UNIVERSITI PUTRA MALAYSIA

FERMENTATION CONDITION BY YARROWIA LIPOLYTICA FOR ERYTHRITOL PRODUCTION

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ABSTRACT

Erythritol is a type of sugar alcohol that can be used as a sugar replacement or sweetener. It is a member of the group known as sugar alcohols. A meal consisting of erythritol has close to close to twice as much energy as one consisting of sucrose. Erythritol is an excellent sugar replacement for diabetics since it has either no effect or very little effect on the release of insulin. The use of glucose as a substrate in the synthesis of erythritol is the most common method. With the assistance of yeast *Yarrowia lipolytica*, an erythritol production is being carried out in this research with the use of glucose as the substrate. The fermentation experiment was conducted by using the bioreactor with parameter profiles of 250 rpm for agitation speed, temperature at 30°C, 1.5 L/min of airflow, pH-controlled at 5.5 and 0.2% antifoam. At the end of the 50-hour fermentation procedure, the maximum amount of erythritol that was generated was reported to be 98.45 g/L. However, at 54-hour of fermentation, the amount of erythritol that was created began to diminish as a result of the depletion of glucose. The Monod method was utilised in order to arrive at the values for the saturation constant and the maximal specific growth rate. The highest possible specific growth rate for *Yarrowia lipolytica* was calculated to be 0.059 $hour^{-1}$.

ABSTRAK

Erythritol adalah sejenis alkohol gula yang boleh digunakan sebagai pengganti gula atau pemanis. Ia adalah ahli kumpulan yang dikenali sebagai Alkohol Gula. Hidangan yang terdiri daripada erythritol mempunyai hampir dua kali lebih banyak tenaga sebagai satu yang terdiri daripada sukrosa. Erythritol adalah pengganti gula yang sangat baik untuk pesakit kencing manis kerana ia tidak mempunyai kesan atau sedikit kesan terhadap pembebasan insulin. Penggunaan glukosa sebagai substrat dalam sintesis erythritol adalah kaedah yang paling biasa. Dengan bantuan yis *Yarrowia lipolytica*, pengeluaran erythritol sedang dijalankan dalam kajian ini dengan penggunaan glukosa sebagai substrat. Eksperimen penapaian dijalankan dengan menggunakan bioreaktor dengan profil parameter 250 rpm untuk kelajuan agitasi, suhu pada 30 °C, 1.5 l/min aliran udara, pH dikawal pada 5.5 dan 0.2% antifoam. Pada akhir prosedur penapaian 50 jam, jumlah maksimum erythritol yang dihasilkan dilaporkan 98.45 g/L. Walau bagaimanapun, pada 54 jam penapaian, jumlah erythritol yang dicipta mula berkurang akibat daripada kekurangan glukosa. Kaedah Monod telah digunakan untuk mencapai nilai -nilai untuk pemalar tepu dan kadar pertumbuhan khusus maksimal. Kadar pertumbuhan spesifik tertinggi untuk yarrowia lipolytica dikira menjadi 0.059 1/jam.

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LIST OF ABBREVIATIONS

μ	Specific growth rate
μ_{max}	Maximum specific growth rate
DCW	Dry cell weight
HPLC	High Performance Liquid Chromatography
K_S	Saturation constant
PPP	Pentose Phosphate Pathway
P_m	Maximum erythritol concentration
Q_P	Volumetric productivity
S	Substrate
t	time
$Y_{P/S}$	Erythritol yield on consumed glucose
$Y_{X/S}$	Biomass yield on consumed glucose

CHAPTER 1

INTRODUCTION

1.1 Background

The fast transformation of Malaysia's food and beverage sectors has had an impact on the amount of sugar and fat consumed by the country's people. The number of fast-food products that entered the Malaysian market has been increasing in the last decade. According to World Health Organization (2019), Malaysia has the highest percentage of obese and overweight males and females in Asia, at 64 percent and 65 percent, respectively. Among people ages 18 and above, the prevalence of diabetes grew from 11.6 percent to 17.5 percent over a period of nine years from 2006 to 2015. Therefore, sugar-free and low-calorie variants of food and beverages are frequently introduced by the food and beverage industries. The upshot is that many of these items feature sweeteners that can be used in place of ordinary table sugar or other nutritive sweeteners, and some of them are low- or zero-calorie options. Sugar substitutes can be classified into four categories, which are natural sweeteners, artificial sweeteners, novel sweeteners, and sugar alcohol (Mayo Clinic Staff, 2020). Erythritol is one of the alternative sweetener or sugar substitutes that belongs to a group of sugar alcohol. There is nearly twice as much energy in an erythritol meal as there is in sucrose. Since erythritol has no or little influence on insulin release, it makes a good sugar substitute for diabetics. In addition, erythritol is not a causative agent in tooth decay, either.

In nature, erythritol is a naturally occurring chemical compound. Seaweeds and fungi produce it as a metabolite or storage chemical, and a variety of common fruits, such as

melons, grapes, and pears, contain it as well. Other than that, fermented foods and beverages typically include erythritol as well. (Moon *et al.*, 2010). Erythritol can be produced through chemical and biotechnological methods. However, erythritol is now produced commercially by the fermentation process, usually with the help of yeast-like fungus genera. In addition, some lactic acid bacteria produce it. Yeast-like fungus genera have similar pathways to erythritol production. Erythritol is most commonly synthesized using glucose as a substrate. In this study, an erythritol production is using glucose as substrate with the help of yeast *Yarrowia lipolytica.*

1.2 Problem Statement

Yeast that isn't like any other because of its biotechnological potential, *Yarrowia lipolytica* has attracted much attention. *Yarrowia lipolytica* is a sugar alcohol and functional sugar producer in the food and beverage industries with intriguing features. Even though *Yarrowia lipolytica* has been commercially used in the fermentation process, there is less information about the growth kinetics. Previous study was done by using the yeast *Yarrowia lipolytica* that was produced as a result of the development of a straightforward medium that was made from the by-products of industrial waste. However, the current study is using the yeast *Yarrowia lipolytica* which has been granted as GRAS (generally recognized as safe) to be used in the industry. Growth kinetics are important because products can be accurately predicted using the growth-kinetic model of cell growth. Cell concentration is directly inversely proportional to the growth rate in growth kinetics, an autocatalytic reaction. Direct and indirect methods are used to determine the cell concentration. Approaches that use dry weight, turbidity (optical density), plate counts, and other direct methods to determine cell mass concentration and

density include these. The concentrations of proteins, DNA, and ATP are used to measure cell density indirectly (Sakthiselvan *et al.*, 2019). This study can obtain a limiting factor since glucose is used as a substrate. Moreover, the information through this study can be used as a scale-up in the industries that involves the fermentation process in their production by using glucose as a reference medium. In addition, the industries can change to other carbon sources such as bakery wastes, glycerol, and others.

1.3 Objectives

The objective of this study are as follows:

- To evaluate the production of erythritol by *Yarrowia Lipolytica* that is classified under GRAS (generally recognized as safe) by using glucose as a substrate.
- To study the kinetic parameters of yeast *Yarrowia Lipolytica in* erythritol fermentation.

1.4 Scope of Study

Five chapters make up this thesis. The introduction or Chapter 1 provides an overview of the study's origins and aims. Erythritol as a sugar substitute is described in detail. This chapter also contains the problem statement and objectives of the study.

Chapter 2 summarizes prior research and theories on the erythritol production process. As erythritol plays an important role in food and pharmaceutical industries, this chapter explains the advantages of erythritol. To better understand the behavior of *Yarrowia Lipolytica*, a group of kinetic models is included in this chapter to discuss in detail.

Specifically, Chapter 3 discusses the general materials and methods that were employed in this research, which include microorganisms, fermentation in a batch bioreactor, and general analysis methodologies of performance in this study.

The bioreactor fermentation process and conditions are described in detail in Chapter 4. In addition, this chapter examined and studied fermentation kinetic behaviours.

Conclusion and recommendations for future work are provided in the final chapter, which is concise and to the point.

CHAPTER 2

LITERATURE REVIEW

2.1 Erythritol Background

The Scottish scientist John Stenhouse (1809-1880) made the discovery of erythritol in 1848. He was interested in the medical and technical advances that were spurred on by new discoveries of chemical compounds in plants. His contributions to sugar production, dyeing, impregnation, and tanning are numerous and noteworthy. When it comes to purifying the air and eliminating unpleasant odours, he is most well-known for his charcoal air filters and breathing masks.

Stenhouse conducted research on the lichen *Roccella Montagnei*, which originated in southern Africa, in the mid-19th century. He was eventually successful in generating crystalline crystals of the drug, which he termed erythritol after the different chemical methods that he used to generate them. Pseudo-orcin, as the chemical was originally known by Stenhouse, has an extremely sweet taste, according to Stenhouse. While being heated on platinum foil, it burns with a blue flame and emits a faint caramel-like scent. Both water and alcohol are capable of dissolving the material (Petersson and Barregren, 2019).

Traces of erythritol were discovered in blackstrap molasses that had been fermented by yeast in 1950, exactly one hundred years after Stenhouse's discovery of the chemical. This resulted in the development of the process that is currently used to manufacture erythritol. The fermentation of glucose results in the production of erythritol, as opposed

to other polyols, which are created from different sugar types by adding hydrogen (Petersson and Barregren, 2019).

