EFFICIENCY STUDIES OF BIOBUTANOL PRODUCTION FROM PRETREATED LEMONGRASS LEAVES USING *Clostridium beijerinckii* SR1

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"SPECIALLY DEDICATED TO MY MOM, DAD, GRANDMA AND MY LOVING WIFE KANMANI. A SMALL GIFT FOR YOUR ABUNDANT AND ENDLESS LOVE AND CARE MY DEAR WIFE."

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ABSTRACT

The increase in energy consumption and the limited supply of petroleum fuels has raised interest toward development of biobutanol production from cellulosic materials. This research performed the production of biobutanol using hydrolysate from pretreated lemongrass leaves in addressing the petroleum depletion issue and serves as an efficient process for green fuel technology. Chemical composition analysis shows that lemongrass leaves are made up of cellulose, hemicellulose and lignin with solid fraction of 47.21%, 32.41% and 13.17% (w/v), respectively. Screening for suitable pretreatment methods resulted in the highest reducing sugar production of 61.05 g/L employing physico-chemical pretreatment method (autoclaving at 121°C for 15 minutes in 2% (v/v) of sulphuric acid) followed by enzyme saccharification using Celluclast and Novozyme enzyme cocktails at concentration of 3% (v/v) each using 7% (w/v) of lemongrass leaves. Application of central composite design (CCD) on enzyme saccharification further improved reducing sugar production to 80.1 g/L equivalent to 1.31 fold improvement compared to that before optimization. A total of 13 bacteria strains were isolated anaerobically from various sources using rolling tube technique and anaerobic chamber. Strain SR1 which was further identified as *Clostridium beijerinckii* SR1 using 16S rRNA technique exhibited ability to produce highest butanol of 0.71 g/L. Optimization of biobutanol production in batch culture using general factorial design (GFD) for screening of suitable nitrogen source and CCD for optimization of fermentation parameters resulted in improved biobutanol production of 1.40 g/L which was 1.97 fold higher compared to before optimization. Application of CCD further suggested that temperature, pH, reducing sugar concentration, nitrogen source (meat extract) concentration were fixed at 34.91°C, 6.48, 50.06 g/L and 47.72 mM respectively. An initial pH of 6.48 (without pH control at acidogenic phase) and controlled pH during solventogenic phase at 5.5 gave rise to biobutanol production to 1.52 g/L which was 1.09 fold before application of pH control strategy. Fix mode, constant variable mode and exponential mode of feeding were screened for optimum biobutanol production in fed batch culture. Exponential feeding mode with dilution rate of 0.04 h^{-1} resulted in highest biobutanol production of 1.91 g/L that was 1.26 fold higher compared to batch culture. Application of repeated fed batch system using 60% (v/v) of replacement ratio further improved biobutanol production to 2.46 g/L which was 1.28 fold and 1.62 fold higher compared to fed batch system and batch system respectively. Based on the research performed, pretreated lemongrass leaves have a great potential to be used as a substrate for biobutanol production.

