

OPTIMIZATION OF PRE-TREATMENT
PROCESS OF GLYCEROL RESIDUE FOR
SUCCINIC ACID PRODUCTION

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FOR SUCCINIC ACID PRODUCTION

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ABSTRACT

The purpose of this study is to recover glycerol from the oleo-chemical industry waste through acid pre-treatment process. Pre-treatment intervals parameters (pH, temperature and mass substrate) was obtained from OFAT, while the optimum conditions for the recovery of glycerol obtained by using response surface methodology (RSM). Glycerol concentrations before and after pre-treatment and succinic acid derived from the fermentation process were analyzed using HPLC. FTIR spectrometry was used to identify functional groups of glycerol. The optimum conditions of pH, temperature and substrate mass for glycerol pre-treatment process is at 2.0, 35 ° C and 110.3600 grams respectively. In optimal conditions, the concentration of glycerol after pre-treatment process is 159.3120 g / L. Glycerol is then used in the fermentation by *Escherichia coli* K-12 types to produce succinic acid of 0.6698 g / L, with initial concentration of glycerol, rotational speed and temperature at 19.6700 g / L, 200 rpm and 37 ° C, respectively. Succinic acid production from glycerol derived from the recovery process is 8.67% lower than the production of succinic acid from commercial glycerol. Therefore, this study was able to demonstrate that the glycerol from the oleo-chemical industry waste can be used in the production of succinic acid.

ABSTRAK

Tujuan kajian ini adalah untuk mendapatkan semula gliserol daripada sisa oleo-kimia industri melalui proses pra-rawatan asid. Sela parameter pra-rawatan (pH, suhu dan jisim substrat) telah diperolehi daripada OFAT, manakala kondisi optimum untuk pemulihan gliserol diperolehi dengan menggunakan kaedah tindak balas permukaan (RSM). Kepekatan gliserol sebelum dan selepas pra-rawatan serta asid susinik yang terhasil daripada proses penapaian dianalisa menggunakan HPLC. Spektrometri FTIR telah digunakan untuk mengenalpasti kumpulan berfungsi gliserol. Keadaan optimum pH, suhu dan jisim substrat untuk proses pra-rawatan gliserol adalah pada 2.0, 35 °C dan 110.3600 gram. Pada keadaan optimum, kepekatan gliserol selepas proses pra-rawatan ialah 159.3120 g / L. Gliserol ini kemudiannya digunakan dalam penapaian oleh *Escherichia coli* jenis k-12 untuk menghasilkan asid suksinik sebanyak 0.6698 g / L, dengan kepekatan awal gliserol, kelajuan putaran dan suhu pada 19.6700 g / L, 200 rpm dan 37 °C. Penghasilan asid succinic daripada gliserol yang diperolehi daripada proses pemulihan adalah 8.67% lebih rendah dibandingkan dengan penghasilan asid suksinik daripada gliserol komersial. Oleh itu, kajian ini telah berjaya menunjukkan bahawa gliserol daripada sisa oleo-kimia industri boleh digunakan dalam penghasilan asid suksinik.

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LIST OF SYMBOLS

°C	degree celcius
Ca ²⁺	ion calcium
µm	micrometer
%	Percentage
3-D	Three- dimensional
g mol ⁻¹	gram per mol
g/l	gram per liter
kPa	kiloPascal
h	hour
L	liter
min	minute
ml	milliliter
<i>n</i>	Variable quantity
NAD ⁺	nicotinamide adenine dinucleotide
nm	nanometer
MT	metrik tons
OD	optical density
Pa.s	Pascal per second
rpm	rotation or revolution per minute
R _t	retention time
v/v	volume per volume
w/v	weight per volume
w/w	mass fraction (mass per mass)

LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
2FI	two factor interaction
ANOVA	analysis of variance
ATCC	American Type of Culture Collection
CDW	cell dry weight
CFU	colony forming unit
DM	dried matter
DOE	design of experiment
FFD	full factorial design
FTIR	Fourier Transform Infrared Spectroscopy
H ₂ SO ₄	Sulfuric Acid
HPLC	high performance liquid chromatography
MONG	Matter-non-glycerol
NaOH	Sodium Hydroxide
Na ₂ CO ₃	Sodium Carbonate
OD	optical density
OFAT	one-factor-at-a-time
POME	palm oil mill effluent
SD	Standard deviation
RID	refractive index detector
RSM	response surface methodology

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Succinic acid, also known as amber acid or butanedioic acid, is a member of the dicarboxylic acid family (Isar *et al.*, 2006) having the molecular formula of $C_4H_6O_4$. In 1546, after its first purification of succinic acid from amber by Georgius Agricola, it is now being produced by microbial fermentation (Menzel *et al.*, 1999). Succinic acid is used in a number of applications in the food, pharmaceutical and cosmetic industries (Zeikus *et al.*, 1999; Jain *et al.* 1989). In the transition to a more sustainable renewable energy usage, succinic acid production from glycerol residue has attracted wide attention across the world. Glycerol residue is a promising alternative waste source that can be used to produce succinic acid in an environmental friendly manner by method of pre-treatment and fermentation. Incidentally, in the future, this method could replace chemical synthesis plants to produce succinic acid.