2.2 Erythritol

In the class of sugar alcohols known as polyols, erythritol ((2R,3S)-butan-1,2,3,4-tetrol) is a sugar alcohol that is generated as a result of the hydrolysis of the aldehyde or ketone group in different carbohydrates. There are abundant polyols found in a number of fruits and vegetables such as grapes and mushrooms, as well as in fermented foods such as soy sauce and miso (fermented soy sauce).

Erythritol is a sugar alcohol that performs an unusually important role within the sugar alcohol family. As a result of having only four carbon atoms, it has the lowest molecular weight of any of the sugar alcohols, and as a result, it exhibits slightly different physical and chemical properties from the others. Asymmetrical molecules, such as erythritol, can only exist in one form: the meso-form, which is the most common. It crystallises into anhydrous crystals with a mild sweetness due to the presence of 60–80 percent sucrose. The absence of any aftertaste, on the other hand, has the advantage of allowing it to be blended with more powerful sugars (Regnat *et al.*, 2018).

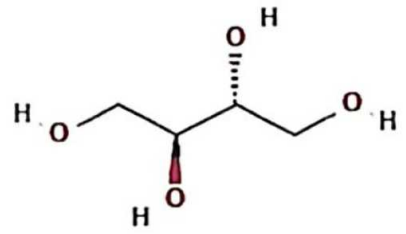

Figure 2.1: Two-dimensional structure of erythritol.

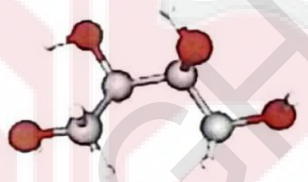

Figure 2.2: Three-dimensional conformer of erythritol.

2.2.1 Erythritol as Sugar Substitute

The effectiveness erythritol as sugar substitutes in the treatment of diabetes has been investigated by researchers. Erythritol have gained popularity not only because of their potential as a sugar alternative and their well-documented favourable effects on oral health, but also because of their wide range of health advantages, which include hypoglycaemic, anti-hyperglycaemic, anti-diabetic, and anti-obesogenic actions. A study by Flint *et al.* (2014) proved that erythritol has been shown to increase endothelial function and reduce central aortic stiffness. In addition, the findings of their study lend credence to

the innovative hypothesis that erythritol may be a preferable non-nutritive sweetener for the dietary treatment of type 2 diabetes.

2.2.2 Erythritol in Food Applications

In food industry, sweeteners can be classified into two different groups which are natural sweeteners and synthetic sweeteners. Polyols are produced as a byproduct of the hydrogenation of reducing sugars because this process results in the creation of an alcohol group in place of the carbonyl group. Polyols are naturally occurring sweeteners that have found application in the food industry (Martău *et al.*, 2020). Because of its ability to lower water activity, act as humectants, and give texturizing qualities, polyols are important in a variety of applications. They are also used as flavouring solubilizers and sugar crystallisation mediators, among other things. They do not cause Maillard reactions, however, because erythritol (polyol) is not a reducing sugar, which means they do not cause Maillard reactions. The vast majority of Maillard reactions result in favourable qualities, such as browning in baked goods, which is sought in many applications. Polyols, on the other hand, are excellent in situations where non-reactivity is desired (such as the preservation of colours, flavours, and enzymes, for example).

2.2.3 Erythritol in Pharmaceutical Industry

Erythritol is not only used in food applications but also in pharmaceuticals industry. In order to manufacture tetranitro erythritol, erythritol is utilised as a starting material. Tetranitro erythritol is a medicine that is used to treat asthma and angina pectoris (Röper and Goossens, 1993). Moreover, because of its low reactivity, minimal moisture absorption, and excellent solubility, erythritol has the potential to be employed in

pharmaceutical applications (Hoashi *et al.*, 2013). Glucagon was administered via inhalation through an erythritol-based powder formulation, according to Endo *et al.* (2005), who found that effective emission of the powder into the lungs was obtained.

2.2.4 Erythritol biosynthesis in yeast

The biosynthesis of erythritol from glucose by yeast is mainly through a pentose phosphate pathway. There are two separate steps to this metabolic process. The first oxidative step produces NADPH and ribulose-5-phosphate. During the non-oxidative phase, eryth-rose-4 phosphate is the end product. The final steps of the proposed route are the dephosphorylation of eryth-rose-4 phosphate to eryth-rose, followed by the reduction of eryth-rose to erythritol using one molecule of NADPH (Rzechonek *et al.*, 2018). Moreover, according to Hootman et al. (2017), hexokinase catalyses the phosphorylation of glucose, which is followed by the oxidation of glucose to gluconate-6-phosphate in the oxidative branch of the PPP. Gluconate-6-phosphate dehydrogenase is responsible for the oxidative decarboxylation reaction, which results in the production of ribulose-5-phosphate. At this stage, the first carbon atom of the glucose is converted into carbon dioxide. In the reductive portion of the PPP, further conversions carried out by transketolase and transaldolase ultimately result in the production of erythrose-4-phosphate. The subsequent process involves first removing the phosphate so that eryth-rose can be generated, and then reducing the eryth-rose to produce erythritol. The biochemical pathway of erythritol from glucose by yeast is shown in the Figure 2.3 along with other various substrates.

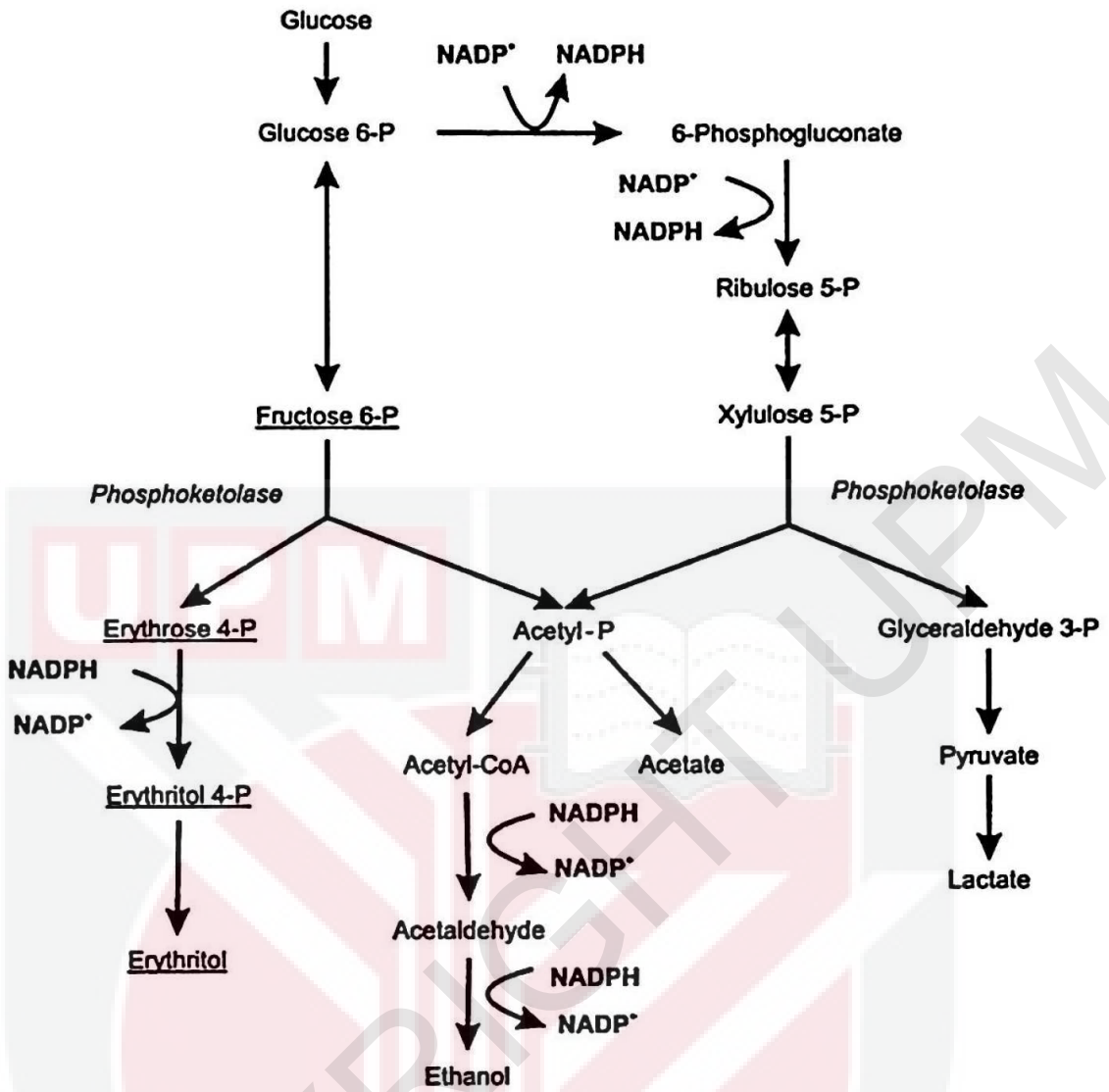

Figure 2.3: Erythritol synthesis in yeast.