ABSTRAK

Peningkatan penggunaan tenaga dan bekalan bahan api petroleum terhad meningkatkan minat terhadap perkembangan penghasilan biobutanol dari bahan selulosa. Dalam penyelidikan ini penghasilan biobutanol dilakukan menggunakan hidrolisat dari daun serai yang diprarawat dapat membantu masalah pengurangan petroleum dan menjadi proses efisien bagi penghasilan bahan api teknologi hijau. Analisa komposisi kimia menunjukkan daun serai terdiri daripada sellulosa, hemisellulosa dan lignin dengan kandungan pepejal masing-masing adalah 47.21%, 32.41% and 13.17% (w/v). Penyaringan kaedah prarawatan yang sesuai menghasilkan gula penurun tertinggi 61.05 g/L menggunakan kaedah prarawatan gabungan fizikal-kimia (autoklaf pada suhu 121°C selama 15 minit di dalam 2% (v/v) asid sulfurik) diikuti sakarifikasi menggunakan koktel enzim Celluclast and Novozyme pada kepekatan 3% (v/v) dengan 7% (w/v) daun serai. Aplikasi *Central Composite Design* (CCD) melalui sakarifikasi berenzim meningkatkan produksi gula penurun kepada 80.1 g/L bersamaan dengan 1.31 ganda peningkatan berbanding sebelum pengoptimuman. Sebanyak 13 strain bakteria dipencilkan dari pelbagai sumber menggunakan teknik gulingan botol dan kebuk anaerobik bagi penghasilan biobutanol. Strain SR1 yang diidentifikasi sebagai *Clostridium beijerinckii* SR1 melalui kaedah 16S rRNA menunjukkan keupayaan menghasilkan biobutanol tinggi sebanyak 0.71 g/L. Pengoptimuman penghasilan biobutanol dalam kultur kelompok menggunakan *General Factorial Design* (GFD) untuk pemencilan sumber nitrogen dan CCD bagi pengoptimuman parameter fermentasi telah meningkatkan penghasilan biobutanol kepada 1.40 g/L iaitu 1.97 ganda lebih tinggi berbanding sebelum pengoptimuman. Aplikasi CCD mencadangkan agar suhu, pH, kepekatan gula penurun, kepekatan sumber nitrogen (ekstrak daging) ditetapkan masing-masing pada 34.91°C, 6.48, 50.06 g/L and 47.72 mM. pH permulaan pada 6.48 (tanpa kawalan fasa asidogenik) diikuti kawalan pH pada 5.5 sewaktu fasa solventogenik meningkatkan penghasilan biobutanol kepada 1.52 g/L iaitu 1.09 ganda lebih tinggi sebelum aplikasi pengawalan pH strategik. Suapan mod tetap, mod malar dan mod eksponen disaring bagi penghasilan biobutanol tertinggi dalam kultur kelompok suapan. Mod eksponen dengan kadar pencairan 0.04 h^{-1} menghasilkan biobutanol tertinggi sebanyak 1.91 g/L iaitu 1.27 ganda lebih tinggi berbanding kultur kelompok. Aplikasi sistem suapan kelompok ulangan menggunakan 60% (v/v) nisbah penggantian meningkatkan penghasilan biobutanol kepada 2.46 g/L masing-masing adalah 1.28 ganda dan 1.62 ganda lebih tinggi berbanding sistem suapan kelompok dan sistem kelompok. Berdasarkan penyelidikan yang dijalankan, daun serai yang telah diprarawat mempunyai potensi untuk digunakan sebagai substrat bagi penghasilan biobutanol

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LIST OF ABBREVIATIONS

2LFD	-	Two-factorial design
ANOVA	-	Analysis of Variance
CCD	-	Central Composite Design
DNS	-	Dinitrosalicyclic acid
FESEM	-	Field Emission Scanning Electron Microscope
FTIR	-	Fourier Transform Infrared Spectroscopy
g	-	Gram
GC	-	Gas Chromatography
GFD	-	General Factorial Design
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
HNO_3	-	Nitric acid
HPLC	-	High Performance Liquid Chromatography
kDa	-	Kilo Dalton
L	-	Liter
min	-	Minute
mL	-	Milliliter
mm	-	Millimeter
MW	-	Molecular Weight
NaOH	-	Sodium hydroxide
N/A	-	Not applicable
nm	-	Nanometer
$^{\circ}C$	-	Degree Celsius

PAGE	-	Polyacrylamide Gel Electrophoresis
RID	-	Refractive Index Detector
RSM	-	Response Surface Methodology
SDS	-	Sodium Dodecyl Sulfate
U/g	-	Unit of enzyme per gram
v/v	-	Volume per volume
w/v	-	Weight per volume
µL	-	Micro liter
µm	-	Micro meter

