Simultaneous Saccharification and Co-Fermentation Using Co-Culture for Conversion of Mango Leaves into Bioethanol

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PAGE

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CCD	Central Composite Design
DNS	Dinitrosalicylic Acid
FPA	Filter Paper Assay
FPU	Filter Paper Unit
FRIM	Forest Research Institute Malaysia
FTIR	Fourier Transform Infrared
GHG	Greenhouse Gas
HPLC	High Performance Liquid Chromatography
INSAT	Institute of Sustainable Agrotechnology
IU	International Unit
IUPAC	International Union of Pure and Applied Chemistry
LAP	Laboratory Analytical Procedure
LC	Lignocellulosic
MYGP	Malt Yeast Glucose Peptone
NREL	National Renewable Energy Laboratory
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
RED	Renewable Energy Directive
RMSD	Root Mean Square Deviation
RSM	Response Surface Methodology
SEM	Scanning Electron Microscope
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
TSS	Total Soluble Solids
XRD	X-Ray Diffraction
1G	First-Generation
2G	Second-Generation

LIST OF SYMBOLS

cm	Centimeter
°	Degree
℃	Degree Celsius
g	Gram
h	Hour
kV	Kilovolt
λ	Lambda (Wavelength)
L	Liter
µL	Microliter
mL	Milliliter
mg	Milligram
mm	Millimeter
mM	Millimolar
min	Minute
M	Molar
nm	Nanometer
%	Percentage
rpm	Revolutions per minute
w/v	Weight to volume ratio
w/w	Weight to weight ratio

Pensakaridaan Serentak dan Ko-Fermentasi Menggunakan Ko-Kultur untuk Pertukaran Daun Mangga kepada Bioetanol

ABSTRAK

Persaingan makanan berbanding bahan api telah mendorong minat negara terhadap bioetanol sekunder, yang dihasilkan dari biojisim lignoselulosa (LC). Daun mangga (*Mangifera indica*) telah diakui sebagai sisa pertanian yang tidak boleh dimakan berpotensi dijadikan substrat untuk bioetanol berasaskan LC kerana kandungan polisakarida yang tinggi. Selain itu, pensakaridaan serentak dan ko-fermentasi (SSCF) dikenali sebagai keadah effective untuk meningkatkan produksi bioetanol sekunder ini. Namun demikian, ko-kultur untuk menukar kedua-dua gula heksosa dan pentosa dalam daun mangga secara serentak kepada bioetanol masih belum ditemui. Oleh itu, kajian ini menekankan pada pertukaran daun mangga kepada bioetanol menggunakan *Saccharomyces cerevisiae* dan *Trametes versicolor* melalui SSCF. Pada mulanya, perbandingan komposisi biojisim antara varieti daun mangga menunjukkan Harum Manis merupakan sumber bioetanol yang paling wajar kerana mengandungi holoselulosa (78.73 %), kepekatan gula fermentasi (163.83 ± 5.48 mg/g biojisim) dan bioetanol (0.21 ± 0.02 g/L) yang lebih tinggi berbanding Chokanan dan Sunshine. Seterusnya, kajian ini bertujuan untuk menjelaskan perubahan struktur daun manga Harum Manis selepas pra-rawatan kimia tunggal (asid atau alkali) dan berperingkat (asid-alkali atau alkali-asid). Pra-rawatan berperingkat asid-alkali dengan kesan pendeligninan paling ketara (86.97 ± 1.26 %) membolehkan biojisim menghasilkan komposisi holoselulosa, kandungan gula fermentasi dan kepekatan bioethanol yang tertinggi iaitu masing-masing pada 95.26 %, 415.02 ± 7.01 mg/g biojisim dan 1.57 ± 0.06 mg/mL. Di samping itu, pencirian struktur mengesahkan bahawa biojisim selepas pra-rawatan asid-alkali merupakan substrat yang paling sesuai untuk pengeluaran bioetanol dalam kajian ini. Seterusnya, selulase dan xilanase telah disintesis daripada *Aspergillus niger* dengan aktiviti enzimatik masing-masing pada 0.413 ± 0.014 FPU/mL dan 0.514 ± 0.009 IU/mL dalam kajian ini. Kedua-kedua enzim ini telah terbukti sesuai untuk sakarifikasi enzimatik semasa penghasilan bioethanol. Selepas itu, kajian ini juga menunjukkan bahawa *S. cerevisiae* dan *T. versicolor* serasi sebagai ko-kultur untuk meningkatkan ko-fermentasi glukosa dan xilosa dalam daun mangga kepada bioetanol dengan ketara. Dengan ini, penghasilan bioetanol berdasarkan SSCF yang ditentukan telah disaring dan dioptimumkan. Hasilnya, keadaan optimum pada suhu: 30.68 °C, pH: 5 dan masa inkubasi: 5.08 hari telah menyumbang kepada produksi bioetanol maksimum sebanyak 79.59 ± 0.32 %. Akhirnya, kinetik pengeluaran bioetanol ini dikaji. Model Logistik dan model Modifikasi Gompertz ditemui untuk memahami sintesis sel ko-kultur, penggunaan substrat, dan pembentukan produk yang berlaku semasa pengeluaran bioetanol ini. Secara keseluruhannya, potensi penambahbaikan untuk proses SSCF ini dapat diterokai menggunakan pandangan kinetik yang diperolehi untuk pertukaran daun mangga kepada bioetanol secara berkesan.