2.3 Glucose

Glucose is the most essential form of carbohydrate sustenance that the body may have in order for it to carry out its functions appropriately. During a condition of fasting, gluconeogenesis and glycogenolysis are crucial for maintaining blood glucose levels. However, during a fed state, the bulk of circulating glucose is obtained from the meal. The majority of the glucose that is consumed comes from more complex carbs, which, during the digestion process, are reduced into monosaccharides rather than being taken directly from the source of the glucose (as glucose). The remaining half of the total carbohydrates in the diet is primarily composed of simple sugars and other simple carbohydrates.

Polysaccharides account for roughly half of the total carbohydrates that are present in the diet. A disaccharide that is made up of glucose and fructose, sucrose is responsible for approximately two-thirds of the sugar that is consumed in one's diet. As a monosaccharide, glucose is distinguished from other sugars by the fact that it cannot be broken down further through the process of hydrolysis. It is further categorised as a hexose due to the presence of a six-carbon skeleton, and as an aldose due to the presence of an aldehyde group on carbon 1. It is also classed as an aldose due to the presence of an aldehyde group on carbon 1. The aldehyde group condenses with the hydroxyl group, resulting in the formation of a hemiacetal ring structure in the presence of glucose. The ring structure of glucose is responsible for many of the reactions that occur (McMillin, 2005).

As soon as glucose enters the body, it circulates through the bloodstream and is carried to the tissues that require energy to function properly. There, glucose is decomposed through a number of metabolic processes, with the energy generated as a result of the process being stored in the form of ATP. When these processes take place in the body, the ATP produced by them is required to fuel virtually every energy-intensive process that requires energy. Eukaryotes obtain the vast majority of their energy from aerobic (oxygen-requiring) activities that begin with the breakdown of glucose molecules in the cell membrane. An initial step in the breakdown of glucose occurs during the anaerobic process of glycolysis, which leads in the production of some ATP as well as the end-product pyruvate, among other things. Reduction, which occurs in anaerobic environments, is responsible for the conversion of pyruvate to lactate. The pyruvate can enter the citric acid cycle under aerobic conditions, resulting in the synthesis of energy-

dense electron carriers, which are then used to aid in the production of ATP at the electrochemical transfer chain (Hantzidiamantis and Lappin, 2021).

2.4 Fermentation Process for Erythritol Production

By breaking down a carbohydrate such as starch or sugar into simpler sugars, an organism can convert a carbohydrate such as starch or sugar into alcohol or acid. For example, yeast obtains energy through fermentation by converting sugar into alcohol, which is subsequently utilised to feed the organism. Bacteria are responsible for fermentation, which is the conversion of carbohydrates into lactic acid (Helmenstine, 2020). Many studies have been made by researchers to produce erythritol by using different yeast strains. With the use of an osmophilic mutant of *Candida Magnoliae*, Ryu et al. (2000) developed a fed batch culture approach that increased erythritol yields by tenfold. Over half of the glucose that was provided was converted into organic acids and cell mass throughout the manufacturing stage, accounting for more than half of the final product's weight. As a result, when the fed-batch technique of conversion was used, just 23 percent of the erythritol conversion yield was obtained. Jeya and colleagues (2009) discovered an erythritol-producing strain of the bacteria *Pseudozyma tsukubaensis* with a high erythritol content and named it P. tsukubaensis KCCM 10356. Their research focused on the features of a novel yeast strain that produces a high yield of erythritol (61% g/g) while producing no undesirable by-products such as glycerol or ribitol. Moreover, the fermentation process was successfully scaled up from seven to fifty thousand litre fermenters for the purpose of industrial production of erythritol. Moreover, in a study by Kim et al. (2000), the erythritol produced by *Torula sp.* outperforms the competition in a number of ways. First and foremost, this is the first time that *Torula sp.* has been shown

to manufacture erythritol. Second, among the erythritol-producing microorganisms tested, the maximum output of erythritol was produced by a strain of *E. coli.* In addition, because this strain did not produce undesirable by-products such as glycerol and ribitol, it has the potential to be used on an industrial scale.

Recently, it was discovered that *Yarrowia lipolytica* is also a potent producer of erythritol, which was previously unknown. According to Tomaszewski-Hetman and colleagues (2020), an innovative low-cost technology for erythritol production by the yeast *Yarrowia lipolytica* was developed based on the invention of a straightforward medium made from industrial waste by-products. The bacteria *Yarrowia lipolytica* A-6 was able to create 108.0 g/L of erythritol at a rate of 1.04 g/L/h and a yield of 0.45 g/g of the medium, which comprised just 220 g/L of crude glycerol produced from biodiesel production and 40 g/L of maize steep liquor in a production process. Furthermore, when a portion of crude glycerol was exchanged for a portion of the crude fraction of fatty acids in the two-steps procedure, a concentration of erythritol (108.1 g/L) that was comparable to that obtained in the first stage was obtained. Table 2.1 shows summary of the previous researchers on production of erythritol.

Table 2.1: Erythritol production by using different yeasts

No.	Yeast	Findings	References
1	*Moniliella* sp. N61188-12	The generation of erythritol from batch fermentation of strain N61188-12 in jar fermenters at two scales, 250–2000 L, in 35 percent glucose and 1 percent yeast extract was 151.4 g/L and 152.4 g/L, respectively.	Lin et al. (2010)
2	*Yarrowia lipolytica*	This is the first report that describes using *Yarrowia lipolytica*'s to manufacture erythritol and utilize industrial raw molasses and glycerol. This process was using 2-stage fermentation. The findings of this investigation, the growth of the yeast Y. lipolytica had the potential to produce 52–114 g/L of erythritol. The productivity ranged from 0.58 to 1.04 g/L/h, whereas the yield ranged from 0.26 to 0.57 g/g. The yield of the final biomasses ranged from 17 to 41 g/L.	Mirończuk et al. (2015)

3	*Yarrowia lipolytica* Wratislavia K1.	This study used peptone as nitrogen source for erythritol production from glycerol by Wratislavia K1 strain of Y. lipolytica. Their best results were obtained by using peptone at a concentration of 2 g/L and sodium chloride at a concentration of 5 percent. This combination resulted in the production of 18.2 g/L of erythritol, which corresponded to a yield of 0.23 g/g, a volumetric productivity of 0.11 g/(Lh), and a specific production rate of 0.010 g/(gh)	Tomaszewska (2012)

2.5 Factors affecting erythritol production in fermentation process

There are few factors affecting erythritol production in fermentation process. According to few research, pH and temperature and aeration among the factors that impact the initiation and progression of fermentation.

2.5.1 pH and temperature

The pH of the solution has a substantial impact on the yield of the result. In all media containing varying quantities of sugars, a medium pH of 5.5 resulted in the highest production yield in all cases. *Saccharomyces cerevisiae* is an acidophilic organism, which

means that it thrives in acidic environments and pH range that is best for yeast growth can range from 4 to 6. Even though reducing the pH of the medium has been shown to greatly reduce the proliferation and metabolism of contaminating bacteria, it has also been shown to reduce the efficiency of converting sugars by yeast, resulting in a reduction in yield during fermentation. A greater sugar content mash can be set at the optimal pH range for yeast production, which is 5.0-5.5, allowing the bacteria to be regulated efficiently and the maximum amount of yield production by the yeast to be reached (Narendranath and Power, 2005). Moreover, when cultivated in a glucose-based medium, the *Yarrowia lipolytica* strain demonstrated greater productivity at a pH of 5.5 (Carly and Fickers, 2018). Thus, the best pH for fermentation process is 5.5.

In yeast, the rate of growth different depending on the temperature. At 25 and 30°C, the typical growth curve with a sequence of short-lag, exponential, stationary, and decline phases was observed. However, at temperature of 35°C, a significant amount of yeast died. Temperature affects fermentation kinetics, which was responsible for determining the chemical makeup of the final product (Torija *et al.*, 2003). Phisalaphong et al. (2006) reported that the effect of temperature on *S. cerevisiae* fermentation is illustrated by the fact that the biomass and production yield rates were slightly increased by increasing the isothermal control temperature from 30 to 33°C, but the rates were significantly decreased at 35°C. When the temperature is higher than 35°C, cell growth and output yield are significantly reduced. According to Gonçalves et al. (2014), the highest temperature at which *Yarrowia lipolytica* can thrive is lower than 34 degrees Celsius. Hence, temperature of 30°C is the most suitable for fermentation process by yeast *Yarrowia lipolytica*.

2.5.2 Aeration

Aeration's purpose in fermentation is to maintain the integrity of the culture broth while simultaneously supplying oxygen to and removing carbon dioxide from microbial cells that are suspended in the culture broth. Aeration rate is frequently a determinant of the rates of cell development and product synthesis. In order to determine the effect of dissolved oxygen on the formation of erythritol, two distinct tactics were used: constant aeration and stirring rates and regulated dissolved oxygen concentration. The findings by Machado (2018) revealed that the dissolved oxygen concentration in the medium is a critical component that determines the erythritol production of the bacteria. The batch run at high constant stirring and aeration rates, 900 rpm and 3 vvm, yielded the highest concentration of erythritol (32 g·L-1), yielded the highest yield (0.43 g·g-1), and productivity (0.44 g·L-1·h-1) of any of the experiments.

