ethanol fermentation at high temperature in tropical countries, where average day-time temperatures are usually high throughout the year. To achieve high temperature fermentation, it is necessary to use an efficient yeast strain that can tolerate high temperature (Eidpum *et al.,* 2012).

Eidpum *et al.* (2012) investigated the high temperature ethanol fermentation by comparing between *K. marxianus* and *S. cerevisiae*. They found that *S. cerevisiae* more effective to produce bioethanol in operating temperature range of 33ºC to 37ºC while *K. marxianus* more effective to produce bioethanol at high temperatures range 40ºC to 45ºC.

Malaysia is one of the tropical country in the world that average day time temperatures are usually high throughout the year. To effectively utilize the condition of the environment, *K. marxianus* may be more efficient microorganism used for the bioethanol fermentation as it can growth at high temperature.

2.5 Factors affect the bioethanol production

There are several factors that affect the bioethanol production such as pH, temperature and substrate concentration. The effects of these factors depend on the characteristics of the microorganism that was used in the production of bioethanol. In this study, two kinetic parameters, temperatures and pH, were investigated.

2.5.1 Effect of pH

pH is one of the important kinetic parameters that affect the microorganism's growth and production rates. Yeast was found to prefer acidic condition for the optimum growth and production. Lin and Tanaka (2006) found that most yeast strain can grow in the pH range 4.5 to 6.

Manikandan *et al.*, (2008) investigated the effect of pH on bioethanol production from banana peel waste using *S. cerevisiae*. They conducting the experiment in the pH range of 3.5 to 5.5 and the results show that highest ethanol production was at pH 4.7 (9.2 g/L). In another study, Manikandan *et al.* (2010) also using *S. cerevisiae* for the investigation of ethanol production but they used corn flour as the substrate instead of banana peel. This time they found that the optimum pH for the ethanol production was 5.5 with the production of 49.037 g/L. Their results revealed that different substrates will affect the optimum pH for ethanol production even though similar microorganism was used.

K. marxianus is a type of yeast that have similar pH range to grow as *S. cerevisiae*. Limtong *et al.* (2007), had investigated the effect of pH on bioethanol production from sugar cane juice using *K. marxianus*. They studied the pH range of 4 to 5.5 and found at pH 5 and pH 5.5, the highest production of bioethanol (8.7% (w/v) and 8.5% (w/v) respectively) could be obtained. Most of the other study that used *K. marxianus* as the misroorganism for bioethanol production used constant pH 5 for the fermentation. Similarly, Eiadpum *et al.* (2012) they using blackstrap molasses and cane juice as the main substrate for ethanol fermentation and they also set constant the pH of fermentation at 5 with though different culture temperature were used.

There is not much investigation for the pH study of *K. marxianus* was done. Since it is a type of yeast, it is predicted that the optimum pH will lie between 4.5 to 5.5 in acidic region.

2.5.2 Effect of temperature

Temperature plays a main role in the fermentation that depends on the microorganism type. Microorganism have been classified according to the optimum temperature for growth, psychrophiles optimum at < 20ºC, mesophiles optimum from 20ºC to 50ºC and thermophiles optimum at > 50ºC.

Manikandan *et al.* (2010) studied the ethanol production by *S. cerevisiae* using corn flour as the substrate at different temperature from 28ºC to 36ºC. They found that the optimum temperature for the ethanol production was at 30ºC (63.04 g/L). In another study, Kosugi *et al.* (2010) using similar microorganism but in oil palm trunk sap substrate. They found that the fermentation was almost complete after 12 h that reached stationary phase and glucose was thoroughly consumed after 24 h. The fermentation was conducted at 30ºC and produce 30 g/L ethanol concentration. Yeast was the microorganism that have optimum grow at 30ºC to produce ethanol.

Oda *et al.,* (2010) investigated the ethanol fermentation using sugar beet juice and crude cheese whey using *K. marxianus* at constant pH 5 for fermentation. Their results showed that ethanol was produced at slower rate at 30ºC (70 mg/ml) if compared to temperature at 33ºC (90 mg/ml) to 37ºC (100 mg/ml). Eiadpum *et al.* (2012) studied the co-culture of *K. marxianus* and *S. cerevisiae* for their abilities to improve the production and stability of ethanol fermentation. From this study, they found that *K. marxianus* was able to produce ethanol at high temperature of 40-45ºC in the sugarcane medium, while *S. cerevisiae* was more effective in producing ethanol at 33ºC to 37ºC. Thermotolerent microorganism was efficient to produce high bioethanol than the mesophile microorganism that cannot survive in the high temperature.

From the research above, it can be concluded that optimum temperature in the producting of bioethanol is very much depend on the type of the microorganism.

2.6 Kinetic study on effect of pH and temperature

Kinetics is the study of changes in a physical or chemical system. Evaluation of kinetic parameters is essential for process scale-up. This research's objective was to investigate the kinetic study of bioethanol production from oil palm trunk sap (OPTS) by wild strain *K. marxianus*. The parameters that were investigated in this study consist of specific growth rate (μ), ethanol production rate (r_p) and substrate consumption rate (r_s).

Dodic' *et al.* (2012) studied the kinetic on ethanol production from sugar beet raw juice. They stated that the kinetics of growth could be quantified using Monod's equation and based on values of biomass, bioethanol and fermentable sugars concentration measured every 2 h throughout the process, the calculations for biomass production rate (r_x), fermentable sugars consumption rate (r_s), bioethanol production rate (r_p) and specific growth rate □ were made. From their result the fermentable sugars consumption rate and bioethanol production rate reached their maximum at 10 h (7.19 g/l.h and 4.17 g/l.h, respectively).

Limtong *et al.* (2007) studied the production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *K. marxianus*. They found that sugar utilization further confirmed the low ethanol production and yeast cell growth. Ehen the experiment was conducted in the condition where the highest ethanol production and growth were obtained, gave the lowest sugar concentration remaining at the end of fermentation (7.05% w/v). However, under condition with the lowest ethanol production and growth, the highest concentration of remaining sugar (10.35% w/v) was observed. This show that when highest remaining sugar at the end of fermentation was observed, the sugar consumption for ethanol production was lower. From these study, the highest consumption rate was at pH 5 an temperature 35ºC which was the highest growth rate and the highest ethanol formation rate at the similar condition.

CHAPTER 3

METHODOLOGY

3.1 Raw Materials and Chemical

The raw material in this research was oil palm trunk sap (OPTS) that was collected from Jengka, Pahang. Pure culture of wild type *Kluveromyces marxianus* was used in this study. Table 3.1 shows the chemicals that were used in this study.

Table 3.1: Chemical used in this study

No.	Chemical	Brand
1	Bacto-Tryptone	OXOID
2	Yeast extract	OXOID
3	Sodium chloride (NaCl)	Sigma Aldrich
4	Agar powder	Sigma Aldrich
5	Sodium hydroxide (NaOH)	Merck
6	Hydrochloric acid (HCl)	Merck
7	Standard ethanol (HPLC solution)	Merck
8	Standard glucose (HPLC solution)	Sigma Aldrich
9	Standard sucrose (HPLC solution)	Sigma Aldrich
10	Standard fructose (HPLC solution)	Sigma Aldrich

3.2 Medium preparation

Nutrient agar, nutrient broth and oil palm trunk sap (OPTS) were used as medium in this study. For solid medium, the composition of agar containing 20 g/L agar, 10 g/L peptone, 5 g/L yeast extract and 5 g/L of glucose according to Yeast extract, Peptone & Glucose (YPD medium) ingredients. The nutrient agar was being autoclaved (Hirayama HV110 Hiclave) at 121°C for 15 minutes followed by cooling. After cooling, the nutrient agar was poured into the sterilized petri plate. The plates were left undisturbed until the agar solidifies (Liu *et al.*, 2010). Nutrient broth was prepared by mixing the same ingredients of YPD medium as in nutrient agar but excluding agar powder.

Oil palm trunk obtained from oil palm plantation. The trunk was cut about 7 cm thick and the inner part of the disk was taken. The sap was collected by pressing the disk. This sap was centrifuged at 6,000 rpm (Eppendorf Centrifuge 5810R) for 15 minutes to remove the debris that containing in the sap and the supernatant was stored at -20ºC before use.

3.3 Microorganism preparation

K. marxianus was cultured in the nutrient agar to get the single colony using streaking method. The agar plate was incubated in the incubator Memmert model 100 - 800 upside down at 30°C for 15-20 hours to prevent the vapor mix with the bacteria. To maintain the pure culture supply continuity for longer time, the strain was cultured in the glycerol stock at -80°C.