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CHAPTER 1

INTRODUCTION

As the crude oil prices started to rise tremendously from the beginning of the 1970s due to the “oil crisis” together with the uncertainty of petroleum supplies and emergent of environmental concern, the world have started to pay more attention towards production of biofuel. The anaerobic fermentation of various polyoses obtained from cellulosic materials especially agricultural waste to solvent via ABE fermentation (acetone-butanol-ethanol) has been reported and commercially used since the World War 1 and 2 (Kandhola *et al*., 2017; Qureshi *et al.,* 2008). However, less interest was shown towards microbial fermentation process compared to petrochemical industries due to tedious process and it’s not economically visible. The development of biotechnology filed however in the recent years had contributed to the enhancement of the biological process towards ABE fermentation using cellulosic materials obtained from agricultural waste to reduce the cost for raw materials. Since then, substantial research efforts on ABE production had been carried out in multiple fields, namely in fermentation technology, to improve solvent yields, low volumetric productivities and final product concentration.

ABE fermentation gained interest of researchers worldwide and attracts more investors as the solvents produced possess many applications in industries. Biobutanol is an important chemical with many applications in the plastic industry and as a potential fuel extender. This chemical has many characteristics, which make it a better fuel extender than ethanol (Wu *et al.,* 2013). Biobutanol also has good potential to be used as an extractant in the food and flavor industry. They are several areas in ABE process that can be continuously improved to obtained high yield of solvents. They are (i) improvement of strains, (ii) development of fermentation techniques (iii) improvement of pretreatments. A large number of solventogenic clostridia have been reported over the years (Gholizadeh, 2010). A promising route to improve ABE-fermentation is the development of metabolic and genetically-modified clostridia with increased solvent production due to reutilization of carboxylic acids accumulated during the acidogenic phase of carbohydrate uptake, and increased resistance to product inhibition.

Lignocellulose is a major structural component of woody and non-woody plants. It consists of three major structure, cellulose, hemicellulose and lignin. The effective utilization of all the three components would play a significant role in the economic viability of cellulosic biobutanol. In recent years, Malaysia has performed research on utilization of various lignocellulosic wastes such as empty fruit bunch (Ibrahim *et al*., 2015), paddy straw (Rahnama *et al*., 2014) and sago pith residues (Linggang *et al*., 2013). To implement a successful biobutanol production process, the first impediment to be resolved is the efficient removal of lignin and hemicellulose through a cost effective pretreatment process (Wi *et al.,* 2015). The selection of pretreatment should take into consider of overall suitability of feedstock, enzymes and organisms to be used (Sarkar *et al*., 2012). Although various polyoses can be obtained from pretreated lignocellulosic materials, a suitable pH regulation is important to increase solvent production as the efficiency of enzymes produced for ABE fermentation by the strain are sensitive towards external factors such as pH. Very few studies are reported on pH control strategies for ABE fermentation which utilizes pretreated lignocellulosic materials.

Batch reactors are usually preferred in the industry due to its simple mode of operation while reducing the contamination risk. However, the productivity obtained in a batch reactor is generally low due to the lag phase, product inhibition effects, cleaning, sterilizing, and re-filling the reactor. The preparation time and lag phase can be surpassed by using continuous operation and the problem of product inhibition can be resolved through the incorporating an *in situ* product removal system (Veettil *et al.*, 2016). To overcome substrate inhibition and to increase the substrate concentration, fed-batch mode of operation with intermittent or continuous feeding of nutrients has been used for biobutanol production. A fed-batch culture by feeding butyric acid and glucose has been studied in an acetone–butanol–ethanol (ABE) fermentation using *Clostridium saccharoperbutylacetonicum* N1-4 introduced by Tashiro *et al.* (2004). Thus, various fermentation techniques besides traditional batch system are being researched worldwide. Suitable fermentation system depends on choices of microorganism, substrate, downstream process and feasibility.