2.6 Kinetic Model

It is possible to obtain information about the kinetic-metabolic properties of a fermentation process using fermentation models. Aside from this, they can be utilised to assist in the control and optimization of the fermentation process in general. The rates of glucose intake, erythritol generation, and population increase of the microorganism *Yarrowia lipolytica* may also be predicted using a kinetic model, according to the researchers.

2.6.1 Specific Growth Rate

Specific growth rate is the most important metric to regulate during the fermentation process because it depicts the dynamic behaviour of microorganisms. It is also the most difficult parameter to govern. As long as the substrate is present in adequate proportions, it is possible to indirectly manage the extracellular environment by employing specific growth rate as a control parameter. This is generally true in most situations, although it is not always the case. Using proper models based on cell growth kinetics, it is possible to estimate the specific growth rate of a given cell and this can be expressed as,

$$r_x = \mu X \qquad (1)$$

or

$$\frac{dX}{dt} = \mu X \qquad (2)$$

where r_x is the rate of cell growth, μ is the specific growth rate (h^{-1}), x is the cell concentration ($g\ L^{-1}$), and t is the time.

2.6.2 Monod Model

Monod's model (1979) connects the growth rate to the concentration of a single growth-controlling substrate, S, through the use of two parameters: the maximum specific growth rate (μ_{max}) and the substrate affinity constant (Ks). The Monod equation states that,

$$\mu = \frac{\mu_{max} S}{K_S + S} \qquad (3)$$

Where S is the concentration of the limiting substrate (g L^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), and K_s is the saturation constant (g L^{-1}).

2.6.3 Lineweaver-Burk method

The Monod equation was turned into an equation for a straight line by Lineweaver and Burk. From the method, cell growth saturation coefficient, K_s and μ_{max} can be predicted and the equation can be represented by taking reciprocals of both sides of the equality sign,

$$\frac{1}{\mu} = \frac{K_S}{\mu_{max} S} + \frac{1}{\mu_{max}} \qquad (4)$$

The Lineweaver-Burk plot of $1/\mu$ versus 1/S and yields a straight line with a slope equal to K_s/μ_{max} and a y-intercept equal to $1/\mu_{max}$.

CHAPTER 3

METHODOLOGY

3.1 Microorganisms and Media

Microorganism used was osmophilic yeast, *Yarrowia lipolytica* (DSMZ 705662) and was brought from Leibniz Institute DSMZ – German Collection of Microorganism. The yeast was kept at 4°C in a chiller. In order to get the *Yarrowia lipolytica* stock culture ready for fermentation, it was grown by using streak plate technique for isolation on Yeast Peptone Dextrose agar plate at 30 degrees Celsius and was incubated for twenty-four hours in an incubator. This was done in order to achieve a healthy development of yeast. For broth preparation, broth powder was weighed and put into a conical flask that has distilled water in it. After ensuring that broth powder had a sufficient amount of time to fully dissolve in the distilled water, it was afterwards poured into bottles and sterilised using an autoclave. Thus, medias used for inoculation of starter fermentation culture were Yeast Peptone Dextrose (YPD) broth and agar (BioBasic, Canada). For the YPD media, Yeast extract (10 g/L), Peptone (20 g/L), and D-glucose (20 g/L) were used to make the broth, and an additional agar (15 g/L) was used to make the agar plate for the experiment.

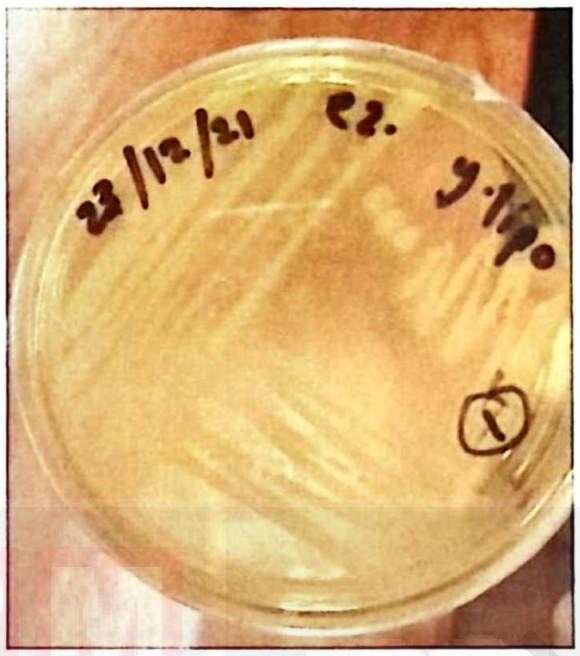

Figure 3.1. *Yarrowia lipolytica* on Yeast peptone dextrose agar.

3.2 Inoculum Preparation and Medium Composition

When preparing the inoculum, a single colony of *Yarrowia lipolytica* on yeast peptone dextrose agar was picked up and inoculated with 100 mL broth in a 250 mL Erlenmeyer flask. Afterwards, the inoculum was incubated at 30°C in a shaker incubator with an agitation speed of 200 rpm for 24 hours. Fermentation media was prepared that consists of 10 g/L glucose, 0.5 g/L ammonium sulphate, 0.5 g/L magnesium sulphate heptahydrate, 0.5 g/L potassium dihydrogen phosphate, 30 g/L sodium chloride and 0.5 g/L yeast extract. After 24 hours, the inoculum then was transferred to fermentation media.

Figure 3.2. Incubation of inoculum in a shaker incubator.

3.3 Batch Fermentation in Bioreactor

A 2-L stirred tank bioreactor with 1.5-L working volume was used in this experiment (Figure 3.3). After assembled the bioreactor, the system was checked up to make sure that it can be operated smoothly. Afterwards, the vessel of the bioreactor was autoclaved with and without the media. Autoclave was done to sterilize the equipment. Jacketed glass vessels with stainless steel top plates and baffle inserts were used to construct a bioreactor culture vessel. Control systems for pH, temperature, dissolved oxygen tension (DOT) foam, and mixing were installed in the bioreactor to monitor the process. To introduce acid, alkali, foam, inoculums, and aeration, the top plate has holes cut out for pH and dissolved oxygen tension electrodes. The peristaltic pump controlled the addition of acid, alkali, antifoam, and feed medium. Temperature in the fermenter was controlled by an external thermostat jacket with a circulation pump, while pH was controlled by the injection of 1M NaOH to the growth medium. 0.2 percent antifoam was

used to control the foaming that occurred throughout the fermentation process. An impeller installed in the middle of the stainless-steel top plate allowed for easy mixing. A six-blade turbine impeller and a rotary shaft were used to stir the mixture. The bioreactor's monitoring and control systems were all shown on a single screen. The system was operated with parameter profiles of 250 rpm for agitation speed, temperature at 30°C, 1.5 L/min of airflow, pH-controlled at 5.5 and 0.2% antifoam. In addition, the bioreactor was operated for 72 hours (3 days). According to Carly and Fickers (2018), when grown in a medium consisting of glucose, the *Yarrowia lipolytica* strain showed significantly increased levels of productivity at a pH of 5.5. Therefore, pH was controlled at 5.5 during the experiment. In addition, Goncalves et al. (2014) stated that the highest temperature at which *Yarrowia lipolytica* may survive and grow is lower than 34 degrees Celsius. Hence, temperature of 30°C is the best temperature for the yeast to generate high productivity of erythritol in this study. After 72 hours, the bioreactor system was disassembled to clean all the parts manually or by using sonicator bath.

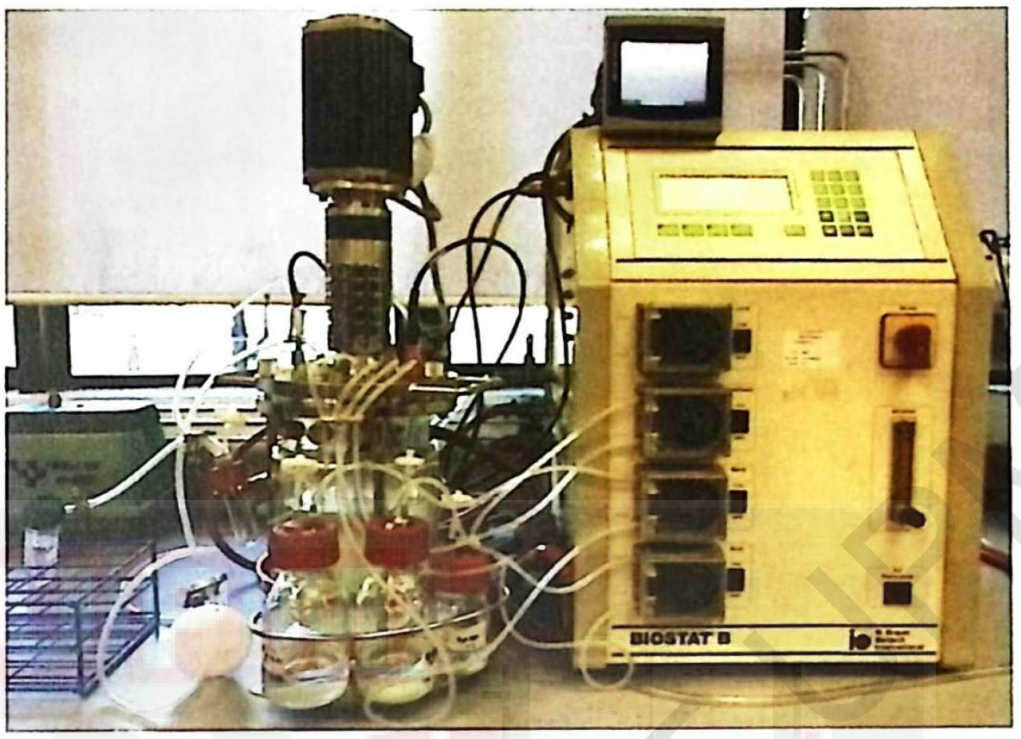

Figure 3.3. 2-L bioreactor with 1.5-L working volume.

