



**DEEP EUTECTIC SOLVENTS PRETREATMENT  
OF *LEUCAENA LEUCOCEPHALA* FOR  
BIOETHANOL PRODUCTION USING  
*KLUYVEROMYCES MARXIANUS* UNIMAP 1-1**

by

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## LIST OF ABBREVIATIONS

AFEX	Ammonia Fiber Expansion
ATR	Attenuated Total Reflection
CBP	Consolidated Bio-Processing
DEs	Deep Eutectic Solvents
EFB	Empty Fruit Bunch
FTIR	Fourier Transform Infrared Spectroscopy
GHG	Green House Gases
GMOs	Genetically Modified Organisms
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
ILs	Ionic Liquids
KBP	Kertih Biopolymer Park
LCC	Ligno-cellulose complex
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation

## LIST OF SYMBOLS

$^{\circ}\text{C}$	Degree Celsius
%	Percentage
M	Molar Concentration
$\lambda$	Ionic conductivity
$\mu$	Viscosity
T	Temperature
t	Time

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**PRARAWATAN *Leucaena leucocephala* MENGGUNAKAN PELARUT  
EUTEKTIK DALAM (DES) UNTUK PENGHASILAN BIOETANOL  
MENGGUNAKAN *Kluyveromyces marxianus* UniMAP 1-1**

**ABSTRAK**

Pengeluaran bioetanol selulosa menggunakan pelarut eutektik dalam (DES) sebagai pelarutan pra-perawatan merupakan langkah yang baik dalam menghasilkan gula fermentasi. Ia boleh mengatasi kelemahan menggunakan pra-perawatan asid dan alkali konvensional yang boleh merosakkan alam sekitar. Selain itu, DES mampu melarutkan lignin, membuka matriks biojisim kompleks, dengan itu membolehkan serangan enzim terhadap struktur selulosa dan hemiselulosa. Walau bagaimanapun, pelbagai kombinasi DES perlu dipilih dengan bijak kerana kesesuaian dan keserasian DES sangat bergantung kepada biojisim dan proses tertentu yang terlibat. Selain itu, keadaan optimum yang tidak sama antara hidrolisis dan fermentasi telah menghalang proses sakarifikasi dan penapaian serentak (SSF) untuk berjaya dalam penghasilan bioetanol. Justeru, kajian ini dijalankan untuk menyaring dan mengkaji gabungan DES yang sesuai untuk digunakan dalam pra-perawatan *Leucaena leucocephala*. Untuk menjadikan pra-perawatan *L. leucocephala* berkesan, beberapa faktor yang mempengaruhi pra-perawatan dan hidrolisis enzim perlu dioptimumkan sebelum digunakan dalam proses fermentasi dan dengan itu hasil bioethanol boleh dinilai. Dalam kerja ini, 30 gabungan DES telah disintesis dan disemak melalui beberapa kriteria seperti sifat fizikal-kimia mereka dan kestabilan serta keserasian terhadap *L. leucocephala*, enzim selulase dan *Kluyveromyces marxianus* UniMAP 1-1. Setelah itu, DES yang terbaik telah dipilih dan kemudian telah digunakan dalam beberapa proses pra-perawatan untuk mengoptimumkan faktor-faktor yang mempengaruhi proses pra-perawatan seperti jumlah biojisim, penambahan air dan kepekatan enzim. Keadaan yang dioptimumkan kemudian digunakan dalam proses fermentasi menggunakan *K. marxianus* melalui proses SSF dan hasil bioetanol yang dihasilkan telah dinilai menggunakan kromatografi cecair prestasi tinggi (HPLC). DES terbaik yang telah dipilih ialah ChCl:Gly pada perbandingan molar 1:2 dengan konduktiviti pada 1.86 mS/cm, kelikatan pada 258.3 cP dan pH 4.11. ChCl:Gly (1:2 M) juga menunjukkan keserasian dan kestabilan yang terbaik terhadap enzim selulase dengan mengekalkan kebanyakan aktiviti selulase pada 7.6 FPU selama 48 jam inkubasi. Ia juga menunjukkan keserasian yang baik terhadap *L. leucocephala* dan *K. marxianus* dengan menunjukkan penyesuaian optima dalam kedua-dua keadaan. Jumlah biojisim yang optimal ialah pada 10% (w/v), penambahan air pada 25% (v/v) dan kepekatan enzim pada 30 FPU diperlukan untuk mendapatkan glukosa yang tinggi. Hasil maksimum bioetanol daripada kajian ini ialah 0.162 g/g dengan kecekapan penukaran pada 31.68% menunjukkan bahawa *K. marxianus* yang baru diisolasi ini boleh digunakan dalam proses fermentasi. Ringkasnya, DES boleh berfungsi sebagai pilihan yang tepat dalam industri bio-penapisan. Ia bukan sahaja mampu melarutkan lignin, tetapi juga berkesan meningkatkan pencernaan selulosa dan menghasilkan glukosa yang tinggi seterusnya menyediakan laluan alternatif untuk mencapai strategi biopemprosesan terkonsolidasi (CBP).

