PRODUCTION OF SUCCINIC ACID FROM BREAD WASTE

NURSHAKIRIN BINTI ROSLEE

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

Faculty of Chemical & Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2014

ABSTRACT

A study of utilising waste bread as a sole nutrient source for the production of a nutrient rich feedstock for the fermentative succinic acid production by *Escherichia coli* has been developed. Waste bread, contains of nutrients, has been hydrolysed into water solution and adjusted its pH was gelatinized by heating the mixture at temperature 97°C. Then, microbial fermentation performed using bread hydrolysis and fermentation medium. Fermentation was carried out in anaerobic flask by using 300 ml working volume. After fermentation at 37 °C optimum temperature of *Escherichia coli* growth and 10 % (w/v) of substrate concentration, the results of HPLC analysis showed that increase in fermentation time will increase succinic acid production. The highest succinic acid concentration was 0.0801 g/L at 60 hours. The effect of substrate concentration, temperature and pH were studied and optimized. The optimum conditions for the succinic acid fermentation using waste bread were using substrate concentration of 15% (w/v), temperature at 30 °C and pH 6.5.

ABSTRAK

Satu kajian yang menggunakan sisa roti sebagai sumber nutrien tunggal untuk pengeluaran bahan mentah nutrien yang kaya untuk pengeluaran asid succinic melalui kaedah penapaian oleh bakteria *Escherichia coli* telah dijalankan. Sisa roti yang, mengandungi nutrien, telah dihidrolisis ke dalam larutan air dan diselaraskan pH menjadi gelatin dengan memanaskan campuran pada suhu 97 ° C. Kemudian, penapaian mikrob dilakukan dengan menggunakan hidrolisis roti dan medium penapaian. Penapaian telah dijalankan dalam kelalang anaerobik dengan menggunakan isipadu 300 ml. Selepas penapaian, dengan menggunakan suhu optimum pembiakan *Escherichia coli* pada 37 °C dan 10 % (w/v) kepekatan substrat, keputusan analisis HPLC menunjukkan bahawa peningkatan masa penapaian akan meningkatkan pengeluaran asid succinic. Kepekatan tertinggi asid succinic adalah 0.0801 g/L pada jam ke 60. Kesan kepekatan substrat, suhu dan pH telah dikaji dan dioptimumkan. Keadaan optimum untuk penapaian asid succinic menggunakan roti sisa ialah dengan menggunakan kepekatan substrat pada 15 % (w/v), suhu di 30 °C dan pH 6.5.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

°C	degree Celcius
hr	hours
min	minutes
(w/v) %	(weight of sample / volume of solvent) %
mL	mili Liter
g	Grams
L	liter
g/L	gram/Liter
rpm	Rotation per minutes

LIST OF ABBREVIATIONS

E. Coli	*Escherichia Coli*
SA	Succinic Acid
DNS	Di-nitro Salicylic Acid
HPLC	High Performance Liquid Chromatography
LDH	Lactate dehydrogenase
PFL	Pyruvate:formate lyase
PTS	Phosphotransferase System
ME	Malic Enzyme
OAA	Oxaloacetate

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Succinic acid is one of the functional chemicals that known as butanedioic acid while historically known as spirit of amber which is a group of dicarboxylic acid. It is a natural constituent of plants, animals and microorganisms and also acts as an intermediate compound in the tricarboxylic acid cycle (TCA). Formerly, succinic acid has been used in Europe as a natural antibiotic and general curative for centuries. It is a powerful antioxidant that helps fight toxic free radicals, shown to stimulate neural system recovery and build up the immune system. Succinic acid also has been used as flavouring agent for food and beverages, also intermediate for various industrial pharmaceuticals. Pu Zheng et al. (2010) states that succinic acid has been recognized as one of the most important platform chemicals in many reports.

Currently, succinic acid has been synthesized from petrochemical based by catalytic hydrogenation of maleic acid which is one of fossil origin. However, recent analysis showed that the production of succinic acid from petrochemical feedstock may cause serious pollution and health problems including cancer and endocrine disruption (interference with hormones within body). Consequently, due to increasing concerns on both limited fossil origin and some problems, fermentative productions of succinic acid are recently attracting many attentions. Therefore, efforts have been made to improve the efficiency of succinic acid production in terms of yields, productivities, concentrations, and product recovery. In the new era of sustainability, renewable biomass is considered to be a viable replacement compared to petrochemical raw for succinic acid production. With a change in raw material, traditional refineries will be replaced by bio refineries with the aim of producing an equivalent range of fuels and chemicals.