3.4 General Experiment Plan

Figure 3.4 depicts an overview of the experimental work carried out in this study.

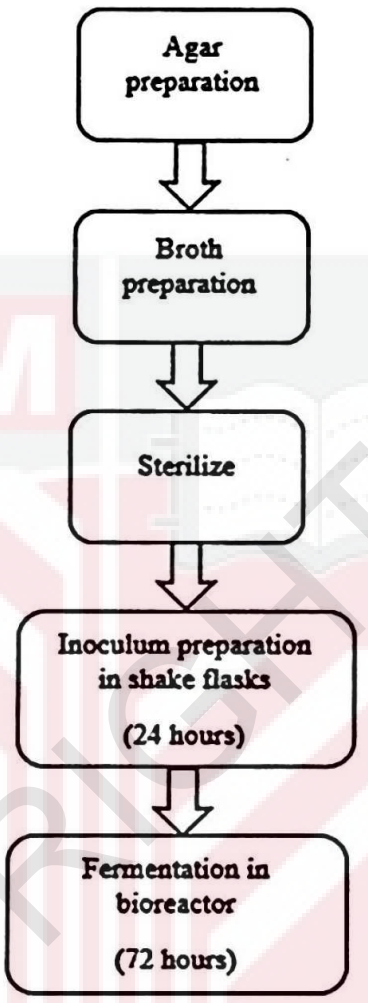

Figure 3.4. The flow diagram of the experiment works.

3.5 Analytical Procedure

Four 10 mL culture samples were collected from Day 0 to Day 3 for chemical analysis during the fermentation process. The supernatants from two of the tubes were collected after centrifugation and were kept at -20°C in a chiller. A glucose and erythritol concentration were then determined using the supernatant that had been collected. The pellets were employed to measure dry cell mass. The optical density was measured in two more tubes to determine cell concentration.

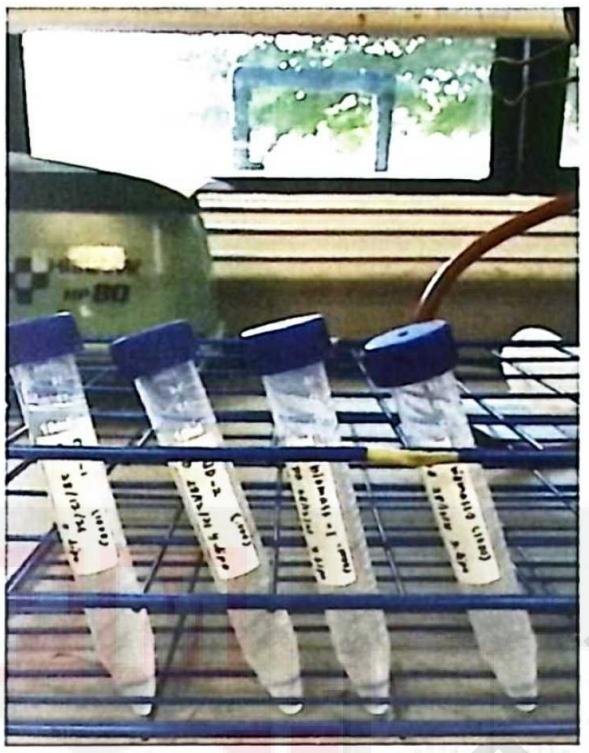

Figure 3.5. Samples collected at the required time interval.

3.6 Cell Concentration

Optical density at 600 nm and fermentation media as a blank were used to estimate cell concentration. With the use of a pipette, the samples from two tubes were transferred into a plastic cuvette. The UV-visible spectrophotometer was then used to measure the cell density's absorbance.

3.7 Determination of Glucose and Erythritol Concentration

Two tubes of samples were centrifuged at 5000 rpm for 10 mins. The supernatants then were filtered with a 0.22 μm membrane filter. The supernatants obtained then were used to measure the concentration of glucose and erythritol. The concentrations were measured by using Agilent High-Performance Liquid Chromatography (HPLC) using RPM monosaccharides Phenomenex Pb^{2+} column. The mobile phase consisted entirely of water and had a flow rate of 0.6 mL/min. At a temperature of 75 °C, 20 uL of the sample

was introduced into the system, and it was identified using the RI detector. The sample was put into the system at a temperature of 80 °C in order to get an accurate reading of the glucose concentration.

3.8 Determination of Dry Cell Weight (DCW)

After centrifuge, the pellets obtained then were used to measure the dry cell weight. The pellets were dried in an oven at 105°C for several hours until they reached a constant weight.

Figure 3.6. Oven dried the samples at 105°C.

3.9 Determination of Specific Growth Rates, Maximum Specific Growth Rate and Saturation Constant

Specific growth rates, μ can be determined by using equation (2) where *dX* is the difference in cell concentration, *dt* is the time difference for each time interval and *X* is a cell concentration in a specific time. By dividing *dX* over *dt* and bringing *X* to the other side, the specific growth rates, μ can be ascertained. The Monod growth model is a basic model for describing microbial growth and explaining the growth kinetics of erythritol fermentation in this investigation (Mohamad, N. L., 2018). Thus, the specific growth rate versus substrate concentration can be plotted according to the Monod equation where μ is directly proportional to S. Maximum specific growth rate, μ_{max} and saturation constant, K_S can be determined based on the specific growth rate graph. Lineweaver and Burk transformed the Monod equation into a straight-line equation. The Lineweaver-Burk method plot the graph of $1/\mu$ versus $1/S$ with a slope equal to K_S/μ_{max} and a y-intercept of $1/\mu_{max}$. The approach predicts the cell growth saturation coefficient, Ks, and μ_{max}.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Evaluation on Erythritol Yield

The study was done under the fermentation process to produce erythritol in a bioreactor by using *Yarrowia lipolytica*. The system was run with the following parameter profiles: 250 revolutions per minute for the agitation speed, 30 degrees Celsius for the temperature, 1.5 liters per minute for the airflow, 5.5 for the pH control, and 0.2 percent antifoam. Figure 4.1 below shows the yield of erythritol concentration during 72 hours of fermentation.

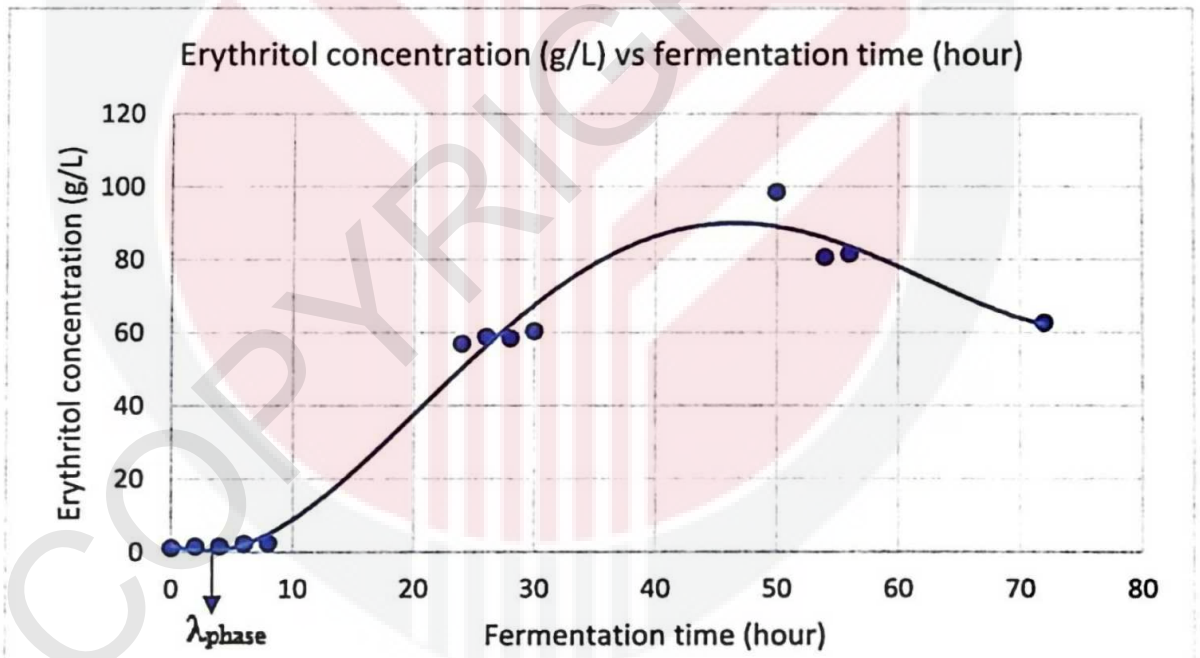

Figure 4.1: Yield of erythritol concentration from fermentation of Y. *lipolytica* in 2-L stirred tank bioreactor.