**DEEP EUTECTIC SOLVENTS (DES) PRETREATMENT OF *Leucaena leucocephala* FOR THE PRODUCTION OF BIOETHANOL USING *Kluyveromyces marxianus* UniMAP 1-1**

**ABSTRACT**

Cellulosic bioethanol production using deep eutectic solvents (DESs) as the pretreatment solvent is a promising step in producing fermentable sugars. It can overcome the drawbacks of using conventional acid and alkaline pretreatment that could be harmful to the environment. Moreover, DESs are capable of solubilizing lignin, opening up the complex biomass matrix, thus allowing enzymatic attack towards the cellulose and hemicellulose structure. However, wide range of DESs combination need to be chosen wisely due to the suitability and compatibility of DESs were strongly depend on the specific biomass and process involved. In addition, non-identical optimum condition between hydrolysis and fermentation have hindered simultaneous saccharification and fermentation (SSF) to be successful in bioethanol production. Hence, this study was conducted to screen the suitable combination of DES mixture to be used in pretreatment of *Leucaena leucocephala*. In order to effectively pretreat *L. leucocephala*, several factors that influence the pretreatment and enzymatic hydrolysis need to be optimized before being used in fermentation process, thus the yield of bioethanol can be evaluated. In this work, 30 combination of DESs were synthesized and screened through several criteria such as their physicochemical properties and the stability and compatibility towards *L. leucocephala*, cellulase enzyme and *Kluyveromyces marxianus* UniMAP 1-1. After that, the best selected DES was then underwent several pretreatment processes to optimized factors influencing pretreatment process such as solid loading, water addition and enzyme concentration. The optimized conditions were then used in the fermentation process using *K. marxianus* via SSF and the yield of bioethanol produced was evaluated using high performance liquid chromatography (HPLC). The best screened DES was ChCl:Gly at 1:2 molar ratio with conductivity at 1.86 mS/cm, viscosity at 258.3 cP and pH 4.11. ChCl:Gly (1:2 M) also shows remarkable compatibility and stability with cellulase enzyme by retaining most of cellulase activity at 7.6 FPU during 48 h incubation. It also shows potential compatibility towards *L. leucocephala* and *K. marxianus* by showing an optimal adaptation in both conditions. The optimum solid loading was at 10% (w/v), water addition at 25% (v/v) and enzyme concentration at 30 FPU were necessary to obtain the highest glucose possible. The maximum bioethanol yield from this study was at 0.162 g/g with 31.68 % conversion efficiency which showed that this newly isolated *K. marxianus* can be used in fermentation process. In short, DESs could serve as a great option in biorefineries, not only capable in solubilizing lignin, but also effectively improve subsequent cellulose digestion and glucose yields, thus providing alternative route to achieve consolidated bioprocessing (CBP) strategy.