Simultaneous Saccharification and Co-Fermentation Using Co-Culture for Conversion of Mango Leaves into Bioethanol

ABSTRACT

The food versus fuel competition has spurred nation's interest on secondary bioethanol, which is produced from lignocellulosic (LC) biomass. Mango leaves (*Mangifera indica*) have been recognized as abundantly available non-edible agricultural waste that could be a LC based bioethanol substrate for their high polysaccharide content. Besides, simultaneous saccharification and co-fermentation (SSCF) is identified as an effective method to maximize this secondary bioethanol production. However, a feasible co-culture to simultaneously convert both hexose and pentose sugar in the mango leaves into bioethanol is yet to be discovered. Therefore, the present work emphasizes on conversion of mango leaves into bioethanol through co-culture of *Saccharomyces cerevisiae* and *Trametes versicolor* via SSCF. Initially, a comparison of biomass composition among mango leaves varieties indicated that Harum Manis mango leaves are the most desirable bioethanol crop for its highest holocellulose content of 78.73 %, maximum concentration of fermentable sugar (163.83 ± 5.48 mg/g of biomass) and bioethanol (0.21 ± 0.02 mg/mL) compared to Chokanan and Sunshine. Subsequently, this study aimed to elucidate the structural changes of Harum Manis mango leaves after single-stage (acid and alkaline) and two-stage (acid-alkaline and alkaline-acid) chemical pretreatments. Acid-alkaline pretreatment with the maximum delignification effect (86.97 ± 1.26) facilitated the biomass to yield the highest holocellulose composition, fermentable sugar content and bioethanol concentration of 95.26%, 415.02 ± 7.01 mg/g and 1.57 ± 0.06 mg/ mL, respectively. Additionally, the structural characterizations also validated the acid-alkaline pretreated biomass to be the most feasible substrate for bioethanol production in this study. Next, cellulase and xylanase with enzymatic activity of 0.413 ± 0.014 FPU/mL and 0.514 ± 0.009 IU/mL, respectively, were synthesized from *Aspergillus niger* in this study. These enzymes were proven to be viable for enzymatic saccharification during the bioethanol production. Thereafter, this study also demonstrated that *S. cerevisiae* and *T. versicolor* were compatible as a co-culture to significantly enhanced the co-fermentation of glucose and xylose in the mango leaves into bioethanol. With this, the designated SSCF based bioethanol production was screened and optimized. Consequently, the optimum condition at temperature: 30.68^{o}C, pH: 5 and incubation time: 5.08 days yielded a maximum bioethanol production of 79.59 ± 0.32 %. Lastly, kinetic study of this bioethanol production was evaluated. The Logistic model and Modified Gompertz model were discovered to proficiently understand the co-culture cell synthesis, substrate consumption, and product formation that occurred during the bioethanol production. Overall, the scale up potential of this SSCF process could be explored using the obtained kinetic insights for an efficient conversion of mango leaves into bioethanol.