Based on the Figure 4.1 and Figure 4.2, the amount of erythritol produced at early stage was 1.1096 g/L meanwhile glucose concentration detected was 7.7464 g/L. Erythritol concentration started to increase to 56.8839 g/L after 24 hours of fermentation. Based on Figure 4.2, glucose concentration keeps on decreasing as erythritol concentration on Figure 4.1 increases. In the meantime, cell concentration as shown on Figure 4.3 was increased as fermentation hour increased. This is because during the process of respiration, cells convert glucose into ATP, which then gives the cell the energy it needs to carry out its operations (Flores *et al.*, 2018). In other words, cells concentration increased due to the rate of consumption of carbon sources from glucose. Thus, the amount of erythritol as product produced increased. The maximum amount of erythritol produced was 98.4461 g/L reported at 50 hours of fermentation process. However, at some point, the amount of erythritol produced started to decrease due to glucose depletion. During the fermentation, cells were also depending on other nutrients in the media other than carbon sources. According to Kim (2013), in addition to carbon, various other nutrients are necessary for the upkeep of cells, as well as their development, reproduction, and the generation of products. Carbon, nitrogen, phosphate, and sulphur are the components that can be found in a nutrient medium that is well-balanced.

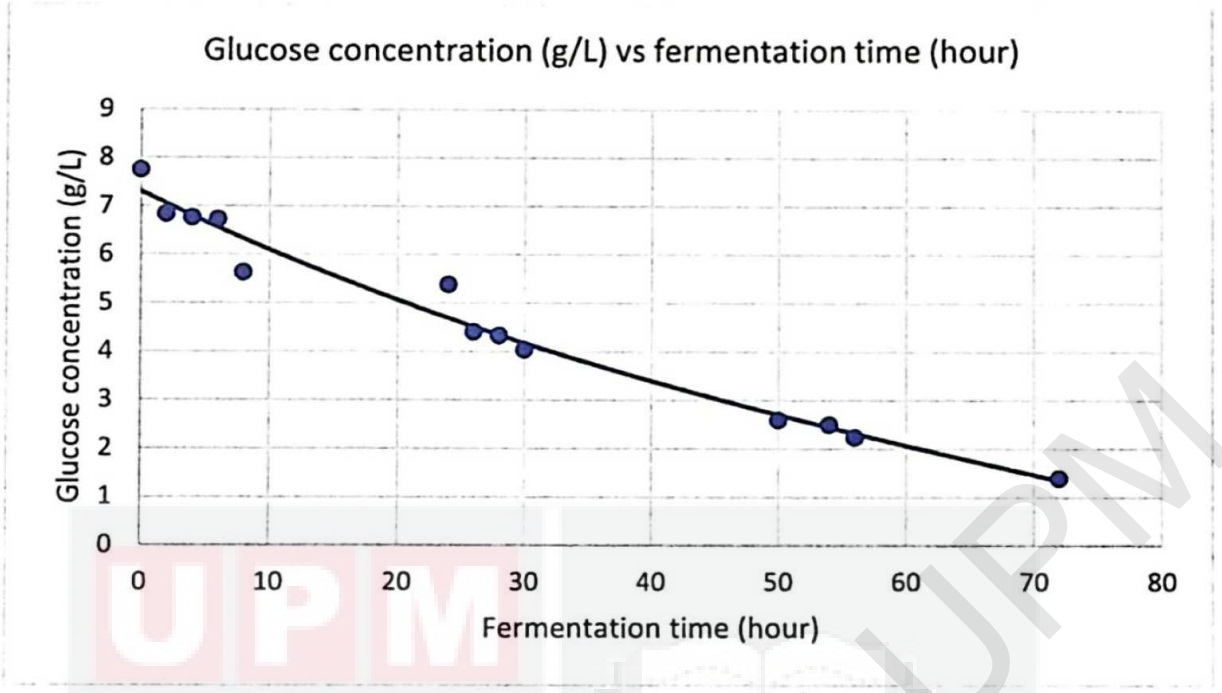

Figure 4.2: Concentration of glucose consumption from fermentation of Y. *lipolytica* in 2-L stirred tank bioreactor.

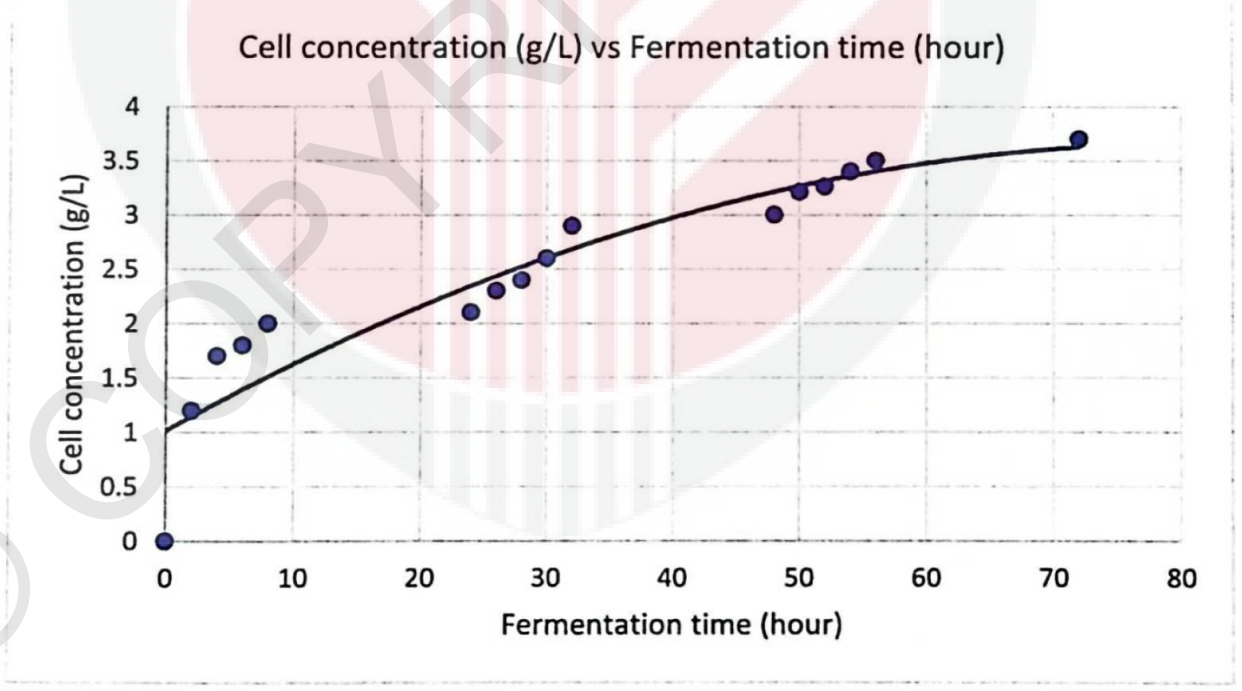

Figure 4.3: Yield concentration of Y. *lipolytica* from fermentation in 2-L stirred tank bioreactor.

Table 4.1 shows the comparison of erythritol yield and erythritol productivity on different substrates. Based on the table, two studies were using the same erythritol producer, *Yarrowia lipolytica* Wratislavia K1 and glycerol as carbon source. The maximum erythritol yield was reported to be 65±0.1 g/L at 73-hour of fermentation and 18.2 g/L at 72-hour of fermentation, respectively. The volumetric productivity was 0.89 g/L/h and 0.11 g/L/h, respectively. Moreover, by using *Yarrowia lipolytica* A16 and glycerol as carbon source, maximum erythritol yield was reported high which was 109.20 g/L at 108-hour of fermentation process. The volumetric productivity was studied to be 0.19 g/L/h. Meanwhile, current study was using *Yarrowia lipolytica* (DSMZ 705662) and glucose as a substrate. This study was reported to accumulate the maximum erythritol yield of 98.45 g/L at 50-hour of fermentation process and volumetric productivity of 1.96 g/L/ which reported to be the highest as compared to the previous studies.

Table 4.1: Comparison of erythritol yield and productivity on different substrates

Yeast	Substrate	pH	Temperature (°C)	P_{max} (gL^{-1})	t_{max} (hour)	Q_P ($gL^{-1}h^{-1}$)	References
Y. lipolytica (DSMZ 705662)	Glucose	5.5	30°C	98.45	50	1.96	This work
Y. lipolytica Wratislavia K1	Glycerol	3.0	30°C	65±0.1	73	0.89	Rakicka et al. (2016)
Y. lipolytica A16	Glycerol	3.0	30°C	109.20	108	0.19	Yang et al. (2016)
Y. lipolytica Wratislavia K1	Glycerol	2.9-3.3	29.5°C	18.2	72	0.11	Tomaszewska, L. (2018)

4.2 Determination of Kinetic Parameters for *Yarrowia lipolytica*

When it comes to monitoring and predicting the fermentation process, kinetic models play a crucial part. These models (Monod kinetic model and Lineweaver-Burk) encompass the kinetics of substrate use, growth, and product formation (Ghimire *et al.*, 2020). Oxygen supply is important for microorganisms' growth in aerobic fermentation, yet some bacteria may be harmed by oxygen toxicity at excessive oxygen concentration (Zhou *et al.*, 2018). Thus, during the fermentation in the bioreactor, oxygen supply in the vessel causes the cell growth that also leads to the increase of cell biomass. As a result,

this causes the limitation of oxygen availability in the medium hence elevated the amount of erythritol accumulated and glucose consumption.

