PRODUCTION OF SUCCINIC ACID FROM GLYCEROL AND GLYCEROL WASTE

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ABSTRACT

Succinic acid is extensively used globally for manufacture of synthetic resin, biodegradable polymer, pharmaceutical product and food flavoring. Traditionally, succinic acid produced through petrochemical route. The process is non-renewable and will affected pollution in the environment. Therefore, more attention has been focused towards a friendly environment processes for succinic acid production by way of fermentation process; which is highly recommended nowadays. In this research, studies been done to produce succinic acid through anaerobic fermentation of glycerol waste using *Escherichia coli* and to investigate the effect of each factors that influence succinic acid production. The most influential parameter for succinic acid production obtained via preliminary study of parameter range selection were glycerol concentration, tryptone concentration, sodium carbonate (Na_2CO_3) concentration , inoculums density, pH and incubation period. This experiment has produced 0.973g/l succinic acid using One-factor-at-a-time (OFAT) approach from commercial glycerol sample. These six variables screened to select the most significant factor affected succinic acid production using fractional factorial design (FFD) based on first order model term. Four factors selected while, two factors eliminated for the next stage of experiment. Subsequently, full factor central composite designs (CCD) falling under RSM employed to develop a mathematical correlation model between the significant factors for physio-chemical variables of succinic acid production. The experiment has produced 7.55g/l succinic acid from commercial glycerol sample. Besides, a second order polynomial regression fitted and the model was adequate by R-squared 0.9864. Meanwhile, under the optimum level, it is about 7.34g/l succinic acid being produced from commercial glycerol and 7.32g/l of succinic acid produced from recovered glycerol. Overall, this research shows glycerine pitch can be used safely and effective as a carbon source for *Escherichia coli* to produce succinic acid. In addition, this research provides an exciting opportunity to transform low-grade glycerine pitch into highly demanded chemical such succinic acid.

ABSTRAK

Asid suksinik digunakan secara meluas di seluruh dunia dalam pembuatan resin sintetik, polimer terbiodegradasikan, produk farmaseutikal dan perasa makanan. Secara tradisinya, asid suksinik dihasilkan melalui proses petrokimia. Proses ini tidak boleh diperbaharui dan mencemarkan alam sekitar. Oleh itu, perhatian yang lebih telah difokuskan pada proses pengeluaran asid suksinik yang lebih mesra alam melalui proses penapaian; yang amat digalakkan pada masa kini. Dalam kajian ini, kajian telah dilakukan untuk menghasilkan asid suksinik melalui penapaian anaerobik sisa gliserol dengan menggunakan *Escherichia coli* untuk menyiasat kesan bagi setiap faktor yang mempengaruhi pengeluaran asid suksinik. Parameter yang paling berpengaruh untuk pengeluaran asid suksinik yang diperolehi daripada kajian awal pemilihan julat parameter adalah kepekatan gliserol, kepekatan tripton , kepekatan natrium karbonat (Na_2CO_3), ketumpatan inokulum, pH dan jangka masa inkubasi (pengeraman). Kajian ini telah menghasilkan asid suksinik 0.973g/l menggunakan kaedah Satu faktor dalam satu masa (OFAT) daripada sampel komersial gliserol. Enam pembolehubah telah disaring untuk memilih faktor yang paling penting yang mempengaruhi pengeluaran asid suksinik menggunakan bentuk faktorial pecahan (FFD) berdasarkan hukum model pertama. Empat faktor telah dipilih manakala dua faktor telah dihapuskan untuk percubaan ke peringkat seterusnya. Selepas itu, faktor keseluruhan pusat reka bentuk komposit (CCD) telah digunakan untuk membangunkan model korelasi matematik di antara faktor-faktor yang signifikan bagi pembolehubah fisio-kimia penghasilan asid suksinik. Ujikaji ini telah menghasilkan asid suksinik sebanyak 7.55g/l menggunakan sampel komersial gliserol. Regresi polinomial peringkat kedua telah digunakan dan model yang terhasil yang telah menepati R-kuasa dua 0.9864. Manakala diperingkat optimum, sebanyak 7.43g/l asid suksinik terhasil daripada gliserol komersial dan 7.32g/l asid suksinik terhasil daripada sampel gliserol terpulih. Secara keseluruhannya, kajian ini telah menunjukkan bahawa sisa gliserol boleh digunakan dengan selamat dan berkesan sebagai sumber karbon untuk *Escherichia coli* dalam penghasilan asid suksinik. Di samping itu, kajian ini menyediakan peluang yang menarik untuk mengubah sisa gliserol bergred rendah kepada bahan kimia permintaan tinggi seperti asid suksinik.