## CHAPTER 1 : INTRODUCTION

### 1.1 Research Background

Alternative energies including water, wind, geothermal and biomass can fulfil the demand of sustainable renewable energy. Renewable energy's share of total primary energy supply was expected to increase from 14 % in 2015 to 63 % in 2050. This equates to an annual growth rate of 1.4 % on average, a six-fold rise over recent years (Gielen et al., 2019). However, not all of these energies were economically feasible. The urge to reduce greenhouse gases (GHG) and waste produced from industries or landfills were the major concern to the world today. The best option that helped to reduce the problem was by using biofuels made from biomass feedstocks (Sadhukhan et al., 2019). In the last few decades, biomass has attracted the attention of many researchers to shift the current fossil fuels-based into biomass-based fuel energy.

Biomass that commonly used for energy production are plants and woods (Barot, 2022), as such resources would not exhaust. The major criteria of biomass to be chosen as a feedstock for biofuel production can be seen by the conversion efficiency of its material into various phases of fuels using biological processes (Ramos et al., 2022; Sadhukhan et al., 2019). Hence, the utilization of biomass for biofuel conversion was a great decision as they can meet the energy demand and at the same time, protect the environment.

Competition for the food sources have limited the usage of raw materials such as corn and sugarcane as bioethanol feedstock. Hence, an alternative to these feedstocks

must be explored. Lignocellulosic materials such as sugarcane residues, rice straw and wheat straws seemed to be favourable to overcome the drawbacks of the corn and sugarcane feedstocks (Suryadi et al., 2022; Soccol et al., 2019). They contained three major components that made up the plant cell walls; lignin, cellulose and hemicellulose (We et. al., 2023; Lou and Zhang, 2021). Cellulose and hemicellulose can be converted into fermentable sugars via hydrolysis. Hence, bioethanol production from lignocellulosic biomass seems to be promising as the crops continue to be produced in agricultural activities.

In the last decade, there has been a growing interest on a plant known as *Leucaena leucocephala*. It would be a great selection for biomass feedstock as it has the ability to grow rapidly and abundantly (Alemán-Ramirez et al., 2022). It is leguminous, which means it is adapted to harbouring nitrogen-fixing bacteria in its root nodules, therefore this plant requires less fertilization (Sutaryono et al., 2019).

In Malaysia, this tree is well known as ‘petai belalang’, as its physical characteristics of the pods and seeds were similar to *Parkia speciose* (petai), but with smaller seeds. The taste is also very similar to each other. Petai belalang was used as a livestock forage, reforestation materials, as well as furniture and construction timber (Husin et al., 2019). In 2015, the East Coast Economic Region (ECER)’s effort to promote the use of cellulosic feedstock from biomass at Kertih BioPolymer Park (KBP) received a major boost with the planting of 40,000 seedlings of *L. leucocephala*. Petai belalang trees would be processed into wood chips, which will then be converted into advanced carbohydrates. The advanced carbohydrates would be processed further using biotechnology to produce various types of bioderivatives, including biofuels (Husin et

al., 2019). Hence, this tree could be the perfect selection for the types of lignocellulosic materials as it can grow wild and almost everywhere.

The current pretreatment method to produce fermentable sugar was not environmental friendly nor cost effective (Samanta et al., 2022). For example, biomass pretreatment using mild alkalines and dilute acids were harmful to the environment as the chemicals cannot be recycled. The waste would be absorbed into the soil, making it alkaline or acidic. The used of harsh chemicals such as sulphuric acid ( $H_2SO_4$ ) can corrode the vessel and production pipes, and at the same time increase the maintenance cost (Fegueiredo et al., 2019). Hence, an alternative solvent for biomass pretreatment was required to reduce the effects that's normally produced when the pretreatments were done using harsh chemicals.

A new green solvent, known as Deep Eutectic Solvents (DESs) has drawn much attention as a substitute for the conventional acids and alkalines. It is not only biodegradable, but also recyclable, making it a suitable solvent for future use (Joarder et al., 2023). DESs have been used in a variety of applications such as reactions, separations and purification (Bashir et al., 2023). In biofuel production, DESs were remarkable in hydrolyzing lignin content in biomass (Xu et al., 2023), allowing more exploration in this kind of pretreatment.