One of the renewable resources that can be used to produce succinic acid is waste bread. Food waste (including bread) being the highest potions of municipal solid waste which stated in municipal solid waste composition in Kuala Lumpur, Malaysia. Utilisation of waste bread which is rich in organic carbon and nitrogen as the renewable feedstock in bio refinery for chemicals and energy production becomes a promising option to target solid waste problem yet can minimize uses of petrochemical raw which commonly act as a major source of chemicals and energy. In fact, nutrients that contained within waste bread can be converted by micro-organisms through fermentations into the desired products, such as functional chemicals like succinic acid. From other studies, it can be proved that the achievement of industrial bio-based succinic acid production is highly dependent on the utilization of a cheaper source of material.

In this research, succinic acid production was conducted by waste bread as the fermentation material and *Escherichia Coli* as the production strain. The fermentation performance was evaluated, and the operation conditions such as substrate concentration, pH and fermentation temperature were also optimized.

1.2 PROBLEM STATEMENT

Nowadays, succinic acid is a bulk chemical which give higher demand of global production. The demand of succinic acid per annum was 16,000 to 30,000 tonnes, and the annual growth is 10% (A. Higson and C. Smith, 2011). However, cost of production has been the main problem in order to produce a lot of succinic acid. Production of succinic acid and other organic chemicals depend mainly of fossilized hydrocarbons. Former researches had derived succinic acid from maleic anhydride, which was produced from n-butane through oxidation over vanadium-phosphorous oxide catalysts (Liu et al., 2008 ; Gascon et al., 2006). Due to lack of fossil feedstock availabilities, the price of fossil fuel feedstock has increase. Therefore, production cost of succinic acids by chemical plants is higher compare with production cost of fermentation process by microorganism. Besides that, second problem for chemical plants are products from petrochemical raw may cause a lot of problem for environment and human health. Due to the increasing concern of human and environmental issues, the development of sustainable alternatives based on renewable raw materials had created (Du, C. et al., 2007). Moreover, another problem is food waste been one of the highest waste compositions in municipal solid waste in Malaysia's commercial district. In order to give solution of petrochemical raw problem, it also can overcome the problem of increasing food wasted in Malaysia.

1.3 OBJECTIVE

The following are the objectives of this research:

o To study the succinic acid production using bread hydrolysis by bacterial fermentation using *Escherichia Coli.*

1.4 SCOPE OF RESEARCH

The following are the scope of this research:

i. To examine the production of succinic acid from waste bread.

ii. To study the effect of substrate concentration, pH and fermentation temperature towards the production of succinic acid.

CHAPTER 2

LITERATURE REVIEW

2.1 OVERVIEW

This chapter discussed about the detail of succinic acid, the properties, current production of succinic acid by petrochemical raw and renewable resources and its applications in industries. Besides that, this chapter also explained about the succinic acid act as biochemical role in citric acid cycle. The previous studies of fermentation that used various strain for produce succinic acid also discussed in this session. Moreover, food waste is one of the contributors on the compositions of municipal solid waste in Malaysia's commercial district.

2.2 INTRODUCTION

Succinic acid, which is a four-carbon di-carboxylic acid important platform chemical, mostly produced by a chemical process that uses liquefied petroleum gas as the starting material. However, due to expensive petrochemical raw and other factors, succinic acid is produced by various microorganisms (S., Raja et al., 2011). Succinic acid is a common organic acid because it can be used as a precursor of many industrially important chemicals in the food, chemical and pharmaceutical industries. By using renewable sources such as waste bread as a carbon source, succinic acid can be produced with much less of the product cost formation.

2.3 BACKGROUND OF SUCCINIC ACID

Succinic acid (SA) is a naturally-occurring di-carboxylic acid compose of four carbon atoms by which having the molecular formula of $C_4H_6O_4$. Succinic acid can be found in plants and animal tissues (Hyohak, S., 2006). It is very important to body. Moreover, it used in the Krebs cycle which is involved in the intermediary metabolic process. David W. (2013) states that the succinate is a component of the citric acid cycle capable of donating electrons to the electron transfer chain. Formerly, the term 'Succinic Acid' is another name for Amber Acid that has been used in Europe. Up until the early 1900's, succinic acid was used in Europe as a natural antibiotic and general curative. Figure 2.1 below showed the molecular formula of Succinic Acid (Baker, 2008).