In Malaysia, up to 160 million tons of agricultural wastes is produced annually (Goh *et al.,* 2010; Ozturk *et al.,* 2017). This includes lemongrass leaves after the cultivation process of the stalks. Malaysia had been known to produce nearly 7612 tonnes of lemongrass leaves in year 2012 (Ang *et al.,* 2015b; Hussin *et al.*, 2015). Lemongrass leaves are one of the most abundant agricultural wastes that contain high lignocelluloses materials in Malaysia. Lignocellulosic materials in lemongrass leaves can be converted into sugars for biobutanol production by suitable pretreatment. This research would be the first attempt in the world for utilization of treated lemongrass leaves for biobutanol production via microbial biotransformation. This invention could be a stepping stone for utilization of agricultural waste to commercial valued products. In the present study, hydrolysate from pretreated lemongrass leaves was used as a source of carbon to produce biobutanol. This study provides a solution for almost all the limitation occurred in previous researchers during ABE production using different lignocellulosic substrate. This includes selection of suitable pretreatment, application of pH control strategic and application of fed batch system for ABE fermentation process.

1.1 Objectives

The objectives of the research are:

1. To screen and optimize suitable pretreatment technique of lemongrass leaves for optimum sugar production.
2. To isolate, screen and identify potential biobutanol producer.
3. To optimize the fermentation medium for optimum biobutanol production in batch culture using general factorial design (GFD) and central composite design (CCD).
4. To develop suitable pH control strategy for optimum biobutanol production in 2L bioreactor.
5. To screen for suitable feeding strategy in fed batch and repeated fed batch culture for optimized biobutanol production.

1.2 Problem Statement

The search for sustainable energy calls for the usage of renewable resources as the feedstock (Bramono *et al*., 2011). Apart from alleviating pollution problems, alcohols converted from the cellulosic biomass are promising alternative fuels such as biobutanol and can be used to fulfill the increasing energy demand (Maddipati *et al*., 2011). ABE fermentation for biobutanol production however requires high cost of raw materials. Since Malaysia is rich in various agricultural resources, there will be fewer tribulations in finding a potential and cost effective substrate to be exploited for ABE fermentation (Aditiya *et al*., 2016). Bioconversion of renewable lignocellulosic biomass to biofuel and value added products are globally gaining significant prominence. In Malaysia, the government support for lemongrass plantation can be seen by help given through MARDI, a government body which was founded to aid in the agricultural sector in the country (Ahamad *et al*., 2013).

During the lemongrass stalk harvesting, 70% of the plant materials were discarded as waste (Hussin *et al*., 2015). The major problem encountered during harvesting of lemongrass is the waste, management system. Due to its content which high in aromatic compounds, the waste does not served as ruminants feed (Kaur and Dutt, 2013). It had been reported that developing countries such as Malaysia find an easier solution such as open burning system without any proper controls to dispose their solid waste such as agricultural waste (Ogawa, 2008). Besides that, the high content of cellulosic components in the lemongrass increased the value of the baggase produced to be utilized (Ang *et al*., 2015b). Thus, this study proposes to utilize the lemongrass leaves which are the major waste as an alternative inexpensive substrate in the production of biobutanol.

1.3 Scope of Research

The scope of this research is to study the biobutanol production from hydrolysate of pretreated lemongrass leaves using locally isolated anaerobic bacteria. Various pretreatment techniques were screened for optimum reducing sugar production. Optimization of enzyme saccharification process using commercial enzyme cocktails was performed using central composite design (CCD). Strains from various sources were isolated and screened for biobutanol production via ABE fermentation. Strain which produced highest biobutanol via ABE fermentation and identified using 16S rRNA method. Suitable nitrogen source was selected using general factorial design (GFD). Optimization of selected parameters such as temperature, pH, incubation period, carbon source concentration and nitrogen source concentration in batch culture was performed employing central composite design (CCD). Selection of suitable pH control strategy during acidogenic and

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