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LIST OF SYMBOLS

°C	Degree Celsius
g	gram
g/l	Concentration (gram per liter)
mm	Millimeter
3-D	Three-dimensional
%	Percentage
gl/h	Productivity (gram liter per hour)
cm^{-1}	A reciprocal centimeter (or wave number)
cSt	centistokes
R_t	Retention time
OD_{550}	Optical density at wavelength 550nm
$Y_{x/s}$	Growth yield
μ_M	Maximum specific growth
n	Variable quantity
w/w	Mass fraction (mass per mass)
v/v	Volume concentration (volume per volume)
w/v	Mass concentration (mass per volume)
rpm	rotation per minute

LIST OF ABBREVIATIONS

NaOH	Sodium Hydroxide
RSM	Response Surface Method
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning electron Microscope
ANOVA	Analysis of Variance
CCD	Centre Composite Design
HPLC	High Performance Liquid Chromatography
FFD	Fractional Factorial Design
Na_2CO_3	Sodium Carbonate
H_2SO_4	Sulfuric Acid
MONG	Matter-non-glycerol
KOH	Potassium hydroxide
SD	Standard deviation

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Succinic acid is a colourless crystalline dicarboxylic acid which has the molecular formula of $C_4H_6O_4$ (Song and Lee, 2006). This chemical has a potential precursor in the chemicals industry and has a high market volume as a versatile building-block chemical, suitable for many uses (Sauer et al., 2007). It has the potential to become a chemical platform for the production of various value added derivatives including surfactant, electroplating, and flavour enhancer in food products, antibiotics, amino acid, pharmaceuticals and vitamins (Zeikus et al., 1999; Lin et al., 2008). It is also widely used to produce well known industrial chemicals such as butanediol (BDO), tetrahydrofuran (THF) and pyrrolidone (Zeikus et al., 1999).

Succinic acid can be synthesized using both chemical and biological processes. Conventionally, succinic acid is being produced from butane through maliec anhydride in petrochemical routes (Zeikus et al., 1999). Nowadays, technology has created green succinic acid from glucose, generated from cane molasses (Lin et al., 2008), wood hyrolysate (Kim et al., 2004) and straw hydrolysate (Zheng et al., 2009).Although there were many researches being done using glucose, an alternative non-food material such as glycerine pitch which originated from biodiesel production plant could also be used as raw material in succinic acid production. Besides, application of this material as carbon source in succinic acid production is relatively new and there is limited information on the process behaviour. This includes factors affecting on the production as well as the optimum condition in producing the succinic acid. Thus, an understanding of anaerobic fermentation process with the application of batch system could be obtained via research and experimentation in order to determine the optimum reaction condition for succinic acid production.