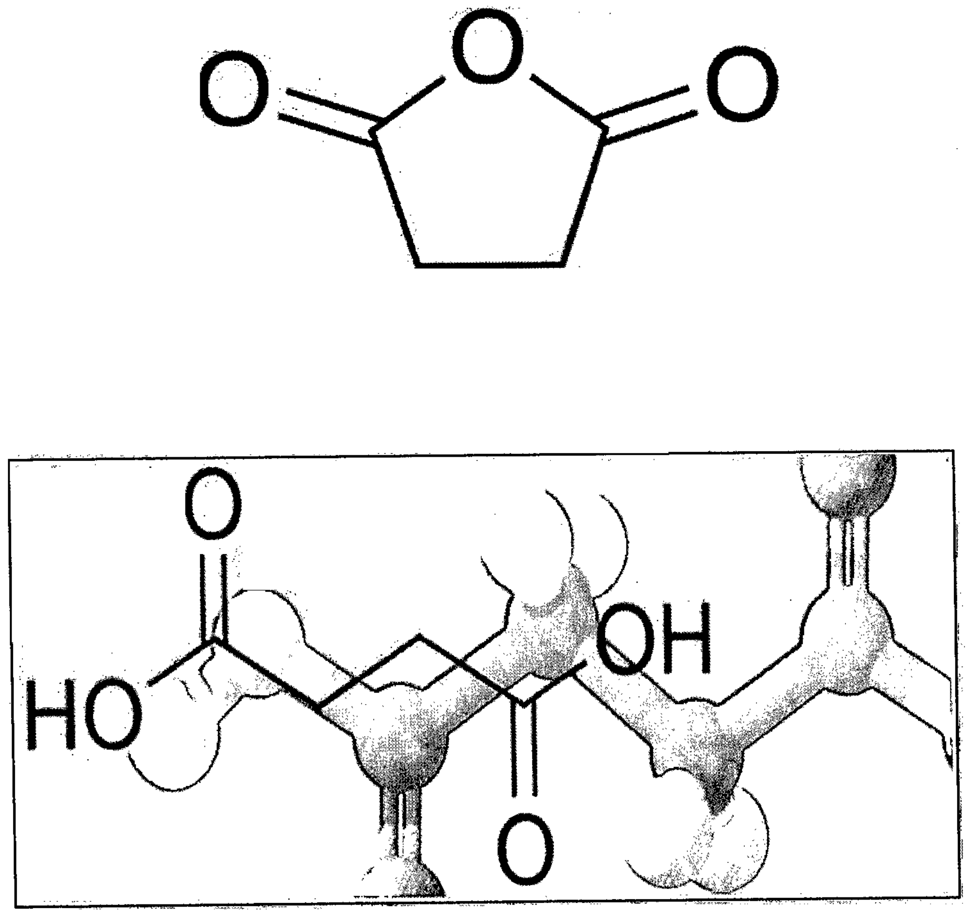

Figure 2.1: Molecular formula of Succinic Acid

2.3.1 Physical and chemical properties

Table 2.1: Physical and chemical properties of succinic acid

CAS Number	110-15-6
Molecular Formula	(CH2COOH)2
Molecular Weight	118.09 g/mol
Melting Point	188°C
Boiling Point	Decomposes at 235°C
Specific Gravity	1.56
Solubility	100g/100ml at 100°C
	7.69g/100 ml at 25°C
Odour	Odourless
Colour	White minute monoclinic prims

Source: Baker (2008)

2.3.2 Biochemical role in the citric acid cycle

The citric acid cycle which is known as the tri-carboxylic acid cycle (TCA cycle), or the Krebs cycle use to develop energy through the oxidization of acetate derived from carbohydrates, fats and proteins into carbon dioxide. It is a series of chemical reactions used by all aerobic organisms. Succinate is a component of the citric acid cycle and is capable of donating electrons to the electron transfer chain by the reaction (Farrukh and Majeed, 2011):

$$\text{succinate} + \text{FAD} \rightarrow \text{fumarate} + \text{FADH2}$$

This is catalysed by the enzyme succinate dehydrogenase (or complex II of the mitochondrial ETC). The complex is a 4 subunit membrane-bound lipoprotein which couples the oxidation of succinate to the reduction of ubiquinone. Intermediate electron carriers are FAD and three Fe_2S_2 clusters part.