3.4 Inoculums preparation

After *K. marxianus* has been grown into a single colony on nutrient agar, 2 loops of the single colony were transferred into 100 ml sterile nutrient broth in 250 ml conical flask. The strain was incubated for 16 hours at 30°C and 150 rpm on rotary shaker until the initial optical density (OD) achieved was 1.5. After 16 hours, the cells were harvested by centrifuging for 10 minutes at 10,000 rpm. Then, cell washing was done to prevent the carryover of spent medium or any waste. The cells were then resuspended with OPTS medium to start the fermentation.

3.5 Fermentation Preparation

Fermentation studies were performed in 250 ml conical flask. 10 % (v/v) of inoculums suspension was transferred to fermentation flask that containing 90 % (v/v) of OPTS medium. Before that, the sap was sterilized in the autoclave at 121°C for 20 min. The medium was incubated on rotary shaker at 150 rpm. Fermentation samples 2 ml were collected and centrifuged to remove cells at every 2 hours for 24 hours. The supernatant was filtered with 0.22 □m Nylon filter membrane to remove any solid particles. All the procedures were repeated at 25°C, 30°C, 35°C, 40°C and 45°C with the fix pH of 5.5 and at a fix temperature of 30ºC with varying of pH at 3, 4, 5, 6 and 7.

3.6 Analytical Method

The growth profile of the *K. marxianus* was monitored by checking the optical density every 2 hours for 24 hours. The absorbance of the sample was measured with a UV-VIS spectrophotometer (HITACHI 1800) at 600 nm. The cell dry weight of the samples was determined by analytical balance.

Sugar components or substrate consumption and ethanol concentration were determined using *high-performance liquid chromatography* (HPLC) equipped with an automatic sampler/injector (Agilent 1200 Series). The column type is REZEX ROA-Organic Acid with the mobile phase of 0.005 N sulphuric acid. The column temperature was controlled at 30°C. The solvent flow rate was maintained at 0.5 mL/min. The peak was detected using refrective index detector (RID). The range for calibration curve for sugar and ethanol were 10 g/L, 20 g/L, 30 g/L, 40 g/L and 50 g/L. These calibration was used to identify the amount of sugar consumption in OPTS and the ethanol concentration that was produced.

The kinetic parameters that were analyzed include μ, r_p and r_s. □ is the specific growth rate of the microorganisms. The specific growth rate can be obtained from the slope of ln X/X_o cell dry weight of the microorganism at log phase of profile.

Volumetric rate of substrate consumption, $r_{s,}$

$$r_S = -\frac{dS}{dt} = -\frac{\mu X}{Y_{X/S}} = q_S X$$

The value of q_s can be obtained from the experimental data through the slope ds/dt that in the plot of total sugar concentration versus time. From the value of slope ds/dt, the value of q_s x was obtained and equal to the rate of substrate consumption r_s.
The specific rate of substrate consumption, q_s

$$q_S = -\frac{1}{X}\frac{dS}{dt}$$

The volumetric rate of product formation, r_p

$$r_P = \frac{dP}{dt} = q_P X$$

The value of q_p can be obtained from the experimental data through the slope dp/dt that was in the plot of ethanol concentration versus time. From the value of slope dp/dt, the value of q_p x was obtained and equal to the rate of product formation, r_p.

The specific rate of product formation, q_P

$$q_P = \frac{1}{X}\frac{dP}{dt}$$

CHAPTER 4

RESULT & DISCUSSION

4.1 Effect of pH and temperature on growth

The effect of kinetic parameters which is temperature and pH towards production of bioethanol were studied using oil palm trunk sap (OPTS) as the substrate and wild type *K. marxianus* as the microorganism. The cell dry weight from the fermentation using conventional nutrient broth was collected as the control for the growth curve of *K. marxianus* as shown in figure 4.1. This growth profile was act as the starter for the inoculums in the large scale with the same optical density and same concentration of microorganism. The cell dry weight was collected every 2 hour interval for 24 hours. As stated by Shuler *et al.* (2002) it consists of lag phase, exponential phase, stationary phase and death phase. From this figure the lag phase occurs in the first 2 hour where the cell was adapting with the new environment in the medium. During this phase, cell mass may increase a little, without an increase in cell number density. The exponential phase starts after 2 hours inoculums. In this phase, the cells have adjusted to their new environment. After the adaptation period, cells multiplied rapidly and cell mass and cell number density increase exponentially with time. This is a period in which all components of cell grow at the same rate. The deceleration phase follows the exponential phase. In this phase, growth decelerates due to either depletion of one or more essential nutrients. The stationary phase starts at 16 hour, which is at the end of the deceleration phase. This is a stage when the net growth rate is zero or the growth rate is equal to the death rate. Then, the last phase is death phase that follows the stationary phase. Some cell death may start during stationary phase. At the end of stationary phase, because of either nutrient depletion or toxic product accumulation, the death phase begins. The lack of nutrient and life span of the *K. marxianus* is the factor of death cell.

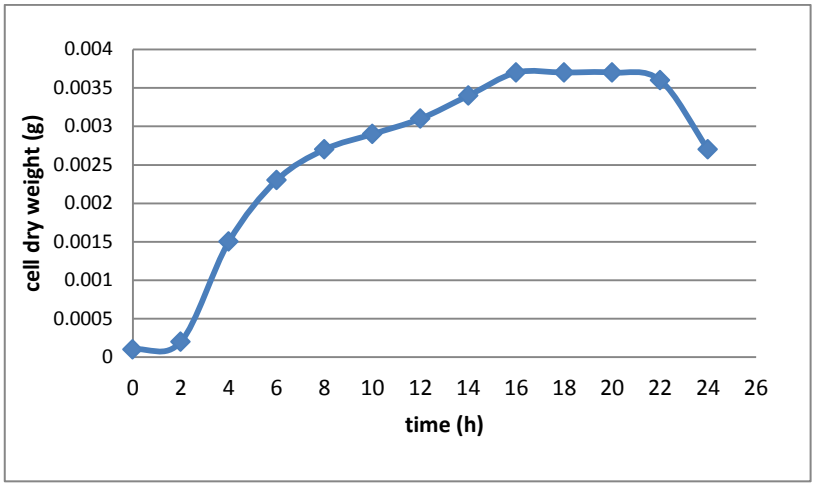


Figure 4.1: Growth profile of K. marxianus in nutrient broth

From this profile in Figure 4.1, it was known that the exponential phase for *K. marxianus* is from 3 to 16 hours. The time of inoculums needed to be ready for fermentation in OPTS is within 16 hour so that cells will not experience log phase during experiment.

Fermentation was carried out in the OPTS by *K. marxianus* for the bioethanol production. The used of OPTS as a substrate in the production of bioethanol would affect the growth of the *K. marxianus* because of different in composition of important nutrient. The effect of the growth of *K. marxianus* in OPTS were studied using different pH (3, 4, 5, 6 and 7) and temperature (25ºC, 30ºC, 35ºC, 40ºC and 45ºC) respectively.

The growth profile of *K. marxianus* in OPTS at different pH was shown in Figure 4.2. The growth of *K. marxianus* was the best at pH 5, where it has the higher growth profile at 16 hour (0.0095 g). This high cell dry weight shows that *K. marxianus* grow very well at pH5.

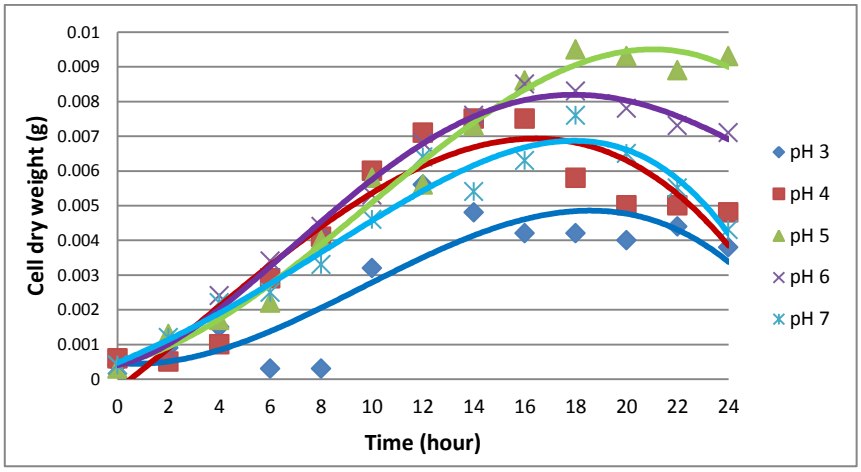


Figure 4.2: Cell dry weight of K. marxianus at different pH

The growth profile of *K. marxianus* in OPTS at different temperature was shown in the Figure 4.3. From this profile, the growth of *K. marxianus* was slightly stationary at 16 h for all temperature. The best growth profile is at 35ºC which has the highest cell dry weight at 16 hour (0.017 g). The higher the cell dry weight indicated the cell could grow very well in the condition.