Bioethanol production from cellulosic materials typically involved many unit operations such as pretreatment, enzymatic hydrolysis and fermentation. These processes could heavily affect the production cost (Ranim et al., 2021). Hence, Simultaneous Saccharification and Fermentation (SSF) method has been introduced to simplify the

process which combines enzymatic hydrolysis and fermentation in one vessel. However, the most obvious limitation was that the optimum condition for the processes were not identical. Optimum temperature for enzymatic saccharification usually occurs at 50°C, while fermentation was at 35°C. Introducing a thermotolerant yeast like *Kluyveromyces marxianus* which is capable in fermenting sugar at higher temperature can overcome the limitation of SSF. Moreover, *K. marxianus* also has been reported to be capable in utilizing both glucose and xylose, thus allowing efficient sugars conversion into ethanol.

In short, this research mainly focused on the pretreatment method of *L. leucocephala* by using the new green solvent, DESs. Successful production of lignocellulosic bioethanol started with complete utilization of its components; cellulose and hemicellulose. DESs was believed to be able to solubilize completely the fibrous lignin and thus open up the structure for the enzymatic hydrolysis on cellulose and hemicellulose structure. To date, this was the first attempt in producing ethanol via SSF fermentation using an isolated thermotolerant yeast, *K. marxianus* UniMAP 1-1 from *L. leucocephala* treated with DES.

## 1.2 Problem Statement

Successful bioethanol production was firstly determined by the type of biomass used in particular process. There were many types of crop available and have proven to be the feedstock for bioethanol production. However, most of them were limited and often a seasonal type of crops which can affect the productivity of bioethanol production. Contrary, *Leuceana leucocephala* was one of the crop with high biomass productivity which easily available throughout the years.

In *L. leucocephala* pretreatment, lignin would hinder the enzymatic attack towards the targeted cellulose and hemicellulose, hence it must be removed from *L. leucocephala* structure. Conventional methods to solubilize lignin usually use harsh chemicals such as alkaline and acid (Basak et al., 2023). These chemicals were non-recyclable and not environmental friendly. Deep Eutectic Solvents (DESs) has emerged as a potential greener solvent that was capable of solubilizing lignin. This was a perfect substitute for the conventional chemical pretreatment that have high capital and processing costs due to the inability of solvent recycling (Rachiero et al. 2022).

Utilizing DESs as a pretreatment solvent in biorefinery was promising as they can be prepared easily by just mixing two components and heated up until homogenous. However, the challenge comes when a large number of possible combinations of hydrogen-bond acceptor (HBA) and hydrogen-bond donor (HBD) need to be chosen wisely (Xu et al., 2023) depending on the chosen *L. leucocephala*. This was due to the fact that improper HBA and HBD combinations would not efficiently solubilize lignin in *L. leucocephala* and also may gave a toxic effect towards the subsequent process.

Production of bioethanol using simultaneous saccharification and fermentation (SSF) could become a pioneer of successful consolidated bio-processing (CBP) which combined enzymatic hydrolysis and fermentation process in a single unit operation. However, their most obvious limitation was that the optimum condition in both processes were not identical. Enzymatic hydrolysis typically runs at 50°C, however fermentation runs at 35°C. For this reason, it would be worthwhile to introduce a thermotolerant yeast capable of fermenting sugar at elevated temperature, such as *Kluyveromyces marxianus* UniMAP 1-1. This isolated strain reportedly capable to grow at 50°C and ferment ethanol

at 47°C (Rahman et al., 2018) which cannot be applied to the commonly used industrial yeast, *S. cerevisiae*.

### 1.3 Objectives

The main objective of this research was to pretreat *L. leucocephala* using green solvent, Deep Eutectic Solvents (DESs) for bioethanol production by an indigenous thermotolerant yeast, *Kluyveromyces marxianus* UniMAP 1-1. The specific objectives were as follows:

- i. To screen the suitable DES mixtures for pretreatment of *L. leucocephala* for subsequent use in Simultaneous Saccharification and Fermentation (SSF).
- ii. To optimize the effects of several influencing factors during DES pretreatment and enzymatic hydrolysis for high fermentable sugar production.
- iii. To evaluate the yield of bioethanol produced by *K. marxianus* UniMAP 1-1 using the fermentable sugar obtained via SSF method.