2.3.3 Current Production of Succinic Acid

Production of succinic acid using petrochemical resources is currently produced. It was derived from maleic anhydride, which is produced from n-butane through oxidation over vanadium-phosphorous oxide catalysts (Liu et al., 2008; Gascon et al., 2006). It is sold at the price of \$5.9 to 9.0 kg−1 depending on its purity. The manufacturing cost is affected by several factors including succinic acid productivity and yield, the costs of raw materials, and the recovery method.

Efforts have been made to improve the efficiency of succinic acid production in terms of yields, productivities, concentrations, and product recovery. Nowadays, succinic acid is a bulk chemical with global production at 16,000 to 30,000 tonnes, and annual growth of 10 % (A. Higson and C. Smith, 2011). In the new era of sustainability, renewable biomass is considered to be a viable replacement compared to petrochemical raw for succinic acid production. With a change in raw material, traditional refineries will be replaced by biorefineries with the aim of producing an equivalent range of fuels and chemicals.

There are the renewable resources used in succinic acid production recorded are cheese whey (Samuelov et al., 1999; Lee et al., 2000), cane molasses (Agarwal et al., 2006), Jerusalem artichoke (Ren et al., 2008), wood hydrolysate (Lee et al., 2003b) and corn straw hydrolysate (Zheng et al., 2009). It can be proved that the achievement of industrial bio-based succinic acid production is highly dependent on the use of a cheaper source of renewable besides the hard work strain of continuous improvement and simplification of the purification.

2.3.4 Application of Succinic Acid

Currently, succinic acid is used as a surfactant, an ion chelator, and an additive in the pharmaceutical and food industries (Zeikus et al., 1999). Succinic acid is a building block that used as a flavouring agent for food and beverages, as an intermediate for various industrial chemicals such as dyes, perfumes, natural antibiotics, lacquers and photographic chemicals.

More importantly, succinic acid act as a raw material for production of 1,4-butanediol, tetrahydrofuran, Nmethyl pyrrolidinone, 2-pyrrolidinone, and gamma-butyrolactone, or for the preparation of biodegradable polymers such as polybutylene succinate and polyamides (McKinlay et al., 2007; Song and Lee, 2006). Figure 2.2 shows succinic acid and its chemical derivatives.