1.2 PROBLEM STATEMENT

Generally, glycerol waste is an alternative substrate being used for succinic acid production because it has become inexpensive and is abundant in the world market. Silva et al. (2009) had reported that an excess glycerol generated might cause environmental problems since it cannot be disposed into the environment. However, to produce succinic acid from glycerol waste directly without performing treatment, is quite difficult as it contains high impurities such fatty acid (soap) and salts (Yong et al., 2001) which might complicate further utilization of glycerol (Hajek and Skopal, 2010). Besides, it is important to gain a concentrated glycerol with the least amount of other chemical substances to get the highest purity of glycerol that could be used as a substrate in the production of succinic acid. Thus, alternative methods of glycerine pitch treatment are being introduced to overcome these problems as proposed by Kongjao et al. (2010) by the ways of treating glycerine pitch using chemical and physical treatment methods. Nevertheless, not many studies had been carried out to optimize glycerol waste fermentation by *Escherichia coli* in experimental design. Prior to the succinic acid production, preliminary works of parameter range selection were first generated, as this part was essential to produce high concentration of succinic acid. Inappropriate selection of parameters range would attribute to low succinic acid production and crucial problem would occur when it is being introduced to the optimization of fermentation condition. The reaction parameters which mainly contributed to succinic acid production were glycerol concentration as carbon sources/substrate, nitrogen sources concentration, pH level, metal ion dosage, reaction time, temperature, agitation and inoculums density. Improper employment of reaction time, pH level and metal ion dosage are being attributed to microbial defective and thus would reduce succinic acid production. However, unsuitable amount of glycerol concentration would contribute to the inhibition of *Escherichia coli* growth and subsequently decrease succinic acid production. The present study focuses in the treatment process of glycerine pitch as well as determining the optimum condition of anaerobic fermentation of glycerol using *Escherichia coli* in producing succinic acid.

1.3 OBJECTIVES OF STUDY

The objectives of this study are:

(i) To treat and to recover glycerol content from glycerine pitch.
(ii) To study the significant variables in succinic acid production
(iii)To optimize glycerol fermentation process in succcinic acid production.

1.4 SCOPES OF STUDY

There are mainly four scopes in this research:

(i) The glycerine pitch was purchased from a local biodiesel plant. Prior to the experiment, the glycerine pitch was being recovered using chemical and physical treatment methods in order to remove impurities and gain the glycerol content. Besides, study effect of pH value on glycerol recovery is also being presented throughout this study.

(ii) The characterization of raw material (glycerol), microbe (*Escherichia coli*) and product were being characterized along the way of this research being conducted. Three glycerol samples (glycerine pitch, recovered glycerol, commercial glycerol) was being characterized by its functional groups (Fourier Transform Infra Red - FTIR), quantitative analysis (High Performance Liquid Chromatography - HPLC), physical observation (color changes) and chemical analyses (water, pH, ash and MONG). The *Escherichia coli* was being characterized based on kinetic profile growth and cell concentration (optical density; OD_{550nm}) while, the succinic acid were being characterized by its functional group (FTIR) and quantitative analysis using High Performance Liquid Chromatography (HPLC).

(iii)The commercial strain of *Escherichia coli* (wild strain, K-12 MG1655; ATCC 700926^{TM}) was being fed into a formulated medium which contained commercial glycerol to produce the succinic acid. The glycerol concentration, tryptone concentration, inoculums density, pH value, incubation period and sodium carbonate (Na_2CO_3) concentration were being studied using one factor at one time approach (OFAT). Followed by screening significant parameter using Fractional Factorial Design (FFD). Finally, the optimum condition of glycerol fermentation was obtained by using Centre Composite Design (CCD) falling under Response Surface Methodology (RSM).

(iv) The generated mathematical model from RSM being confirmed and validated using commercial glycerol and recovered glycerol as carbon source in fermentation process. This stage is purposely done to investigate developed model can also applied using recovered glycerol sample in producing succinic acid. Finally, the optimum condition of succinic acid production using both commercial glycerol and recovered glycerol are presented and being compared to un-optimized fermentation condition.

1.5 RATIONALE AND SIGNIFICANCE

Succinic acid production through the fermentation process is widely discussed and accepted as one of the most recent and advance techniques in biotechnology field over conventional method of petrochemical route. None in the previous journals had reported the employing of experimental design in optimizing succinic acid production using low-grade biodiesel glycerine pitch as a raw material. As glycerine pitch contains high impurities such as ash and salts, it would produce high concentration of succinic acid after specific waste treatment being performed on glycerine pitch sample.