### 1.4 Hypothesis

Deep Eutectic Solvents (DESs) was capable in substituting common solvents used in biorefinery such as alkaline and acid pretreatment. Hence, it is expected that the performance of DES treatment was much better as compared to both conventional pretreatments. Li et al., (2023) mentioned that lignin removal in lactic acid based DES

was at 79.73% while in diluted acid pretreatment was at 85%, however due to the formation of large number of inhibitors such as furfural in acid pretreatment makes it becomes less favourable compared to DESs. *L. leucocephala* on the other hand could become a promising crop in biorefinery for its vast population and easy to grow. The cellulose content was also promising which can be converted into large amount of saccharides. Gopinath et al., (2022) reported that cellulose content found in *L. leucocephala* woods was 59.03%. Additionally, the used of thermotolerant yeast, *K. marxianus* capable in fermenting ethanol at elevated temperature (47°C) (Rashid et al., 2021) would be a possible approach towards implementing CBP to improve the lignocellulosic ethanol production process.

### **1.5 Scope of Research**

This study was designed to develop Deep Eutectic Solvents (DESs) solvent mixtures which would be used in the lignocellulose pretreatment method. It mainly addressed the use of DESs as solvent to solubilize fibrous lignin. *L. leucocephala*'s seeds and pods and were chosen as the biomass feedstock because of its abundancy. Screening of DESs mixture were conducted to know which DES mixture was suitable to be used based on the selected biomass and yeast through the suitability and compatibility test. In this study, six hydrogen bond donor (HBD) were selected including polyols and organic acid and mixed in the range of 1:1 to 1:5 molar ratio of HBA: HBD. The selected DES mixture was then analyzed to know the factors influencing the DESs mixture on biomass pretreatment. Parameters such as solid loading (5, 10, 15 and 20 % w/v), effect of water addition (0, 25, 50, 75 % v/v) and enzyme concentration (10, 20, 30, 40 FPU) were the most important factors that must be included in the analysis. In this study, only cellulase enzyme was used in the saccharification process to evaluate the efficiency of

fermentation on glucose as a carbon source. Evaluation of the yield of bioethanol produced from the DESs pretreatment was carried out by SSF fermentation using an isolated *K. marxianus* UniMAP 1-1 in 100 mL fermentation bottles with 50 mL working volume, and the bioethanol produced was measured using High Performance Liquid Chromatography (HPLC).

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## CHAPTER 2 : LITERATURE REVIEW

### 2.1 Feedstocks for Bioethanol

Future growth requirement for liquid biofuel has expected to reach a minimum of 24 billion liters/year in 2050 (Gielen et al., 2019). Growing interests in biofuel such as bioethanol in this decade has led researchers to find an alternative for new and cheap feedstocks to produce it. The feedstock must not only be low in cost (Awasthi et al., 2023), but it should also be abundantly available (Usmani et al., 2021), safe when used and sustainable to produce (Soccol et al., 2019). Production of bioethanol have raised several issues in terms of feedstocks utilization including the chemical compositions of biomass, cultivation practices, availability of land, emissions of greenhouse gasses, absorption of minerals to ground waters and soils, and uncontrolled use of pesticides (Goswami et al., 2022). Those issues were the concerns of many people including environmental activists hence need to overcome for successful utilization of feedstock.

#### 2.1.1 Feedstocks based on Bioethanol Generations

In the early development of bioethanol production, most people utilized food sources such as grapes and fermented them to get liquor. Since then, many food sources have been used to produce bioethanol. Many years later, feedstocks that were abundantly available came from sugar crops such as sugarcane, sugar beet, and molasses ( Pandey et al., 2022) and starchy agricultural cereals such as grains and maize wheat were discovered (Ummalyma, 2019). These findings has increased the interest in producing bioethanol from these feedstocks and many bioethanol industries have been developed since then.