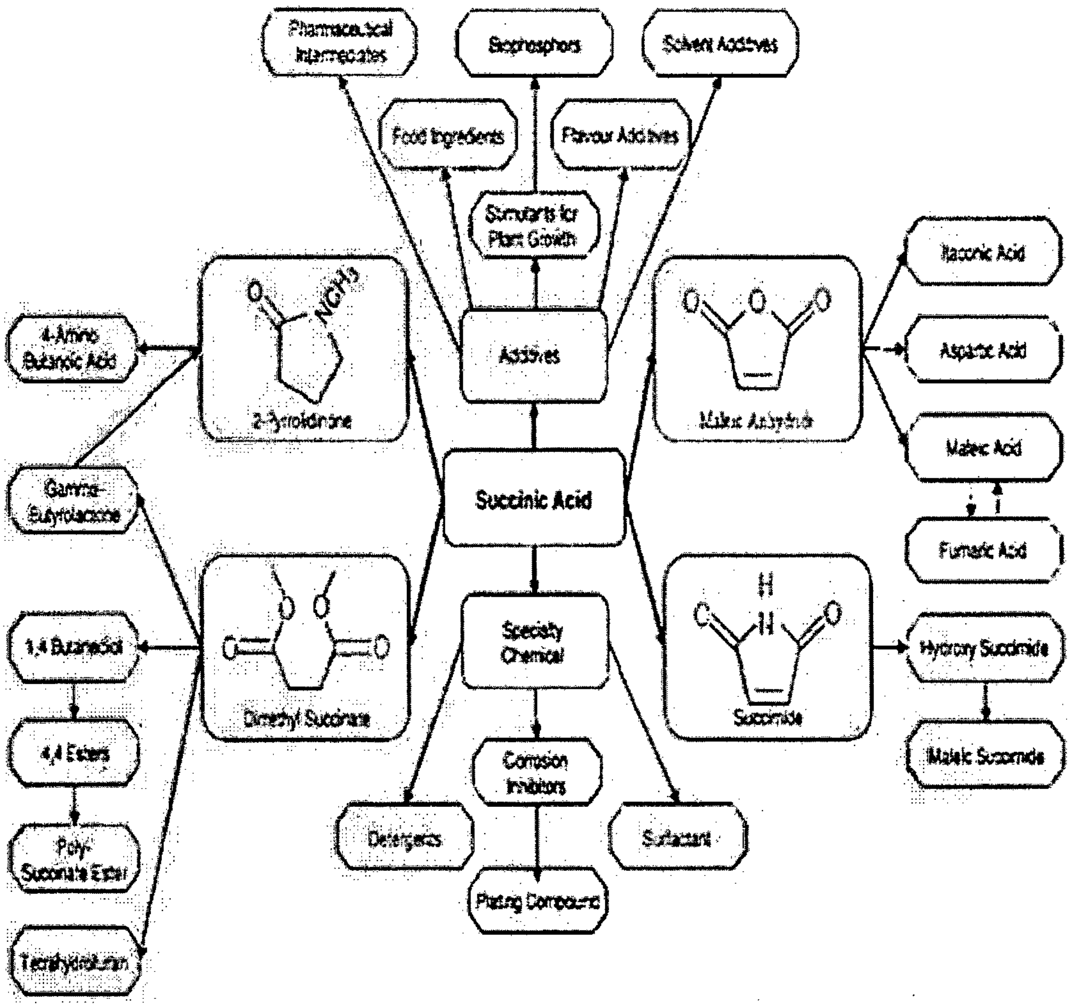

Figure 2.2: Succinic acid and its chemical derivatives

Source: Song and Lee (2006)

2.4 FOOD WASTE

2.4.1 Statistic of waste in Malaysia

Based on the compositions of municipal solid waste in Malaysia's commercial district, from figure 2.3, the highest composition of solid waste is from food or organic waste products which are at 41%, (Kathirvale S. et al., 2003). Food is wasted throughout by a wide range of foods chains such as by farmers, food industry, consumers or caterers. The major factor is due to lack of awareness by humans. Lack of knowledge and confusion about "best before" and "use by" date is the reason why food labels are easy to remove by the people. Moreover, overproduction, product & packaging damage from farmers and food manufacturing being one of the factor.