Besides, this research had utilized *Escherichia coli* as a microbe which is a very amendable to industrial application since it could help to overcome pathogenicity problems, strict anaerobic conditions, unavailability of genetics tools and lack of physiological knowledge that could be found in other microbes (Murarka et al., 2008).Thus, the process would be more helpful when the industrial organism like *Escherichia coli* is being used as biocatalysts (Murarka et al., 2008). Furthermore, anaerobic fermentation under batch system that been employed in this work could be used as a stand point for succinic acid production from renewable resources towards producing commercials succinic acid. In addition, the process being employed is more reliable to scale up into a pilot scale or industrial scale based on mathematical modelling obtained from the experiment.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Annually, industrial residues such as glycerol are produced in large amounts in both oleochemical and biodiesel industries worldwide. This product is normally classified as waste and can be processed in the production of highly demanded product as succinic acid. Succinic acid plays an important role as a precursor in many industrial chemical productions. It has been found in a wide range of applications such as in food industries, pharmaceutical and other important chemical productions such as BDO, THF, and adipic acid. Recent developments in biotechnology have created an impact on succinic acid production. The increase of awareness in environmental issues and the depletion of mineral oil reserves have led to the search for alternatives in making greener succinic acid. One of the prominent solutions in producing this greener succinic acid is through the fermentation process using renewable source that exists in nature such as glycerol. The word fermentation in succinic acid production is described as the process of succinic acid production which involves interaction between mass culture of microorganism and substrate, used under specific condition; either aerobic or anaerobic condition. In this chapter, basic knowledge on succinic acid production through fermentation process would be presented as well as earlier works that are related to this current research.

2.2 FERMENTATION PROCESS

Fermentation process is a biological process that strictly requires sterility during the process, use of cellular enzymatic reactions instead of chemical reactions aided by inanimate catalysts and sometimes operating at higher temperature and pressure.

Fermentation is not necessarily carried out in an anaerobic environment. It can also be run in an aerobic way. In modern fermentation technology, aerobic conditions are maintained in a closed fermenter with submerged cultures. The culture contents are agitated with an impeller and aerated by forcing sterilized air. Table 2.1 presents earlier works on glycerol fermentation which also included the conditions being used.

Table 2.1: Bio-based production of chemical and fuels via glycerol fermentation

Product (concentration, yields*)	**Substrate**	**Strains**	**Culture**	**References**
1,3-Propanediol (30g/l, Y=0.61)	Crude glycerol	*Clostridium acetobutylicum* DG1 (psPD5)	Anaerobic (N_2), continuous	Gonzalez-Pajuelo et al. (2005)
Hydrogen (60mM) Ethanol (90mM, Y=0.69)	Crude glycerol	*Enterobacter aerogenes* HH-101	Anaerobic (N_2), Batch/ continuous	Ito et al. (2005)
Citric acid (77.4g/l, Y=0.69)	Crude glycerol	*Yarrowia lypolitica* NCIM 3589	Aerobic, batch	Imandi et al. (2007)
Succinic acid (4.9g/l, Y=0.59)	Pure glycerol	*Anaerobiospirillum succiniciproducens* ATCC 29305	Anaerobic (CO_2), batch	Lee et al. (2001a)
Butanol (17g/l, Y=0.39)	Pure glycerol	*Clostrodium pasteurianum* DSM 525 (ATCC6013)	Anaerobic (N_2), batch	Biebl et al. (2001)

*yield (mol product per mol glycerol)

2.2.1 Type of Fermentation

Industrial fermentation processes can be divided into two main types. They are batch fermentations and continuous fermentations.

i) Batch fermentation process

Batch fermentation is considered as ‘closed system’. In the course of an entire fermentation, no nutrient is being added into the fermenter except for oxygen, anti-foam agent and pH controller, either in base or acidic form. In general, batch fermentation is being used to produce biomass and primary metabolite. In order to maximize biomass production, optimization of cultural condition for both physical and chemical nutrients is highly recommended. There are a few advantages of batch fermentation such as low contamination risk, able to run different successive phase in the same vessel and close control of genetic stability of microorganism(s). In batch fermentation mode, the systems are normally controlled culture pH, dissolved oxygen tension and temperature. There are many researches which had applied this batch processing system to produce new products. Lee et al. (2008) had successfully produced succinic acid by *Anerobiosprillum succiniciproducens* in batch fermentation of mixed sugar of galactose, glucose and lactose. Furthermore, Jiang et al. (2010) had employed batch fermentation system in producing succinic acid from enzymatic hydrolysate of brewer’s yeast (EBY) by *A. succinogenes* NJ113 with the addition of vitamins to replace commercial yeast extract. After 48h of cultivation under batch mode, it has resulted in 46.8 g/L succinic acid concentration and yield of 68.8% being produced.