CHAPTER 1 : INTRODUCTION

Chapter one presents background study, problem statement, research objectives, and scope of research. Background study is a brief overview on the evolution of bioethanol production. Problem statement reflects on existing problems in the field of bioethanol production to which this research work, studies on mango leaves as lignocellulosic (LC) substrate for the production of bioethanol via simultaneous saccharification and co-fermentation (SSCF) configuration could be a solution. This section validated the significance of the research work. The final part of this chapter is on research objectives and scope that specifies the depth of the research work.

1.1 Background Study

In the current waves of globalization, fossil fuels including petroleum, coal, and natural gas are known to be the most dependent non-renewable sources. Owing to the needs of transportation and industrialization that rose tremendously, natural gas and petroleum source have been losing their sustainability gradually (Sathendra et al., 2019; Tan et al., 2020). This awareness has led to the search for an alternative energy resource (Jugwanth et al., 2020; Qu et al., 2020). Through this quest, researchers have devoted their attention to the production of ethanol fuel (Bhardwaj et al., 2020).

Ethanol can be manufactured either through the chemical or microbiological method. The chemical pathway to produce ethanol can be described through the reaction mechanism of ethylene hydration (Mączyńska et al., 2019). The transformation of reducing sugar by selective microorganism into ethanol during fermentation defines the microbiological process (Cao et al., 2020). In fact, the monomeric sugar in the microbiological process usually originates from renewable sources such as agricultural feedstock and plant residuals (de Barros Ranke et al., 2020). Ethanol produced via this microbial fermentation process is known as bioethanol or green petroleum (Mohapatra et al., 2020).

Currently, the bioethanol production process has been gaining intense interest worldwide in the transportation sector (Astolfi et al., 2020; Rabbani et al., 2020; Wang et al., 2020). In fact, bioethanol is the most suitable alternative for fossil fuel. Unlike petroleum, bioethanol is a form of renewable energy, non-toxic and biodegradable (Arpia et al., 2021). In conjunction with its establishment as clean and safe for petroleum replacement, bioethanol is widely used as a biofuel additive to decrease environmental issues especially greenhouse gas (GHG) emission (Balali & Stegen, 2021; Granjo et al., 2020). Renewable Energy Directive (RED) has published a legislative proposal, RED II, which obliges the countries of European Union to have an energy production of at least 27 % from renewable source, whereby 6.8 % should be represented by the biofuel industry by 2030. This requirement is in line with the need of the Fuel Quality Directive to reduce 6 % of GHG emission caused by fossil fuel combustion in vehicles within year 2020 (Garofalo et al., 2020).

At earlier stages, bioethanol manufactured at industrial scale utilized starch and sucrose-based sources such as corn, sugarcane, maize and potato as a substrate. However, as for the present scenario, this first-generation (1G) bioethanol production was not optioned in term of long-term reliability (Adetoyese et al., 2020). This is because the substrates are edible biomass. A continuous usage of these feedstocks could cause incompetence to meet the human food demand (Ben-Iwo et al., 2016; Mishra &

Ghosh, 2020). Subsequently, second-generation (2G) bioethanol production has been introduced in both academic society and biofuel industry. LC biomass such as agricultural residue is aided as substrate for 2G bioethanol production (Bonenkamp et al., 2020).

In this study, the leaves of Mango (*Mangifera indica*) removed during the pruning session were chosen for the secondary bioethanol production. This LC based agricultural left-over is widely accessible with polymeric constituents similar to the conventional starchy or sugary biomass used in bioethanol production. Specifically, the cumulative of cellulose and hemicellulose composition in the mango leaves could be converted into fermentable sugar for bioethanol production (Das et al., 2013; Paul et al., 2020). In addition, these leaves were acclaimed as agricultural waste and discarded through burning (Shi et al., 2020; Singh et al., 2020). Indeed, this make the LC biomass a non-competitive food supply. As a consequence, food versus fuel crisis was resolved (Alayoubi et al., 2020; Lyu et al., 2020; Pachón et al., 2020; Rabbani et al., 2020). Furthermore, this method is a wise practise of waste materials management and could be a great environmental solution to demolish the intolerable condition caused by dumping of waste into landfills (Stoumpou et al., 2020). Besides, this waste to wealth initiative would be a strategy to minimize the expenditures of the raw material and, thus, developing a cost-effective bioethanol production process (Song et al., 2020).