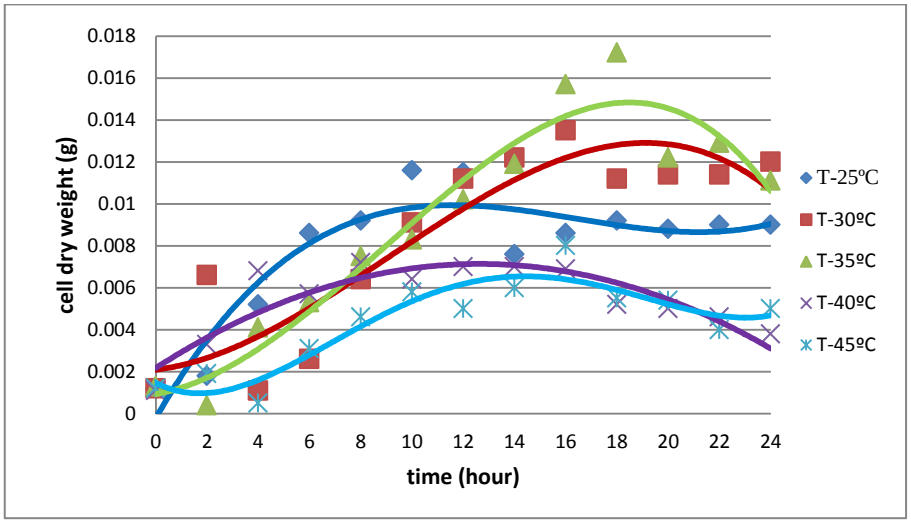


Figure 4.3: Cell dry weight of K. marxianus vs time at different temperature

Ethanol is a primary metabolite of microbial cell growth; thus, a growth associated product. In other words, a better growth will ensure a better or higher production of ethanol. Effect of pH on the specific growth rate was shown in Figure 4.4. At pH 3 and 4, the specific growth rate of *K. marxianus* increased slowly but at low values of 0.2 h^{-1} and 0.28 h^{-1} respectively. *K. marxianus* could adapt very well to pH 5 as the specific growth rate was the highest (0.44 h^{-1}). The specific growth rate dropped tremendously at pH 6 and 7, which is in alkali condition. As the rule of thumb yeast can grow in pH range 3 to 6 (Shuler *et al.*, 2002). Therefore, it is not surprised that specific growth rate fell greatly after pH 6.

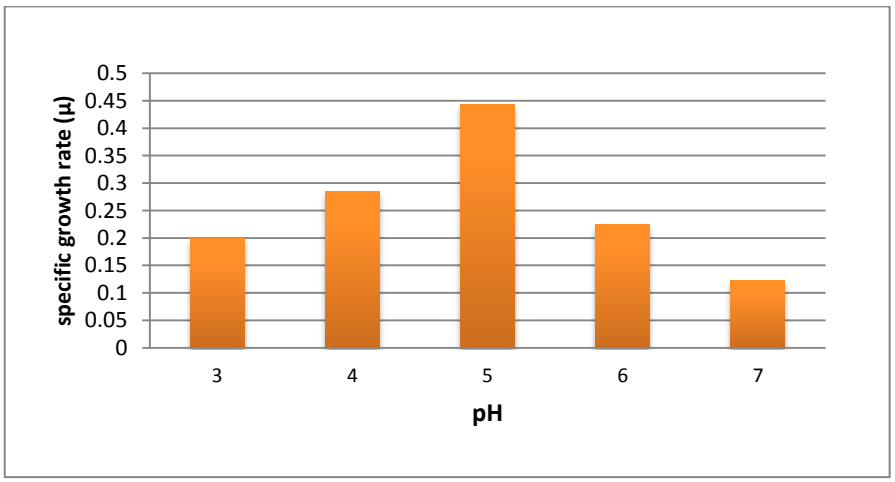


Figure 4.4: Effect of pH on the specific growth rate (μ) of K. marxianus

Figure 4.5 shows the effect of temperatures on the specific growth rate of *K. marxianus*. From the figure, it can be seen that *K. marxianus* can grow at both low and high temperature. *K. marxianus* is the thermotolerent yeast that grows in temperature ranges 25ºC to 45ºC (Matsuzaki *et al*., 2012). At low temperature, the microorganism is not actively growing as the condition was not the optimum. The specific growth rate falls at low temperature because of non-optimal condition for all enzyme involved in the metabolic regulatory mechanisms and the diffusional limitation such as rate of transport of nutrient and product in and out of the cell (Shuler *et al.* 2002). It can be seen that *K. marxianus* growth actively at temperature of 35ºC as specific growth rate was the highest (0.31 h^{-1}) in the range of temperatures studied. At 40ºC and 45ºC the specific

growth rate drops because there was thermal denaturation of protein and breakdowns of important cell structures (Shuler *et al.* 2002). A relatively higher specific growth rate at 40ºC and 45ºC as compared to 25ºC and 30ºC verified that *K. marxianus* is a thermotolerent yeast strain (Eiadpum *et al.*, 2012).

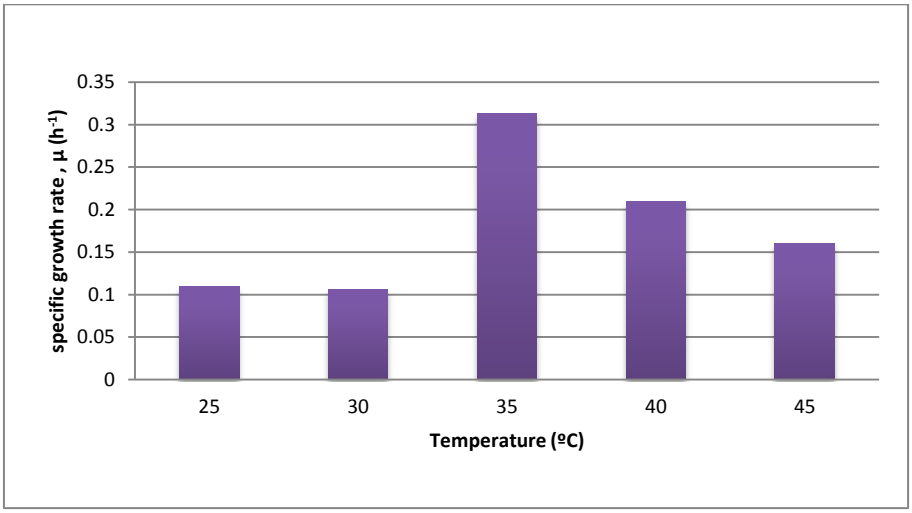


Figure 4.5: Effect of temperatures on the specific growth rate

4.2 Effect of pH and temperature on bioethanol production

The effect of different pH in substrate and different temperature of fermentation by *K. marxianus* towards bioethanol production is presented in Figure 4.6 and 4.7. The effect of ethanol production using OPTS as a substrate and wild type *K. marxianus* was monitored by conducting experiment at different pH (3, 4, 5, 6 and 7) and keeping all the parameters constant at temperature 30ºC. The results are shown in figure 4.6. Bioethanol concentration at 0 to 4 hours is lower and as the time increased the bioethanol production also increased. At 16 hour, the ethanol production for all pH was slightly leveled off. The higher production of ethanol was at pH 5 with ethanol concentration 24.55 g/L.

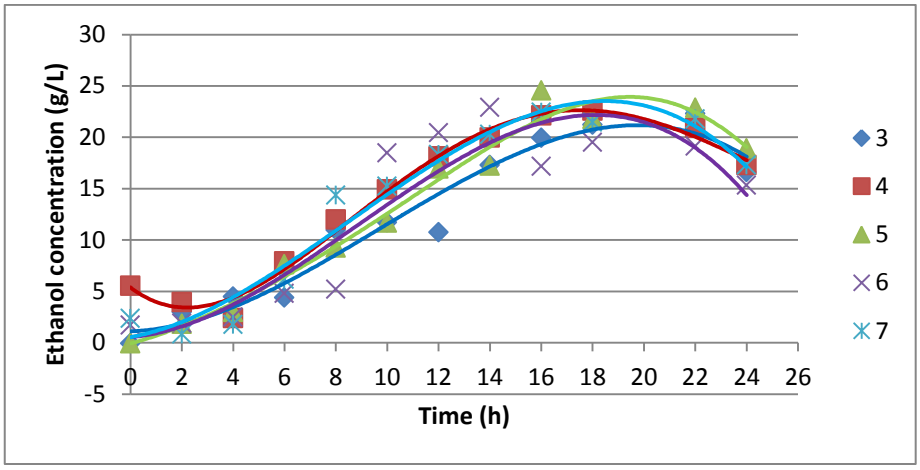


Figure 4.6: Ethanol concentration vs time at pH 3, 4, 5, 6, and 7

The experiment for effect of temperature on ethanol production was conducted at the different incubation temperatures of 25ºC, 30ºC, 35ºC, 40ºC, and 45ºC by keeping all other parameters constant at pH 5. From Figure 4.7, it can be seen that the highest ethanol concentration was obtained at 16 hour of fermentation at 35ºC (45.06 g/L). At lower and higher temperature, the ethanol was also produced but in the lower concentration. Further increment of fermentation time lower the ethanol concentration as some of the ethanol may be vaporized due to its highly volatile nature.