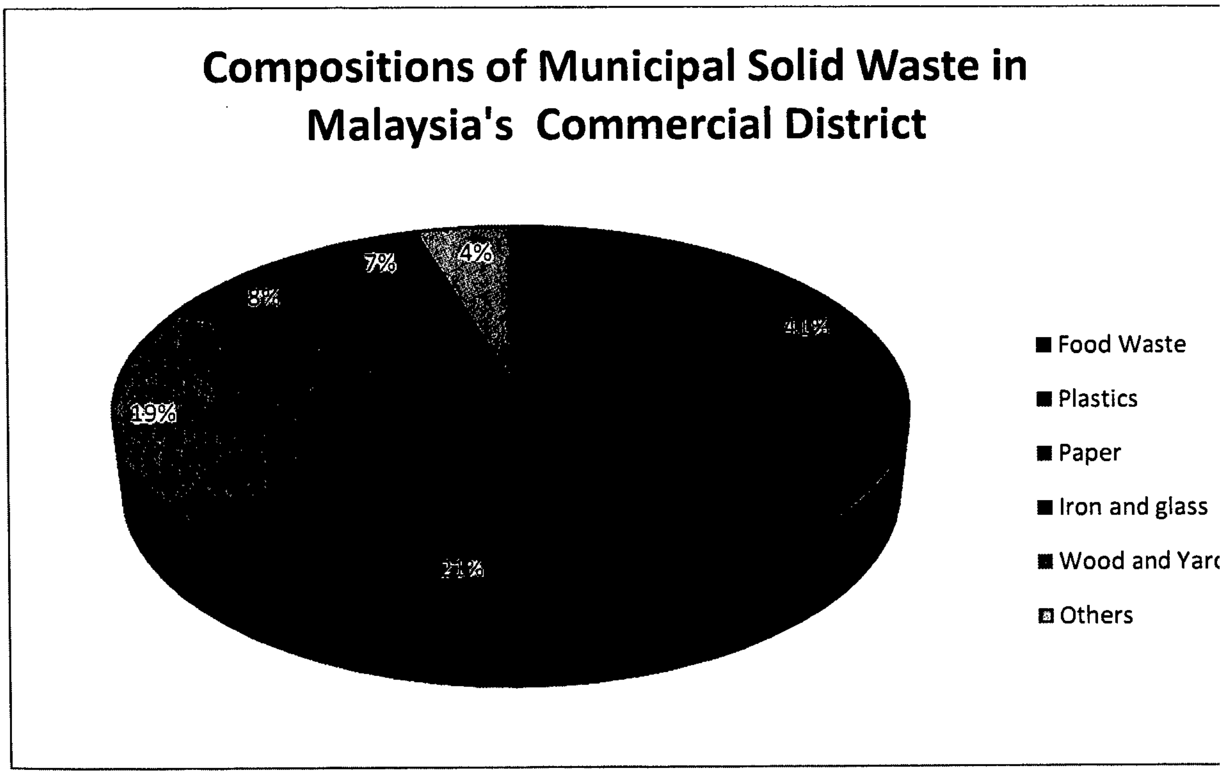

Figure 2.3: Compositions of municipal solid waste in Malaysia's commercial district

2.4.2 Waste bread composition

There is evidence that the rest of the bread is the greatest thing in the waste bin users. It is massively downgraded from high-quality food to feed or remove. It has an expiration date, so, usually bread expiry date just thrown in the trash (N.A, 2010). The main reason's people for throwing away bread because of the bread was left on the plate after a meal, passed its date, looked, smelt or tasted bad and it went mouldy. Therefore, there are researches have been done to manage waste bread since it has many advantages. Within food waste, the nutrients contained can be converted by microorganisms into desired products. Besides that, waste bread is rich in energy, low in fibre but high in vitamins which also has led to reduction in feed cost compare to other waste. Table 2.2 shows the composition of waste bread which is rich in organic carbon and nitrogen. Kent, et al. (1994) states that 100 g of white breads contains around 50 g carbohydrate, 37 g of water, and around 8 g protein which make 95% of the total weight. From this composition, it makes bread an excellent source of nutrition.

Table 2.2: Waste bread composition per 100 g bread.

Moisture	22.3 g
Starch (dry basis)	59.8 g
Total organic nitrogen (dry basis)	1.56 g
Protein (TN × 5.7) (dry basis)	8.9 g
Total phosphorus	Trace

Source: C.J.L. et al. (2012)

Figure 2.4: Waste bread from industrial food.

2.5 FERMENTATION OF SUCCINIC ACID

2.5.1 Succinic Acid Production through Fermentation

Succinic acid, when produced through fermentation, converts glucose to succinic acid along part of the reductive cycle tricarboxylic acid (TCA) cycle (Lee et al., 2002). Figure 2.5 depicts reactions and enzymes in the normal fermentation process that turns glucose to succinic acid.

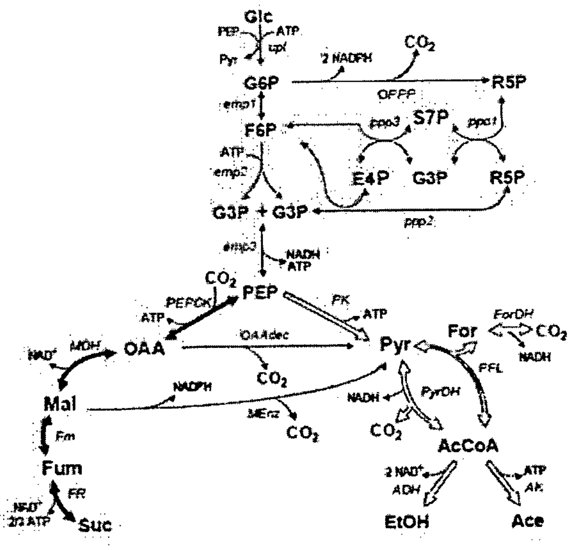

Figure 2.5: Metabolic pathway of a typical succinic acid producing microorganism

Source: McKinlay and Vieille, (2008)