Since mango leaves are used during the bioethanol production, a combination of enzymatic saccharification and fermentation is commonly suggested to overcome the complexity of the LC biomass. Among various integrated fermentation technologies, SSCF approach has emerged to be the most effective method for the 2G bioethanol production (Chen et al., 2017; Liu & Chen, 2016). In SSCF, saccharification enzymes added into the media convert the LC carbohydrates converts into fermentable sugars such as hexose and pentose sugar. Simultaneously, the inoculated hexose and pentose sugar fermenting mono or co-culture microbial system yields the bioethanol. The overall process of SSCF takes place in one vessel (Panahi et al., 2020). This process is an advanced version of the initially discovered method of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In precise, the SSCF process has been experimentally proven to record a higher bioethanol yield in comparison to SHF and SSF. This is because, unlike, SHF and SSF, in SSCF, both the pentose and hexose sugar available in LC biomass could be utilized without an end-product inhibition during the process (Amoah et al., 2019; Liu et al., 2017; Qin et al., 2017).

To date, there has been certainly insufficient discussion of the research works conducted on SSCF based bioethanol production from mango leaves. Although previous studies have been reported on mango waste residues as a substrate for bioethanol production, the focus was only on implementation of SHF and SSF methods (Das et al., 2013; Jahid et al., 2018). Hence, this study is an investigation on the feasibility to maximize the utilization of the fermentable sugar in mango leaves for production of bioethanol via SSCF configuration.

1.2 Problem Statement

Mango leaves is an agricultural lignocellulosic left-over with limitless accessibility for secondary bioethanol production. The conversion of mango leaves into bioethanol requires inclusion enzymatic saccharification and fermentation processes after the delignifcation process to overcome the structural recalcitrance. At first, SHF process was implemented whereby the enzymatic hydrolysis conducted prior to fermentation led to end-product inhibition which resulted a low bioethanol yield. These obstacles contribute to the initiation of SSF method in which enzymatic hydrolysis is carried out in parallel with the fermentation process to convert only glucose into bioethanol. Conversely, xylose in LC biomass cannot be utilized for bioethanol production using SSF configuration. Hence, SSCF was introduced for its strategical approach which combined enzymatic hydrolysis and co-fermentation of glucose and xylose to boost the bioethanol yield.

However, previous studies on SSCF process have reported selective utilization of glucose over xylose which triggered decrease in bioethanol yield. Despite a number of attempts through metabolic as well as evolutionary engineering to facilitate the SSCF process, the xylose fermentation capacity of the microbial strain is inhibited by the presence of glucose. Based on these constraints, a viable microbial co-culture system to execute the bioethanol production via SSCF process is still in search. Although *Saccharomyces cerevisiae* is widely incorporated into LC bioethanol production, this yeast strain can only metabolize glucose into bioethanol. Hence, it would be worthy to co-culture *S. cerevisiae* with a xylose-utilizing microorganism that could maximize conversion efficiency of fermentable sugar derived from mango leaves into bioethanol. Nevertheless, concerns on the growth conditions adaptibility and fermentation compatibility of the microbial strains should be addressed prior to attempting this SSCF approach for bioethanol production from mango leaves.

1.3 Research Objectives

The research objective is divided into two which are the general objective and the specific objectives.

1.3.1 General Objective

In general, this study is structured to analyse the prospective of *M. indica* leaves as a LC substrate for the bioethanol production via SSCF co-cultured with *S. cerevisiae* and *Trametes versicolor*.

1.3.2 Specific Objectives

1) To investigate the biochemical composition and structural modifications after chemical pretreatment on the selected variety of mango leaves for bioethanol production.
2) To examine the hydrolytic potential of on-site synthesized saccharification enzymes along with the compatibility of *S. cerevisiae* and *T. versicolor* for bioethanol production.
3) To screen and optimize the process parameters of bioethanol production using SSCF configuration.
4) To evaluate the kinetic of the proposed bioethanol production process using unstructured kinetic models.