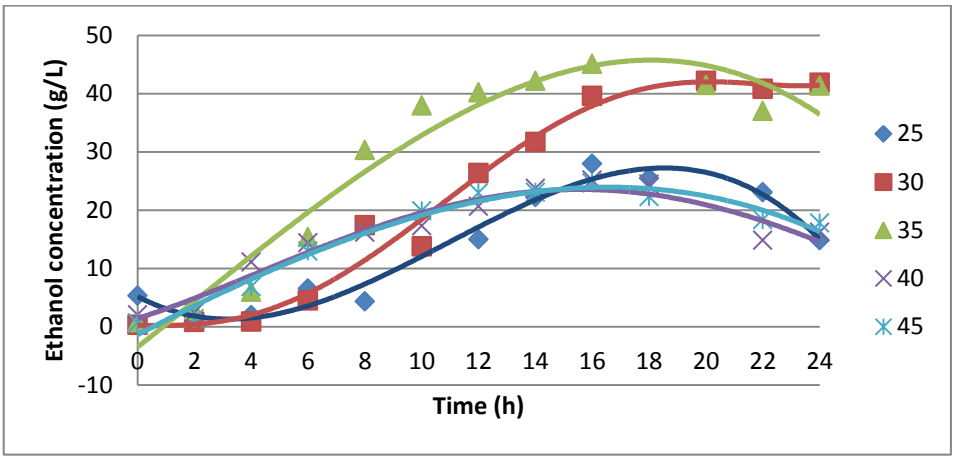


Figure 4.7: Ethanol concentration vs time at temperature 25°C, 30°C, 35°C, 40°C and 45°C

The product formation rate was described by batch culture kinetic as given in equation (1) and (2). The equation indicates that the rate at which product is formed per unit volume is directly proportional to the cell concentration.

$$r_p = \frac{dP}{dt} = q_p x \qquad (1)$$

$$q_p = \frac{1}{X}\frac{dP}{dt} \qquad (2)$$

The value of q_p can obtain from experimental data of product concentration versus time. The value of dp/dt was obtained from the slope. From the value of slope dp/dt, the value of q_p X was gained. To obtain q_p, the slope dp/dt was devide by X at the time measurement, t_m. In this figure the value of slope is equal to the value of product formation r_p.

From the experiment the product formation rate, q_p, at different pH is shown in Figure 4.8. The product formation rate increased as the pH increased from pH 3 until maximum formation rate of ethanol 1.41 g/L.h at pH 5 and decreased as the further increased of pH 6 and 7.

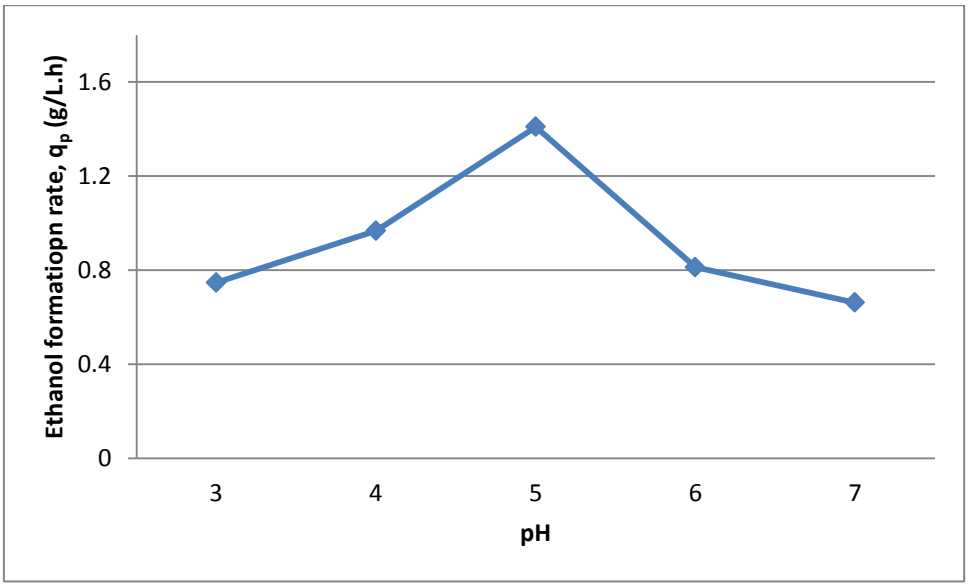


Figure 4.8: Ethanol formation rate at pH 3, 4, 5, 6 and 7

The formation rate at different temperature was increased as the temperature increased from 25ºC until it reached a maximum rate of 0.9 g/L.h at 35ºC as shown in the Figure 4.9. As the temperature increased to 40ºC and 45 ºC the formation rate of bioethanol decreased because of the denaturation of proteins involved in growth and production (Haurowitz F. 2012). They stated that protein denatured when it was heated, treated by alkaline or acid and certain organic solvents, so the ethanol production was low as the temperature increased.

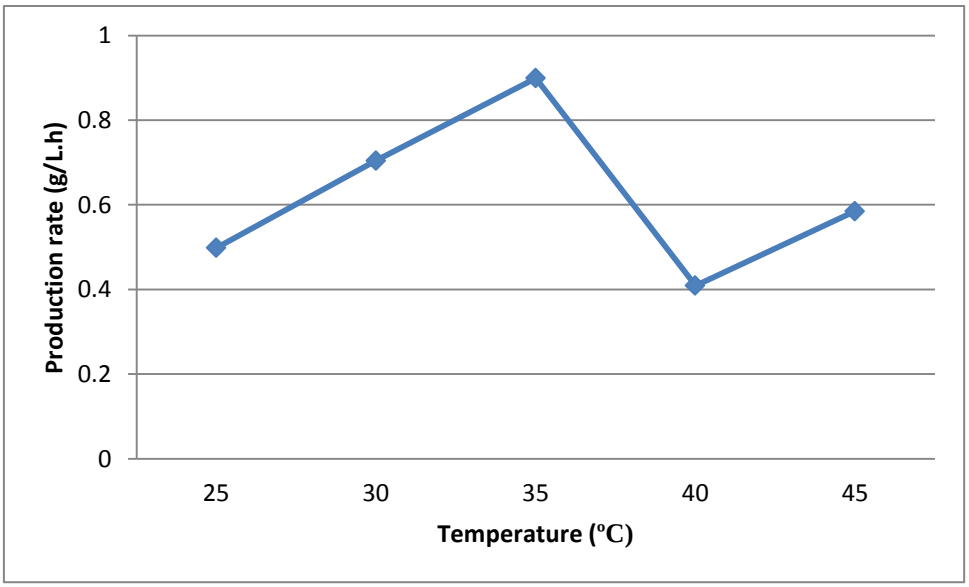


Figure 4.9: Ethanol formation rate at temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC

Dodi *et al.* (2012), was study the kinetic modeling of ethanol production from sugar beet raw juice and *S.cerevisiae*. Their result shows that the bioethanol production rate was depend on the biomass production which is at 10 h of the fermentation, the maximum bioethanol production rate reached 4.17 g/L.h. The high biomass production will produce high bioethanol formation rate.

Manikandan *et al.* (2008) studied the effect of pH and temperature on ethanol production from banana peel waste using mutant strain *S.cerevisiae*. From this research it was found that increased the temperature and pH will increase the ethanol production until it reached maximum value of 9 g/L at 33ºC and 9.2 g/L at pH 4.7. Even though

different species of microorganism and different substrate were used in current study, the optimum temperature and pH for the production of ethanol were very similar. This may be due to the fact that *K. marxianus* is also a yeast strain. However, the results obtained in this study were in contrast with those of Oda *et al.* (2010). By using sugar beet juice and crude cheese whey as the substrate and *K. marxianus* as the microorganim, their result showed that optimum temperature for ethanol production was at 37ºC (100 mg/ml), which is much higher than the results obtained here.