ii) Continuous fermentation process

Continuous fermentation process is a system where the mixed culture is continuously being supplied with fresh nutrients, and volume of culture is being kept maintained by continuous removal of liquid culture at the same flow rate as the feeding rate of fresh nutrients. Hence, this method offers a continuation of growth for a long period of cell cultivation. With this feature, the designated medium could overcome the problem of limitation of substrate concentration, besides the exponential growth phase is being prolonged until an extra substrate is being exhausted on the cell. The uniqueness of this fermentation style is it operates under steady state conditions, where cell growth occurs at constant growth rate and stable environmental conditions. Factors such as pH of the culture, specific growth rate, nutrient and cell concentration, metabolites concentration and dissolved oxygen; are all keep maintained in the continuous culture. Using this kind of fermentation method, continuous system promises important potential advantages in reducing operating and raw material costs. Brethauer and Wyman (2009) mentioned that some continuous fermentation is now being employed for commercial ethanol production from cane sugar and corn. This is to take advantage of higher volumetric productivity, reduced labour costs, and reduced vessel down time for cleaning and filling. In contrast, these systems are more susceptible to microbial contamination and need more advanced operations.

Continuous operation in succinic acid production gives more advantages over batch operation mode. Since the system is fully equipped with continuous media sterilization and aseptic technique operation, the contamination problem to continuous system could also be eliminated (Shuler and Kargi, 2002). Many scientific papers and patents published today deal with the continuous fermentation system on succinic acid production. Research done by Lee et al. (2003b) had performed anaerobic continuous culture of *Mannheimia succiniciproducens* MBEL55E in whey based medium containing Corn Steep Liquor (CSL) which had resulted in succinic acid yield of 69% and productivity as high as 3.9gL/h and Kim et al. (2004) had attempted to use continuous culture of *Mannheimia succiniciproducens* MBEL55E in wood hydrolysate based medium and had produced succinic acid yield of 56% and the productivity being achieved at 1.17gL/h. Besides, another research had shown another type of microbe which posses the capabilities to produce succinic acid through the continuous system. Kim et al. (2009) had successfully produced succinic acid by continuous fermentation of *Actinobacillus succinogenes* sp. 130Z in an external membrane cell recycle reactor. This work had resulted in 16.4g/L production and productivity at 6.63gL/h was achieved. In addition, Oh et al. (2008) had reported anaerobic continuous fermentation of *Mannheimia succiniciproducens* LPK7 yielded 0.38mol/mol of succinic acid and productivity at 1.77gL/h was successfully being attained.

2.2.2 Nutritional and Physiological Requirement in Fermentation Processes

Normally, bacterial growths in fermentation processes require specific nutritional needs which would suit to its type and its physical conditions. The nutrient includes chemicals and elements that used for bacterial growth. These refer to nutritional requirements for energy generation, cell maintenance, reproduction and biosynthesis of cellular. Essentially, the key components for bacterial growth consist of carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, magnesium, iron, calcium, manganese and traces elements. The common physiological functions of the elements are summarized as in Table 2.2. The microbial cells grow under a huge range of physical conditions such as dissolved oxygen, agitation, hydrogen ion concentration (pH) and temperature. For example, a thermophile grows at high temperatures; an acidophile grows at low pH; and osmophile grows at high solute concentration. Water availability in culture medium also becomes a crucial factor in microbial growth.

Table 2.2: Physiological function of principle element for cell growth (Kampen, 1997)

Element	**Symbol (atomic number)**	**Physiological function**
Carbon	C (6)	Constituents of organic cellular material
Oxygen	O (8)	Constituent of cellular water and organic materials, as O_2 electron acceptor in respiration of aerobes