2.5.2 Fermentation by microorganisms

Succinic acid can be produced by microbial fermentation using a number of microorganisms. Many different microorganisms have been screened and studied for succinic acid production from various carbon sources. The common micro-organisms used in the fermentative succinic acid bio-production are *Mannheimia succiniproducens* (Lee et al, 2000), *Actinobacillus succinogenes* (Cho, C. et al, 2012), *Anaerobiospirilium succiniproducens* (Nghiem et al., 1997) and *Escherichia coli* (Wu et al, 2007). These micro-organisms are effective natural succinate producers with high productivity and tolerance to osmotic pressure caused by glucose and succinic acid. H. Song and S.Y.

Lee (2006) states that a wild type *E. coli* primarily ferments glucose to ethanol, formic, acetic and lactic acids with detectable amounts of succinic acid under anaerobic condition.

2.5.2 *Escherichia coli*

Escherichia coli, also known as *E. coli* are a bacterium that belongs to the family *Enterobacteriaceae*. It is commonly found in the gut of endotherms (warm blooded organisms) and have many beneficial functions. One of the functions is *E. coli* can produce vitamin K and B-complex vitamins in human intestines which give essential for human body.

In addition, Andreas Lennartsson (2005) states that carbohydrates are the main energy source for *E. coli,* particularly carbon six glucose molecules. By lowering glucose, *E. coli* get energy and carbon skeletons to produce amino acids, nucleotides, enzymes and other metabolic intermediates required for growth. To use glucose, the bacteria use different metabolic pathways. An important aspect to *E. coli* is facultative anaerobes enable the microorganisms to grow in the presence and absence of oxygen.

2.5.3 *Escherichia coli* as a microorganism in succinic acid production

There are previously studies that carried out on metabolic engineering of *Escherichia coli* for succinic acid production. *E. coli* is one of the others microorganism in this production. Clark states that *E. coli* be a facultative anaerobe and has the advantages of growing fast, simple requirements for nutrients, detailed knowledge about its genetic background, and abundant strains and vectors available for metabolic engineering studies. It proceeds with mixed-acid fermentation under anaerobic conditions, but succinic acid is a minor product. To improve the efficiency of succinic acid production in *E. coli,* one strategy is to block the competition pathways to succinic acid, such as by inactivation of pyruvate:formate lyase (PFL) and lactate dehydrogenase (LDH), the protein EIICBglc in the phosphotransferase system (PTS), malic enzyme (ME) (Stols et al.,1997),to direct the carbon flow to oxaloacetate (OAA) or malate, from which succinic acid is produced.

CHAPTER 3

METHODOLOGY

3.1 OVERVIEW

This chapter will discuss about methodology that has been used in the research about the production of succinic acid from waste bread through microbial fermentation. The product was manipulated by several parameters such as substrate concentration, pH and temperature. The experiment methods will discuss detail in this chapter. Figure below shows the process flow for this experiment.