4.3 Effect of pH and temperature on sugars consumption

HPLC sugar analysis revealed that OPTS contained glucose, sucrose and fructose as the major components. These sugar contents were consumed by *K. marxianus* to produce bioethanol and as its nutrient to grow. At the initial fermentation, the concentration of the glucose, sucrose and fructose were found to be 34.42 g/L, 17.46 g/L and 14.87 g/L respectively. Generally the trend of sugar consumption at different pH and temperature (Appendix B) were nearly the same as Figure 4.10. From figure 4.10 it shows that at the beginning of fermentation, the concentration of sucrose was high and it rapidly decreases as the time increased. Glucose and fructose concentration were found to increase at the first 2 hour because of the sucrose, as the disaccharide sugar, has been dissociated to fructose and glucose before it was consumed. The sugar decreased because it was consumed by the microorganism. The ethanol produced as the sugar concentration lowered. As shown in figure 4.10, glucose was found to be the dominant component of sugar in the OPTS substrate. The sucrose concentration decreased rapidly and it was fully consumed first among the other sugars. Glucose was consumed after sucrose and this was followed by fructose.

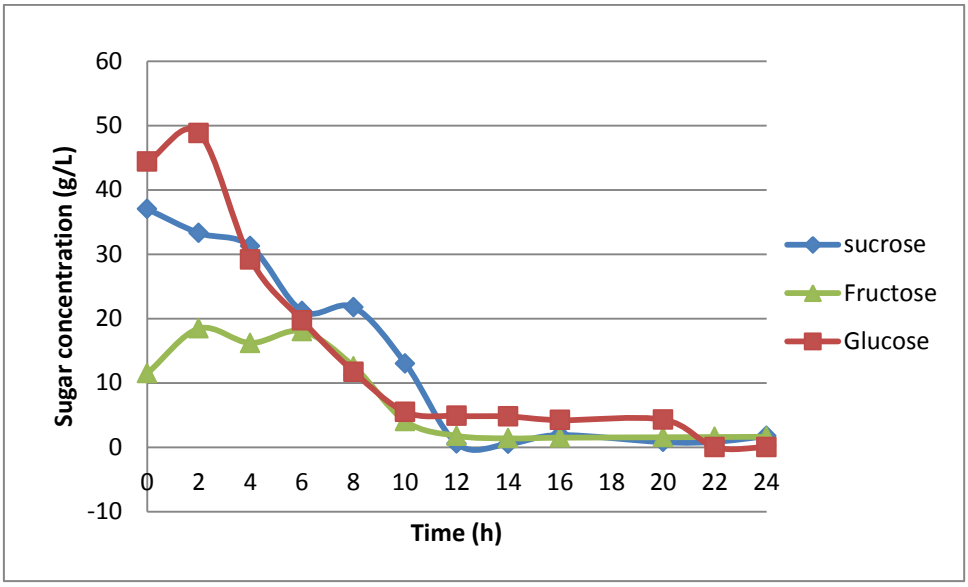


Figure 4.10: Sugar concentration vs time at 35°C

From the previous research towards oil palm trunk sap composition that has been done by Kosugi et al., (2010), they found that glucose was to be the dominant sugar in all parts of the trunk (inner, middle and outer) accounting for approximately 86.9%, 86.3%, and 65.2% (w/v) respectively. Their fermentation was almost complete after 12 hour and sugar was thoroughly consumed at 24 hour.

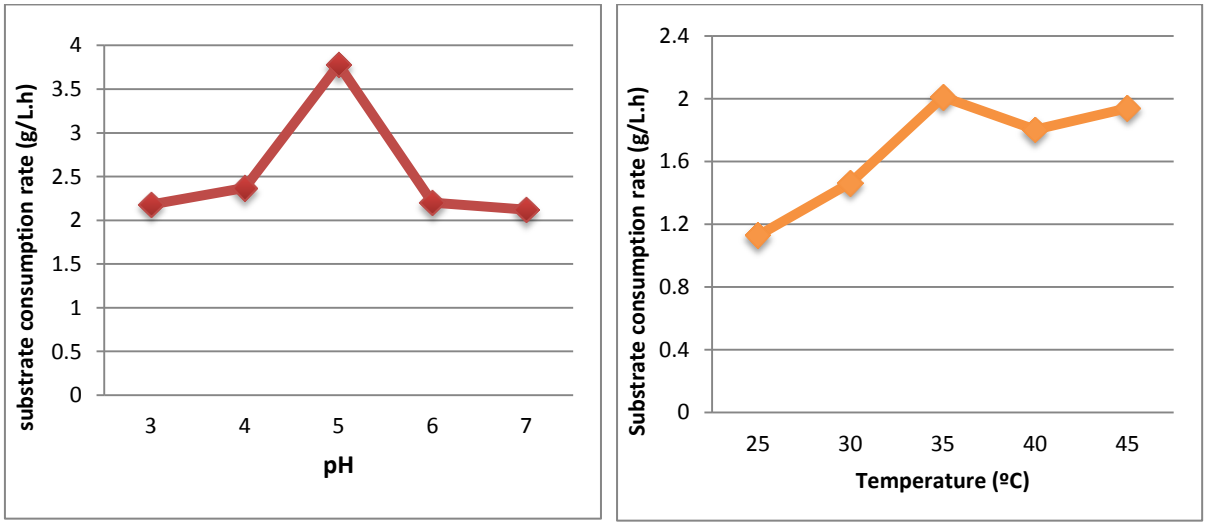
The sugars consumption rate, q_s, was described by batch culture kinetic as given in equation (3) and (4). The equation indicates that the rate at which substrate is consumed per unit volume is directly proportional to the cell concentration.

$$r_s = -\frac{dP}{dt} = \frac{\mu X}{Y_{x/s}} = q_s x \qquad (3)$$

$$q_s = -\frac{1}{X}\frac{dS}{dt} \qquad (4)$$

The value of q_s can obtained from experimental data of product concentration versus time. The value of ds/dt was obtained from the slope. From the value of slope ds/dt, the value of q_s X obtained. To obtain q_s, the value of slope ds/st was divided by the value of X at the time measurement, t_m. In this figure the value of slope is equal to the value of product formation $r_{s..}$

The substrate consumption rates, q_s, at different pH were calculated and presented in Figure 4.11 (a). The substrate consumption rate increased slightly as the pH increased from pH 3 until it reached maximum of rate 3.78 g/L.h at pH 5. When the pH was increased to pH 6 and 7, the substrate consumption rate would decrease. Figure 4.11 (b) shows the substrate consumption rate of sugar at different temperature. The consumption rate increased as the temperature increased from 25ºC until it reached maximum of rate of 2.01 g/L.h at 35ºC. As the temperature increased further from 35ºC, the consumption rate of sugar decreased.



(a) (b)

Figure 4.11: Substrate consumption rate, qs (g/L.h) at different pH 3, 4, 5, 6 and 7(a) and temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC (b)

Limtong *et al.* (2007) studied the production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *K. marxianus*. They found that sugar utilization further confirmed the low ethanol production and yeast cell growth. Ehen the experiment was conducted in the condition where the highest ethanol production and growth were obtained, gave the lowest sugar concentration remaining at the end of fermentation (7.05% w/v). However, under condition with the lowest ethanol production and growth, the highest concentration of remaining sugar (10.35% w/v) was observed. This show that when highest remaining sugar at the end of fermentation was observed, the sugar consumption for ethanol production was lower. From these study,

the highest consumption rate was at pH 5 an temperature 35ºC which was the highest growth rate and the highest ethanol formation rate at the similar condition.

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

This study examined the kinetic parameter that affect the bioethanol production using oil palm trunk sap by *Kluyveromyces marxianus*. Temperature (25ºC, 30ºC, 35ºC, 40ºC and 45ºC) and pH (3, 4, 5, 6 and 7) affect the production of bioethanol, specific growth rate and the consumption of sugar. Product formation rate at different pH and temperature increased as the pH and temperature increased until the maximum of ethanol formation reached 26.75 g/L at pH 5 and 45.06 g/L at 35ºC. Further increased of the pH and temperature would decrease the formation of bioethanol.

5.2 Recommendation

For the further study of bioethanol production, it is highly recommended that investigation on the effect of other parameters, such as different initial substrate concentration and agitation of fermentation should be carried out. This study will be important to gain deeper insight of the process for future commercialization in industrial scale.