Currently, efforts to convert agricultural wastes, especially oil palm wastes, as an energy source are widely investigated in Malaysia. In 2008, Malaysia was the second largest producer of palm oil with 17.7 million tones, or 41% of the total world supply. On another note, palm kernel oil is normally used as a raw material for the production of oleo chemicals via transesterification to produced methyl esters. The oleo chemical industry generates by products that includes glycerol residue (Ooi *et al.*, 2004). Due to the availability of palm oil wastes and the distillation process that generates much glycerol waste, it seems to be a very promising opportunity for an alternative source of renewable energy.

Furthermore, esterification seems to be one of the pre-treatments for glycerol residue (Hayyan *et al.*, 2011) in order to reduce free fatty acids in oils and fats followed by transesterification reaction by using an alkali-catalyst to convert it to glycerol. Nevertheless, less attention has been paid to pre-treatment of agricultural wastes. In fact, limited data has been found for pre-treatment of oleo chemical wastes. In my study, the pre-treatment of glycerol residue will be explored using rotary evaporator.

The focus of this study is on the pre-treatment process to recover glycerol as a substrate for succinic acid production. The parameters involved in the pre-treatment are pH, temperature and mass substrate. The experiments developed were able to successfully explain the behavior of the glycerol recovered, its characteristics and fermentative characteristic of succinic acid produced from the glycerol fermentation process and these are discussed in detail in the coming chapters. Furthermore, a series of batch fermentations with different parameters were conducted and the experimental data was used to estimate parameters and also to validate the experiments. In this research we will also discuss the use of anaerobic fermentation for the conversion of glycerol into higher value products. The succinic acid produced is measured by using HPLC.

1.2 PROBLEM STATEMENT

As a by-product of oleo chemical production, glycerol has now become an abundant and cheap source of carbon. Conversion of glycerol to higher value products will increase the economic viability of the methyl ester production process where the glycerol waste is an alternative substrate for conversion of succinic acid production. Besides, to get a high concentration of succinic acid, the impurities contain in glycerol residue had to be removed. The glycerol residue contains high impurities such as fatty acid and salt which can inhibit the microorganisms (*Escherichia coli*) that help produce succinic acid. Unsuitable methods applied in the recovery process can caused low recovery of glycerol and inhibit the reaction process. Thus, the characteristic study must be performed to study the contents, compound and condition of glycerol residue before performing the chemical and physical treatment methods. Furthermore, not many studies have been carried out to

study the effects on pre-treatment such as the effects of temperature and mass substrate in the recovery of glycerol. Thus, no study has been performed to optimize the glycerol recovery (pre-treatment) in an experimental design. Prior to this, a preliminary study must be performed to select the appropriate range of parameters (pH, temperature, mass substrate) to avoid problems during the optimization process and hence a low recovery of glycerol. Improper employment of temperature and an unsuitable amount of mass substrate can contribute to effects in the reaction process of glycerol recovery. This method and condition is important to set the optimum condition for the recovery process which can generate the highest concentration of glycerol. Inappropriate catalysts such as an acid or a base catalyst added in the recovery process also affects the reaction process. Thus, the selection of a catalyst is important in the recovery of a high concentration of glycerol which will increase the reaction in the pre-treatment process.

1.3 OBJECTIVES

The main objective of this research is optimization of pre-treatment process of glycerol residue for succinic acid production. The specific objectives of this research are:

1. To determine the effects of pH, temperature and mass substrate on glycerol concentration during the pre-treatment process.
2. To identify the optimum condition for the pre-treatment processes which will help recover a high concentration of glycerol.
3. To compare the succinic acid production between glycerol residue, glycerol pitch and commercial glycerol.

1.4 SCOPE OF STUDY

There are mainly five scopes in this research:

- 1) The characterization of raw materials (glycerol residue) and products produced before and after the pre-treatment and fermentation processes being carried out. The glycerol (recovered glycerol, commercial glycerol) was characterized by its functional group (Fourier Transform Infra red-FTIR), qualitative analysis (High Performance Liquid Chromatography-HPLC), the physical observation of glycerol residue, recovered glycerol, commercial glycerol (color changes), and chemical analysis of glycerol residue (moisture content, pH, ash, moisture and MONG content). The characterization of *Escherichia coli* was by analysis based on its kinetic growth profile and cell concentration (optical density OD 550_{nm}) while, succinic acid was characterized by qualitative analysis (HPLC).
- 2) The glycerol residue was used in this study. Prior to its application, the optimum condition for glycerol pre-treatment (pH, temperature and mass substrate) was performed by using the Response Surface Methodology (RSM).
- 3) The glycerol residue was obtained from a local oleo chemical plant. Prior to the application, the glycerol residue was treated by using chemical and physical treatment methods in order to remove the impurities and to purify the glycerol content. This pre-treatment is very important because the glycerol recovered was being used as a substrate for the fermentation process in succinic acid production.
- 4) The recovered glycerol obtained from the pre-treatment process was used in this study. Prior to the experiment, both the recovered and commercial glycerol were used as raw materials in order to compare the production of succinic acid in the next stage the experiment. This phase is very important in order to find the best preparation of glycerol sample as a substrate for succinic acid production by the anaerobic fermentation process.