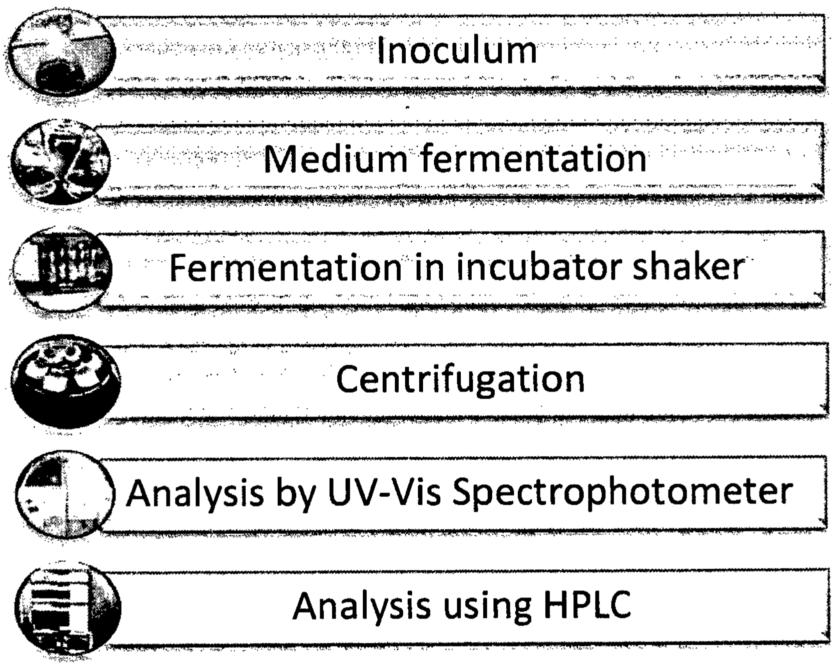

Figure 3.1: Process flow of Succinic Acid Production by Waste Bread

3.2 MATERIALS

3.2.1 Raw Materials

Waste bread was kindly provided by the bread factory around Kuantan, Pahang. Bread was cut into cubes of approximately 1 cm^3 in size and blended for 5 minutes. Then, the bread flour was produced by filtering using 0.5 mm sieve tray. According to Kent (2009), 100 g white bread contains around 50 g carbohydrates, 37 g water and around 8 g protein, which make 95 % of the total weight. This composition makes bread an excellent source of nutrition.

3.3 BREAD HYDROLYSIS

The method of Betiku and Ajala (2010) was employed for the hydrolysis. Appropriate weight of bread flour was measured and mixed with distilled water. To make 8 % slurry, 80 g of bread flour was weighed into 1000 ml distilled water to make slurry. The pH was adjusted to 6.5 with Citrate-phosphate buffer. Then, gelatinization was done by heating the mixture to 97 °C and was held at this temperature for 10 minutes. The slurry was cooled to 60 °C.

3.4 MICROORGANISMS AND MEDIUM COMPOSITION

3.4.1 Microorganisms

The strain used is *Escherichia Coli* was used for succinic acid fermentation were received from Universiti Malaysia Pahang laboratory. The bacterium was maintained on glucose (10% w/v), yeast extract (1% w/v), CaCO3 (2% w/v) and agar slants. The culture was inoculated onto agar slants every month.

3.4.2 Preparation of agar plate

Nutrient agar or nutrient broth is commonly used for the cultivation of a wide variety of microorganisms. For this experiment, nutrient agar which contains Beef Extract (0.3 %), Peptone (0.5 %) and Agar (1.5 %) in water is used to grow *E-coli* bacteria by creates plates for streaking out bacterial colonies. Before starting the experiment, all apparatus are autoclaved at temperature 121 °C for 20 min to kill bacteria.

By preparing 1 litre solution of nutrient agar, in g/L, measure 3 g of beef extract, 5 g of peptone and 15 g of agar and dissolve in 1000 ml of deionized water. Heat the solution on the hot plate heater and mix properly with frequent agitation by magnetic stirrer. Then, the pH of the solution was adjusted to 6.8 ± 0.2 at 25 °C by using 2M NaOH. The medium was autoclaved at temperature 121 °C for 20 min to sterilise the medium.

It is important to autoclave medium or apparatus before starting experiment because the autoclave is a device that uses steam to sterilize equipment and other objects. This means that all bacteria, viruses, fungi, and spores are inactivated. Sterilization by heating is one of the most convenient methods, and it would be effective at the temperature of 121 °C, may not be effective at the temperature of boiling water (100 °C) because many spores can survive at this temperature. Thus, the standard conditions for sterilizing apparatus or small volumes of liquid is to treat them with steam at 1 atm of pressure for 15 min.

After sterilization is complete, take out the beaker from autoclave by using appropriate gloves due to their high temperature. Put the sterilized medium in laminar fume hood. Allow the beakers cool enough so that they can be comfortably handled while wearing vinyl gloves. The work surface of the laminar flow hood should be thoroughly cleaned with 70 % alcohol to kill and prevent contamination of microorganism. Prepare the sterilised petri dishes.

After the sterilized medium and beaker was enough to cool down, take the medium and pours a little into petri dishes. Wait for an hour to let the broth to solidify. The plates were stored at 4 °C in freezer. All actions are doing in aseptic technique, or procedures that maintain the sterility of experimental materials. The objective of this part is to put into practice the method of transferring a stock culture on agar slant to nutrient agar plate aseptically. This is to revive the cells from its inactive state and obtain a pure colony for the following fermentation process.