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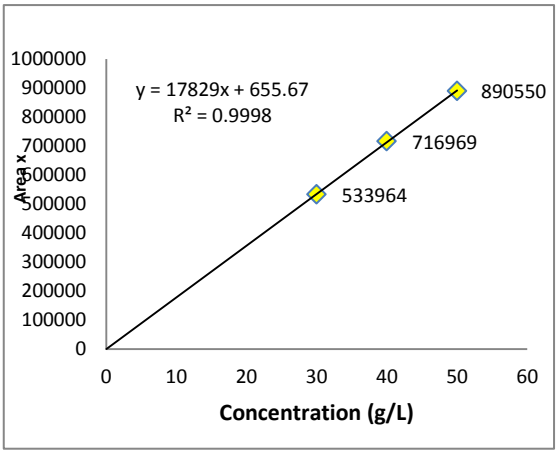
Appendix A

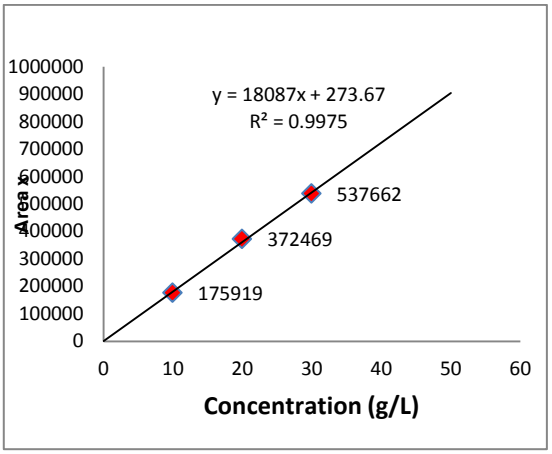
Raw Data

Table A.1: cell dry weight (g) of K.marxianus at temperature 25ºC, 30ºC, 35ºC, 40ºC and 45ºC and pH 3, 4, 5, 6 and 7.

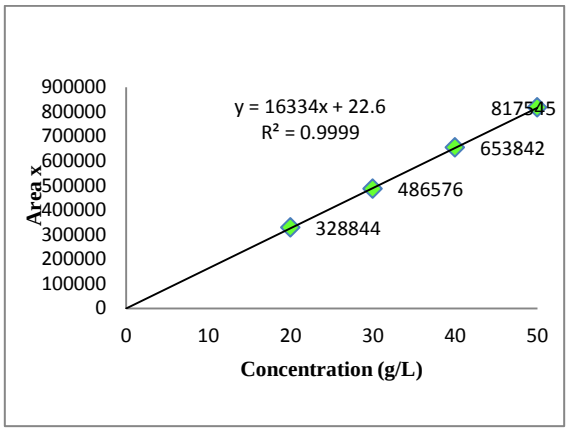
Time (h)	T=25ºC	T=30ºC	T=35ºC	T=40ºC	T=45ºC	pH= 3	pH= 4	pH =5	pH =6	pH =7
0	0.0012	0.0012	0.0013	0.0011	0.0012	0.0004	0.0006	0.0003	0.0004	0.0004
2	0.0018	0.0066	0.0004	0.0033	0.0019	0.0009	0.0005	0.0013	0.0013	0.0008
4	0.0052	0.0011	0.0041	0.0068	0.0005	0.0015	0.001	0.0017	0.0024	0.0022
6	0.0086	0.0026	0.0053	0.0057	0.0031	0.0003	0.0029	0.0022	0.0034	0.0029
8	0.0129	0.0064	0.0075	0.0072	0.0046	0.0003	0.0041	0.004	0.0044	0.0036
10	0.016	0.0091	0.0083	0.0064	0.0058	0.0032	0.006	0.0058	0.0053	0.0046
12	0.0115	0.0112	0.0102	0.007	0.005	0.0056	0.0071	0.0056	0.0068	0.006
14	0.0076	0.0125	0.0119	0.007	0.006	0.0048	0.0075	0.0073	0.0076	0.0054
16	0.0086	0.0135	0.0147	0.0069	0.008	0.0042	0.0075	0.0063	0.0105	0.0063
18	0.0092	0.0112	0.0172	0.0052	0.0055	0.0042	0.0058	0.0095	0.0132	0.0076
20	0.0088	0.0114	0.0122	0.005	0.0054	0.004	0.005	0.0087	0.0091	0.0065
22	0.009	0.0114	0.0129	0.0046	0.004	0.0044	0.005	0.0075	0.0093	0.0055
24	0.009	0.012	0.0111	0.0038	0.005	0.0038	0.0048	0.0089	0.0091	0.0043



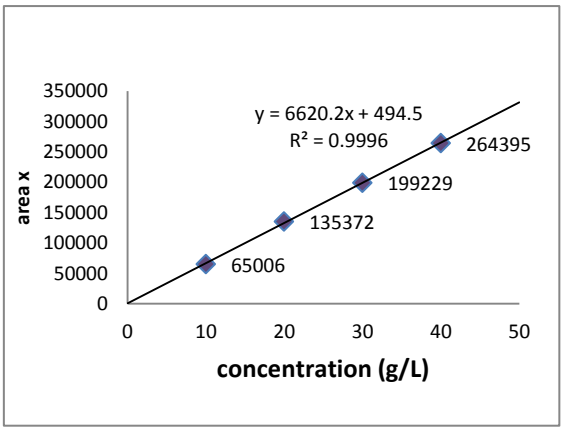
(a)



(b)



(c)



(d)

Figure A.1: Calibration curve of (a)Sucrose (b)fructose (c)glucose (d)ethanol

Table A.2: Effect of specific growth rate at different temperature and pH

temperature (ºC)	specific growth rate (µ)	pH	specific growth rate (µ)
25	0.109	3	0.199
30	0.106	4	0.285
35	0.313	5	0.822
40	0.21	6	0.168
45	0.16	7	0.123

Table A.3: Ethanol formation rate, qp (g/L.h) at different pH and temperature

pH	Product formation rate, qp (g/L.h)	temperature (°C)	Product formation rate, qp (g/L.h)
3	0.74625	25	0.498372093
4	0.966896552	30	0.704615385
5	1.409090909	35	0.899277108
6	0.811764706	40	0.409122807
7	0.661111111	45	0.584782609

Table A.4: Sugar consumption rate, qs (g/L.h) at different pH and temperature

pH	consumption rate, qs (g/L.h)	temperature (°C)	consumption rate, qs (g/l.h)
3	2.17875	25	1.128837209
4	2.369655172	30	1.464835165
5	3.778181818	35	2.013012048
6	2.203529412	40	1.803508772
7	2.123333333	45	1.94