By employing the Monod equation, the particular specific growth rates of *Yarrowia lipolytica* were ascertained. The rate of growth of a cell population's biomass per unit of biomass concentration is referred to as the specific growth rate period (Smith, 2013). According to Liu (2007), the simplest form of Monod kinetics model expresses a functional relationship between the specific growth rate, μ and substrate concentration, S. The specific growth rates of *Yarrowia lipolytica* versus substrate concentration was plotted according to the Monod equation. Thus, the graph plotted is shown in Figure 4.4 and it is reported to increase from a small value to a higher value, which is defined as maximum specific growth rate, μ_{max}. The specific grow rates were increasing as the substrate concentration increased. However, if it has attained the maximum possible specific growth rate, the values of the specific growth rate, which are denoted by, will not be influenced by any increases of substrate concentration and this makes the specific growth rate independent of substrate concentration. Furthermore, the saturation constant, K_S and maximum specific growth rate, μ_{max} were determined by using the Monod approach. The maximum specific growth rate of *Yarrowia lipolytica* determined was 0.0588 1/hr.

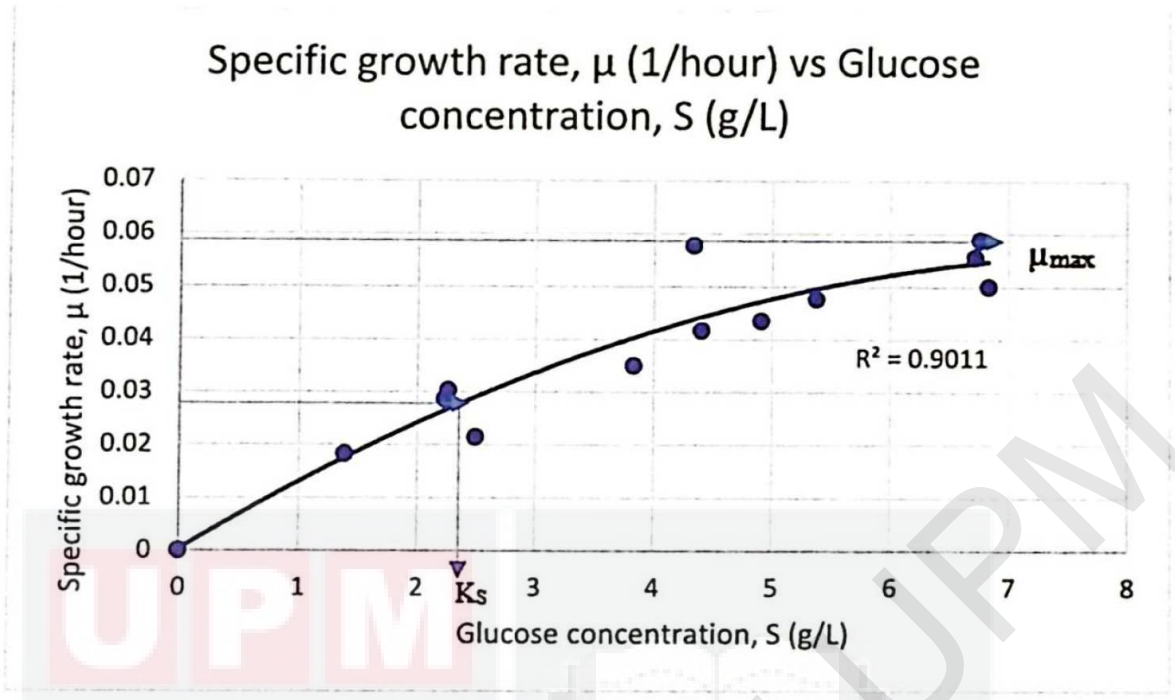

Figure 4.4: Specific Growth Rate according to the Monod equation.

The Lineweaver-burk technique makes use of a linearization mechanism to produce a straight-line plot, which then serves as the basis for determining μ_{max} and K_S. Plotting $1/\mu$ versus $1/S$ should yield a line with slope $K_S/\ \mu_{max}$ and intercept $1/\ \mu_{max}$, as seen in the Figure 4.5 below. If an organism has a very high affinity for the limiting substrate, which indicates that it has a low Ks value, then the growth rate will not be affected until the substrate concentration has declined to a very low level, which will be accompanied by a short decelerating phase for the culture. If, on the other hand, the microbe has a poor affinity for the substrate—that is, if it has a high Ks value—the growth rate will be directly impacted by a relatively high substrate level. This is because a high Ks value corresponds to a low affinity. As a result, the culture will experience a deceleration phase that is somewhat prolonged, which will result in a decreased biomass production (Mohamad *et al.*, 2013). The Ks value obtained from the graph plotted for this experiment was 10.5969.

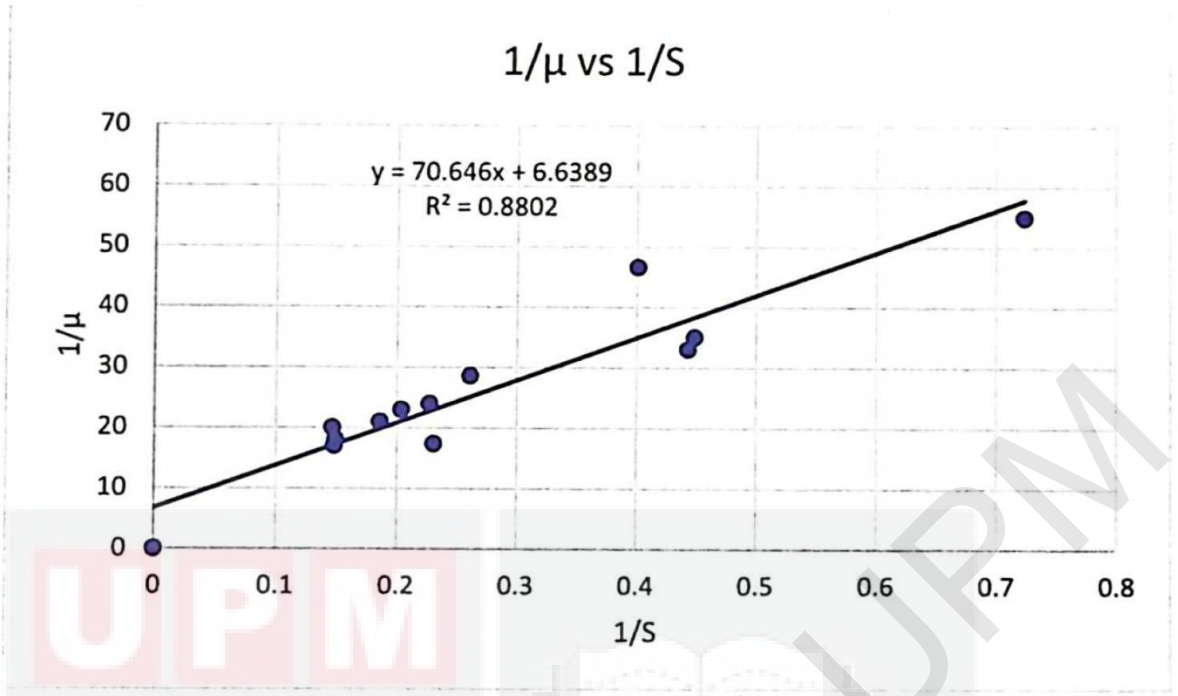

Figure 4.5: Evaluation of Growth Constant by using the Lineweaver-Burk method.

Table 4.2 below shows the comparison of growth constant obtained in the current study by using two different methods (Monod kinetics model and Lineweaver-Burk method). According to the Monod kinetics model, the maximum specific growth rate examined was 0.059 with saturation constant of 0.029. Meanwhile, the maximum specific growth rate and saturation constant by using Lineweaver-Burk method were 0.15 and 10.60, respectively. Furthermore, the R^2 for both methods were studied and obtained through the graphs plotted. By plotting the Monod model, R^2 obtained was 0.90 and 0.89 when using the Lineweaver-Burk method. The value of R^2 obtained by using the Monod equation is more accurate as it has the closest value to 1. Therefore, Monod kinetics model is more suitable to be used to evaluate the two parameters, which are maximum specific growth rate and saturation constant.

Table 4.2: Comparison of growth constant between Monod kinetics model and Lineweaver-Burk method

Methods	Maximum Specific Growth Rate, μ_{max} (1/hour)	Saturation constant, Ks	R^2
Monod kinetics model	0.059	0.029	0.90
Lineweaver-Burk	0.15	10.60	0.89

The process was carried out inside of the bioreactor allowed for an erythritol production rate of 98.4461 g/L to be reached after 50 hours, along with a volumetric productivity of 1.9689 g/L/h and an erythritol yield of 12.7087 g/g. This was accomplished with a volumetric productivity of 1.9689 g/L/h. The kinetic parameters were then summarized in the Table 4.3 below.