- 5) The strain of *Escherichia coli* (wild strain, K-12 MG1655; ATCC 700926TM) was fed into the fermentation medium which contained recovered glycerol and commercial glycerol as a substrate in order to produce succinic acid. Besides, the details in the study of kinetic growth profile will be presented throughout this study.

1.5 RATIONALE AND SIGNIFICANCE

Succinic acid production by way of the fermentation process is well established and accepted as one of the most advanced methods in the biotechnology field to convert glycerol into higher value products based on both chemical and biological transformations. Many patents and scientific papers dealing with succinic acid production by way fermentation using commercial glycerol does exists but none of them report about the production of succinic acid from low grade of glycerol (glycerol residue) as a raw material. In addition, most of the previous studies applied to recover the glycerol from the pre-treatment process but no study had been carried out on the experimental design which optimizes the pre-treatment process to recover the glycerol residue. Thus, the optimization in the pre-treatment process that is employed in this study could be used as a starting point for succinic acid production from glycerol residue resources. Furthermore, the process being employed is more reliable to be scaled up from the pilot plant scale to the industrial scale based on mathematical modeling obtained from the experiment. Most of the previous studies used glycerol waste from biodiesel production as a raw material to recover glycerol but not many studies have been carried out in using glycerol residue from waste products of oleo chemical industries as a raw material. Additionally, none of the previous journals report studies about the effects of temperature and mass substrate in their recovery of glycerol. Thus, in this study the effects of pH, temperature and mass substrate on glycerol concentration was studied in our recovery process by using glycerol residue as a raw material to produce glycerol. The glycerol residue contains high impurities such as salts and fatty acids and it needs a specific study on waste pre-treatment methods to produce a high concentration of succinic acid.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Over the last decade, succinic acid has attracted a great deal of attention worldwide as being an excellent organic compound and also a key compound in producing more than 30 commercially important products which leads to many promising applications (Agarwal *et al.*, 2005).

Succinic acid is usually produced by a chemical process which uses liquefied petroleum gas or petroleum oil as a base material (Song and Lee, 2006). This may lead to a situation whereby the world would feel threatened by potential hazards and environmental pollutions of succinic acid production. However, researchers have proposed that succinic acid be produced by a fermentation process using microorganisms and the base raw material being glycerol (Lee *et al.*, 2000). In order to reduce the formation of by-products, the succinic acid can be produced by using glycerol as a carbon source (Lee *et al.*, 2000).

To produce succinic acid in an environmentally friendly manner, glycerol waste can be used as a base in the fermentation process aided by microorganisms. Glycerol waste is normally obtained from rapidly growing palm-based oleo chemical industries and approximately 70 % glycerol can be easily recovered by conventional chemical treatment (Hazimah and Ooi., 2000).

Table 2.1: The market demand and prices of succinic acid

Price	References
More than 15,000 tons of succinic acid is manufactured yearly and is sold at US\$5.90-8.80/Kg (depending on its purity)	Lee <i>et al.</i> ,2002
\$5.9 to 9.0/Kg (depending on purity)	Song and Lee., 2006
Global succinic acid market is 15,000 tons with an average growth rate of 6-10 % per year	Song and Lee., 2006

2.2 BACKGROUND OF SUCCINIC ACID

Succinic Acid also known as Amber Acid that has been used in Europe as a natural antibiotic and general curative for centuries (Isar *et al.*, 2006). Succinic acid (Butanedioic Acid) is a dicarboxylic acid of four carbon atoms (Lee *et al.*, 1999).

It occurs naturally in plant and animal tissues. It plays a significant role in intermediary metabolism (Krebs cycle) in the human body. Krebs cycle or also called citric acid cycle or tricarboxylic acid cycle is a sequence process of enzymatic reaction when a two-carbon acetyl unit is oxidized to carbon dioxide and water to provide energy in the form of high-energy phosphate bonds. A study by Agarwal *et al.* (2007), reported that many anaerobic and facultative anaerobic microorganisms ferment carbohydrates to form a mixture of acids such as formate, acetate, lactate and succinate as its end product.

These days, succinic acid produced from microbial fermentation has been attracting interests of researchers because of its potential applications. Previously, research has revealed the effects of various microorganisms on succinic acid production (Song *et al.*, 2006). For instance, Agarwal *et al.* (2007) studied the effects of different environmental and nutritional factors on succinic acid production using *Enterococcus flavescens* by of batch fermentation and enzymes involved in acid production. Gonzalez *et al.* (2008) proposed a kinetic model of succinic acid