Appendix B

Sugar concentration and ethanol concentration

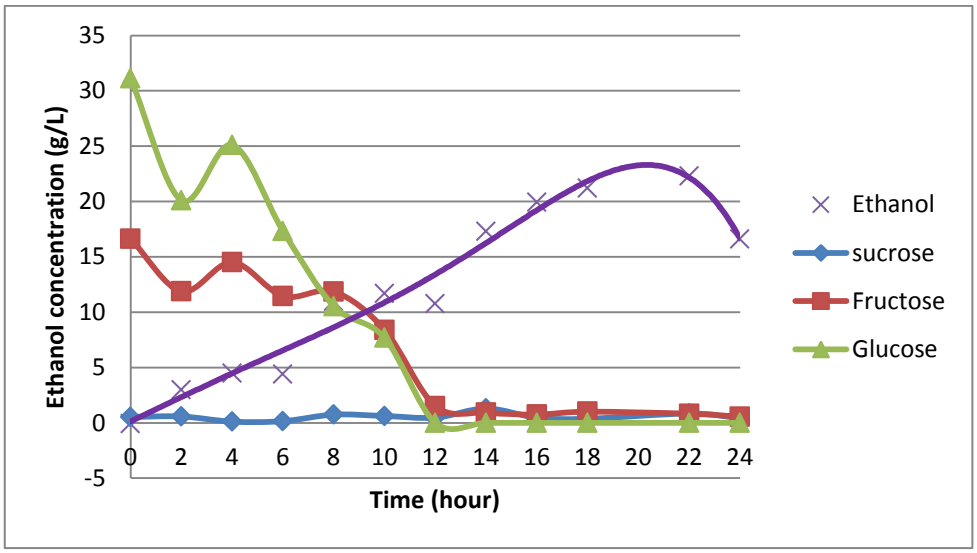


Figure B. 1: Concentration of sugar and ethanol (g/L) vs time (h) at pH 3

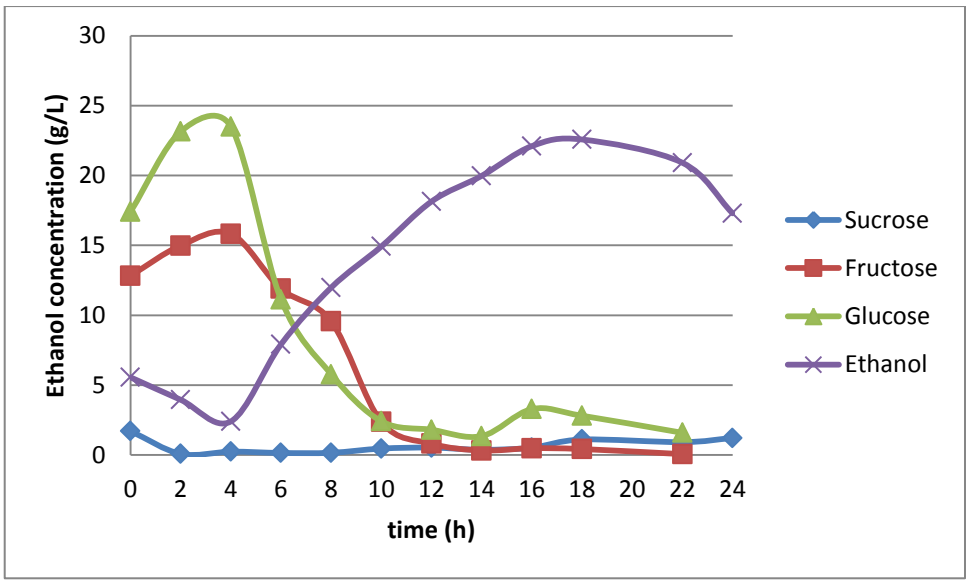


Figure B.2: Concentration of sugar and ethanol (g/L) vs time (h) at pH 4

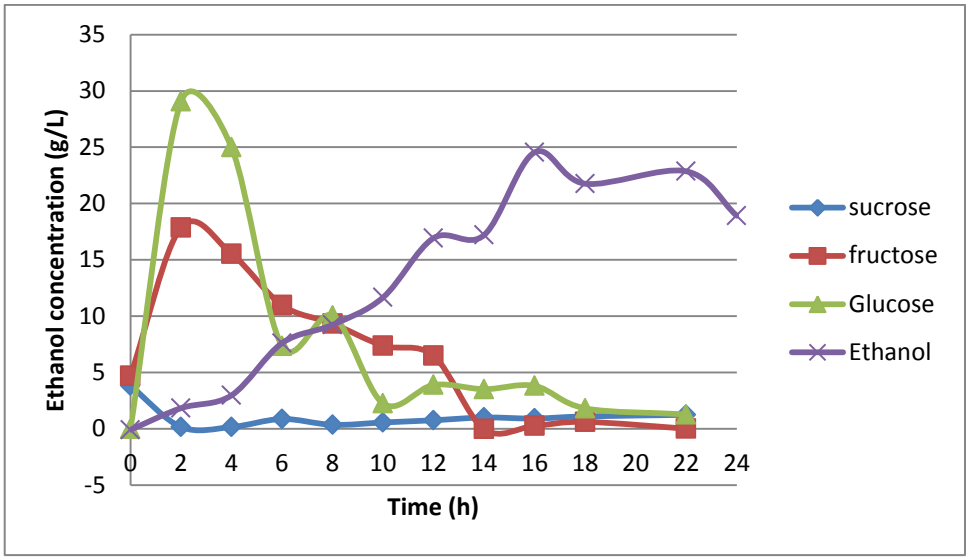


Figure B.3: Concentration of sugar and ethanol (g/L) vs time (h) at pH 5

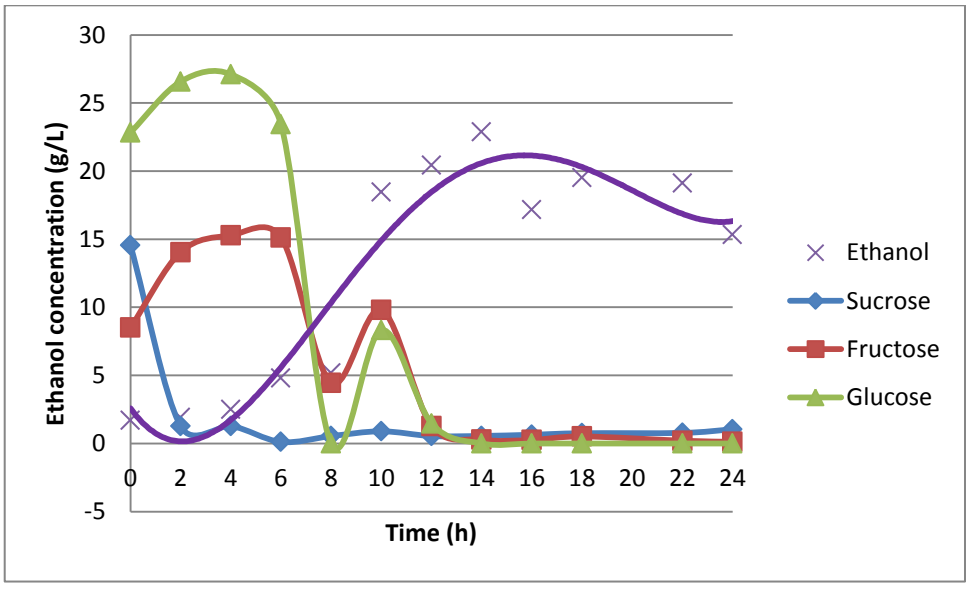


Figure B.4: Concentration of sugar and ethanol (g/L) vs time (h) at pH 6

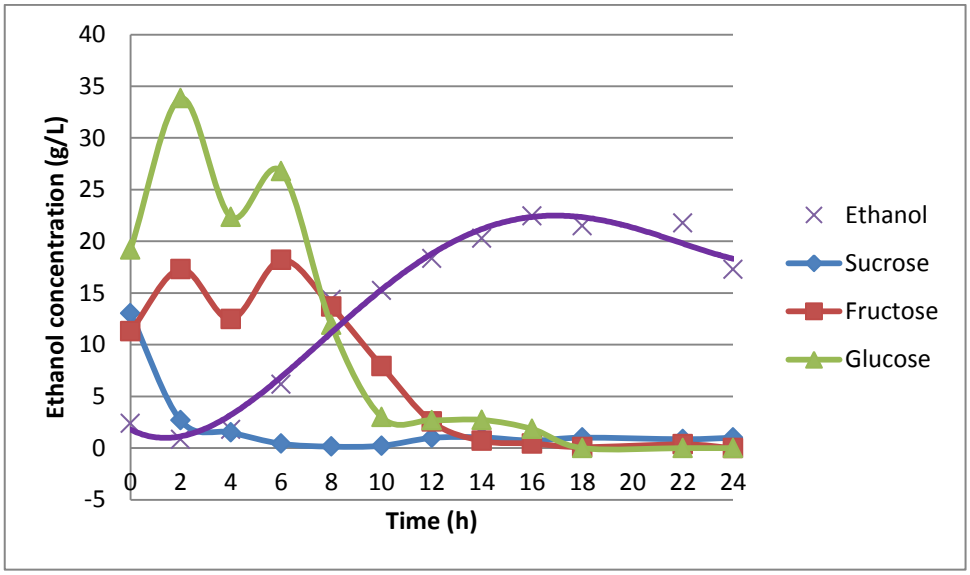


Figure B.5: Concentration of sugar and ethanol (g/L) vs time (h) at pH 7