Table 4.3: Kinetic parameters of experiment conducted with parameter profiles of 250 rpm, 30 °C for the temperature, 1.5 L/min for the airflow, 5.5 for the pH control, and 0.2% antifoam by using *Yarrowia lipolytica*

Parameters	Bioreactor
Initial glucose concentration (gL^{-1})	7.7464
Maximum erythritol production (gL^{-1}), P_m	98.4461
Time to reach maximum erythritol concentration (h)	50
Volumetric productivity ($gL^{-1}h^{-1}$), Q_P	1.9689
Erythritol yield on the consumed glucose ($g\ g^{-1}$), $Y_{P/S}$	12.7087
Biomass yield on the consumed glucose ($g\ g^{-1}$), $Y_{X/S}$	0.4776
Specific growth rate (h^{-1}), μ_{max}	0.0588
Monod cell growth saturation coefficient, Ks	4.1554

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

The fermentation in bioreactor was done with parameter profiles of 250 rpm, 30 °C for the temperature, 1.5 L/min for the airflow, 5.5 for the pH control, and 0.2% antifoam by using *Yarrowia lipolytica.* The fermentation process accumulated a high amount of erythritol as product desired. The maximum production of erythritol was 98.45 g/L at 50 hours of fermentation process. The current study reported to accumulate the highest of erythritol yield among the previous studies. The fermentation conditions with pH-controlled of 5.5 and temperature at 30°C allowed a good condition for the cells which then create a faster growth. Moreover, during fermentation in the bioreactor, the oxygen supply in the vessel promotes cell development, which also increases cell biomass. Therefore, specific growth rate of *Yarrowia lipolytica* keeps on increasing with substrate concentration until it has reached the maximum value. As the graph according to the Monod equation is more fitted, thus it is more suitable to determine the value for maximum specific growth rate and saturation constant. The maximum specific growth rate examined was 0.0588 1/hour and the saturation constant was 0.029.

The results presented in this study were based on only one simple substrate which is glucose and only one equipment used which is bioreactor. Thus, future studies are required to vary the substrates used such us by using simple substrate and complex substrate such as from industry wastes. Thus, the results can be compared to see which substrate used can achieve high productivity of erythritol. In addition, it is recommended to use two different methods which are by using shake flasks and bioreactor. Therefore, the results obtained can be compared and data can be used as a scale up in the future as

well as better understanding of kinetic behavior of cells. Finally, different modes of operation used such as fed batch could be considered in the future to study which mode of operation can results in the higher concentration of erythritol.

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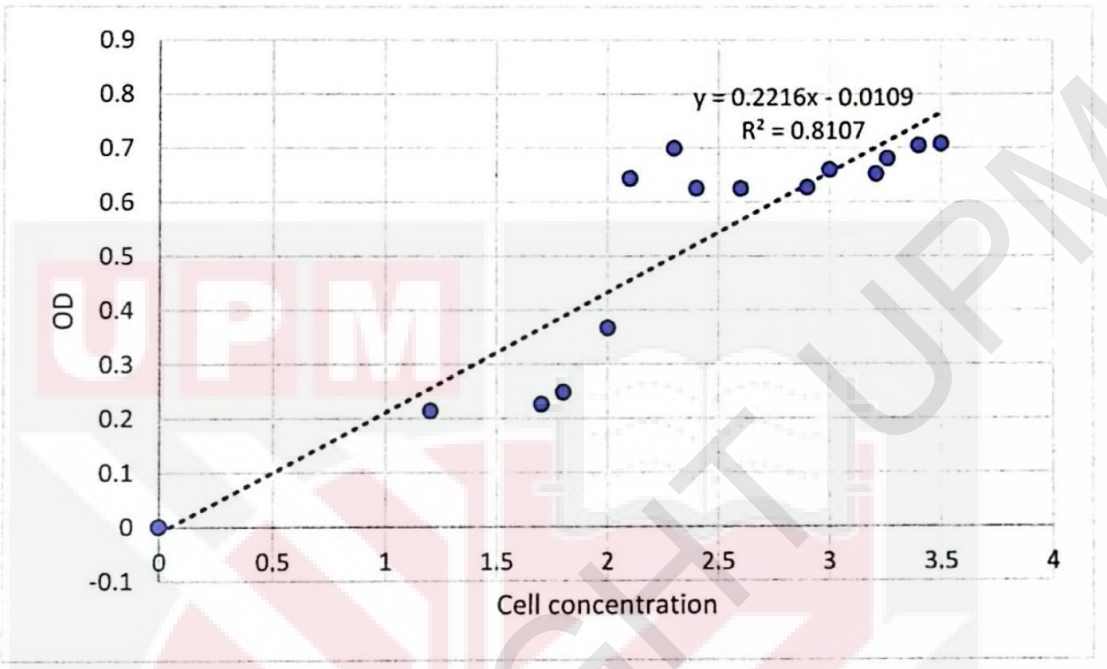
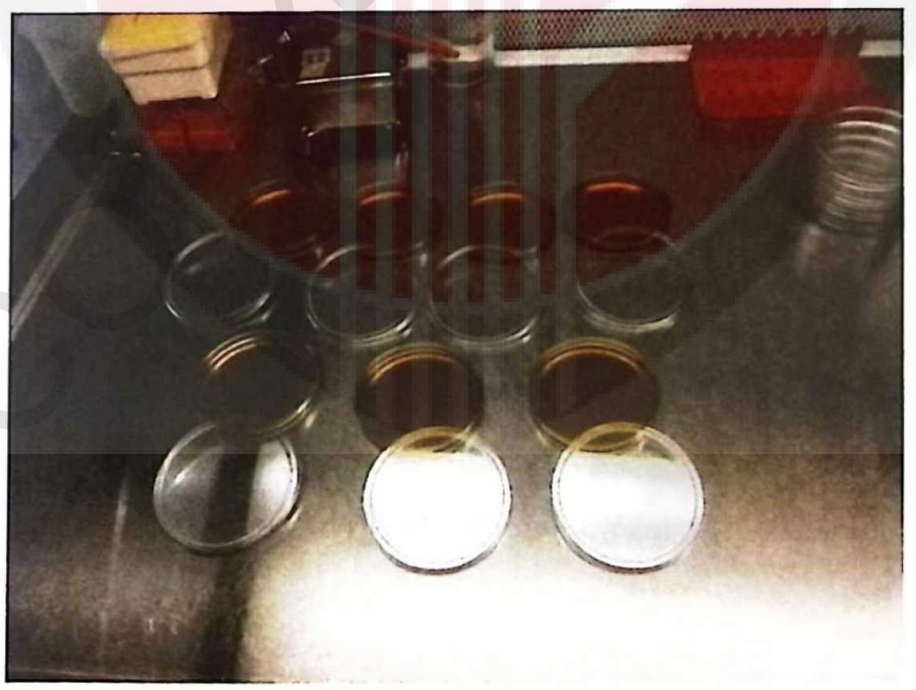
APPENDICES

Calibration curve of dry cell weight and optical density at 600 nm.



Detailed pictures on experimental works.



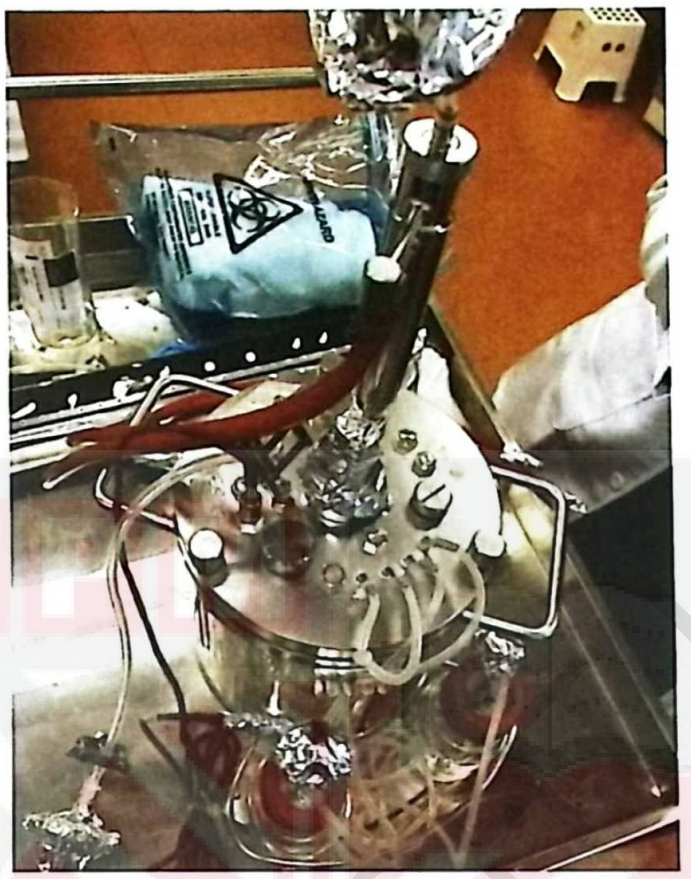
Agar plate preparation

Isolation of y. lipolytica by using streak plate technique

Sterilization of broth and shake flasks

Autoclave

Top plate of jacketed glass vessel and other small parts in a sonicator bath for clean purposes



Top view of plate of bioreactor jacketed glass vessel

Side view of jacketed glass vessel

Full view of 2-L bioreactor with working volume of 1.5-L