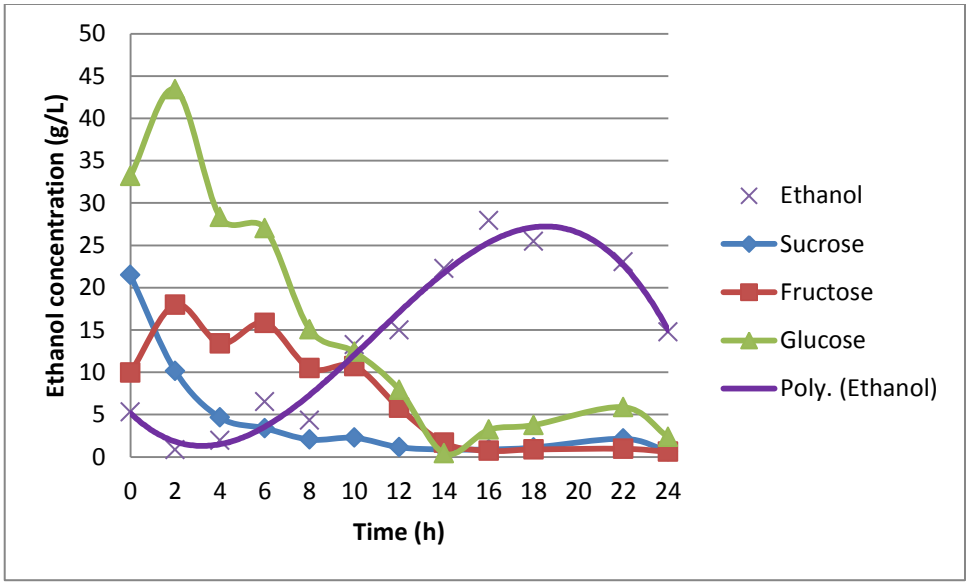


Figure B.6: Concentration of sugar and ethanol (g/L) vs time (h) at 25°C

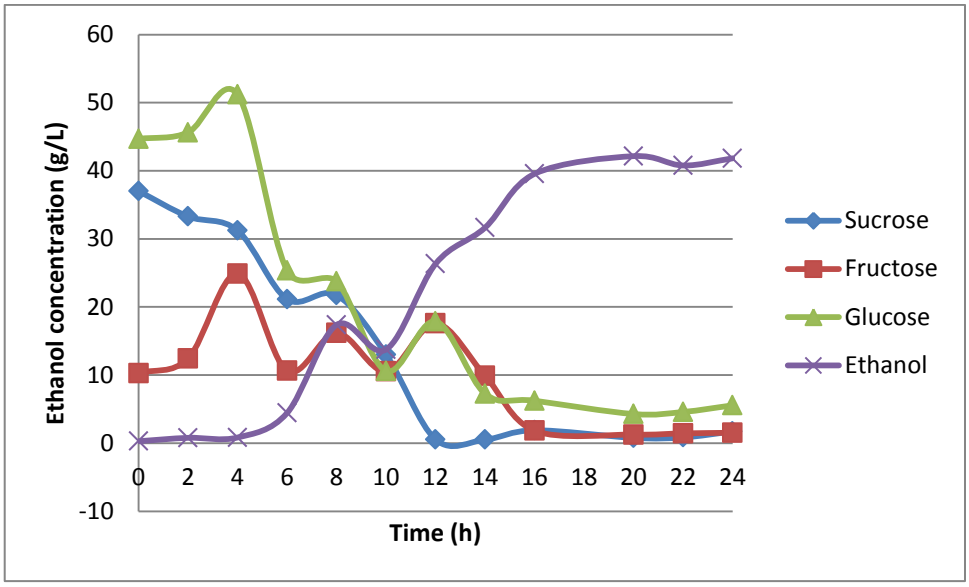


Figure B.7: Concentration of sugar and ethanol (g/L) vs time (h) at 30ºC

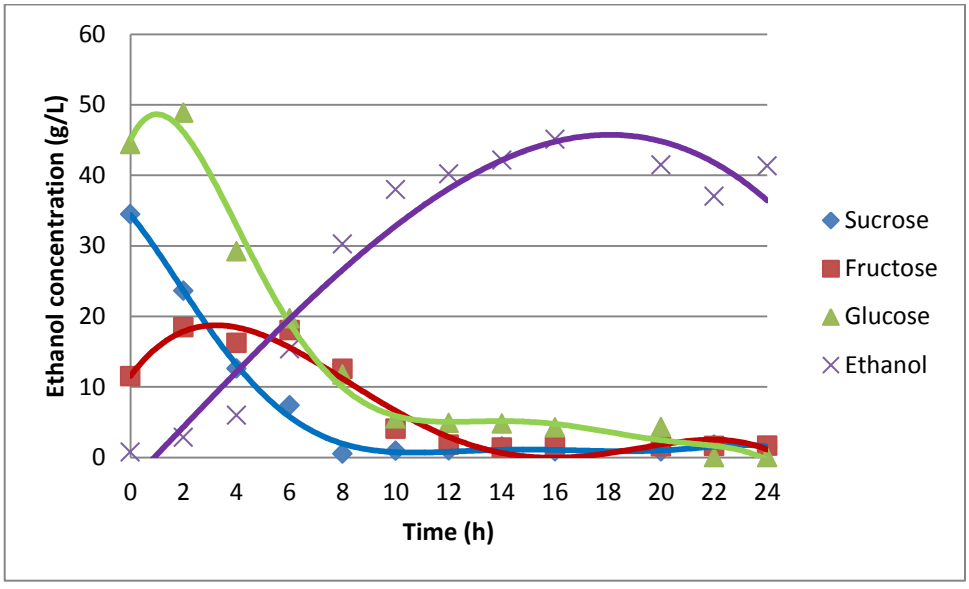


Figure B.8: Concentration of sugar and ethanol (g/L) vs time (h) at 35ºC

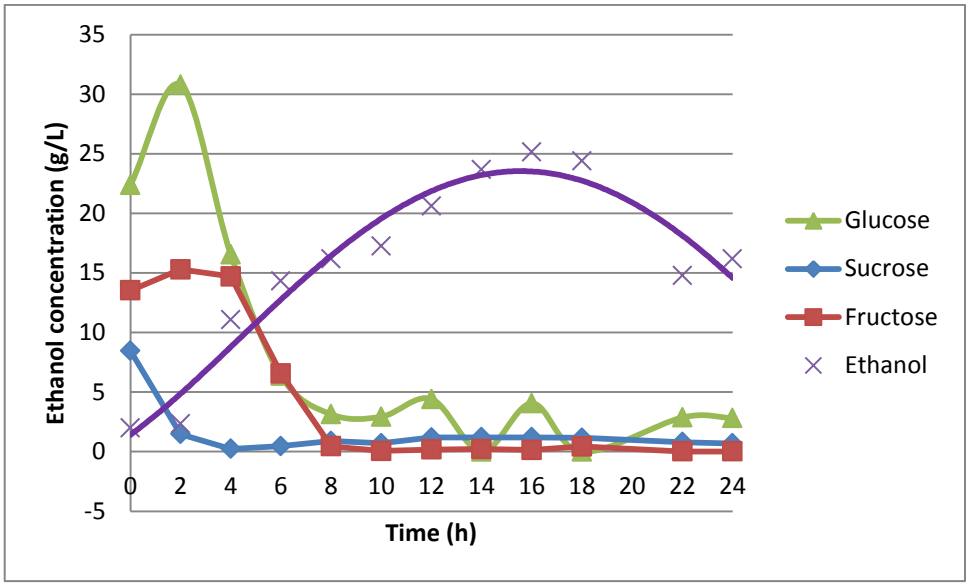


Figure B.9: Concentration of sugar and ethanol (g/L) vs time (h) at 40°C

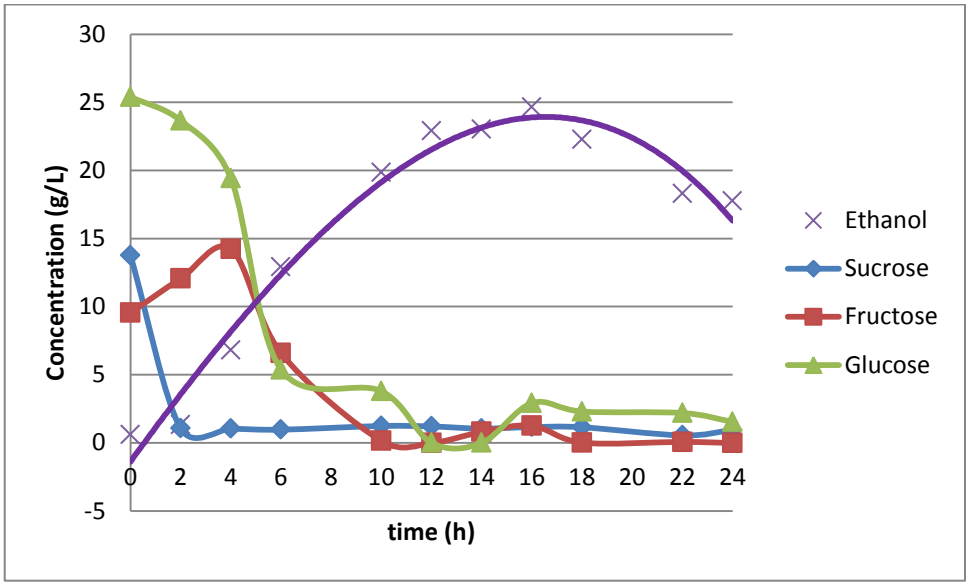


Figure B.10: Concentration of sugar and ethanol (g/L) vs time (h) at 45°C