UNIVERSITI MALAYSIA PERLIS

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Date of birth :09 FEBRUARY 1983 .

Title : STUDIES ON THE THIRD GENERATION OF IONIC LIQUIDS – HALOPHILIC CELLULASE SYSTEM FOR LIGNOCELLULOSE HYDROLYSIS

Academic Session : 2015/2016

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ACKNOWLEDGEMENT

In the name of Allah, the Most Merciful and the Most Compassionate. All absolute praise and thanks are due to Him alone and with His blessings and guidance has enabled me to complete the study, and peace be upon our beloved prophet Muhammad (PBUH). I would like to take this opportunity to express my deepest gratitude to my respected supervisor and "sifu", Assoc. Prof. Dr. Dachyar Arbain, for his thoughtful ideas, priceless and meaningful guidance, constant prayers, encouragement and motivation, patience, never ending support and his endurance to bear with me through all the difficult times from the start of my post graduates studies until the end. This enabled me to complete the PhD program successfully. I am very grateful, proud and honoured to work with him and it was meaningful and enriching experience. Also, I would like to express my sincere appreciation to my respected co-supervisor, Prof. Dr. Parveen Jamal, from the International Islamic University Malaysia (IIUM) for her helpful advice, suggestions, guidance and co-operation throughout the study period. I would also like to thank the late Prof. Dr. Mohamed Zulkali Mohamed Daud, former co-supervisor for all his help and encouragement during the first year of PhD studies. May Allah bless his soul. Further, I could not have completed this thesis without my supervisors' skilful and scholarly assistance.

I would also like to express my special and warm gratitude to my mother for her prayers, support, blessings and encouragement not just throughout the PhD, but through my undergraduate experience. Furthermore, I would like to express my special and warm gratitude to my wife, Rafidah bt Hassan Mydin for her patience, support and prayers throughout this work.

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I would also like to thank to the Ministry of Education (MOE), Malaysia for financing this research under the Fundamental Research Grant Scheme (FRGS 9003-00391) and to the Ministry of Education (MOE) and Universiti Malaysia Perlis for their financial assistance regarding my PhD program under the scholarship scheme for academician (SLAI) scheme.

My sincere thanks also goes to Assoc. Prof. Dr. Enas Muen Nashef from the Chemical and Environmental Engineering (CEE) Masdar Institute for Science and Technology Abu Dhabi, United Arab Emirates and Mr. Ahmad Adlie Shamsuri, from the Institute of Bioscience, University Putra Malaysia for their sincere advice with regards to the preparation, synthesis and design of ionic liquids in this study.

My sincere thanks also go to Dr. Jong Bor Chyan from the Agrotechnology and Biosciences Division, Malaysian Nuclear Agency for his valuable guidance on molecular identification of fungus works and sincere thanks to the late Mr. Burhanudin, Mr. Mustaqim and Mr. Siva, technicians from the School of Chemical Science, University Science Malaysia (USM) for their assistance in the characterization of deep eutectic solvents works.

Last but not least, I would also like to express my sincere appreciation to Mr. Rizo, Mr. Azuwan, Miss Anis, Mr. Nabil, Dr. Mohiuddin, Mrs. Noor Hasyierah, Mrs. Maizatul, Mrs. Afifah, Mr. Radi, Dr. Zarina, Miss Rohazita, Mr. Zulkarnain, Mr. Shahril, Mr. Nasir, Mr. Hafizal, Mr. Humaidi, Mr Zaim, and other colleagues for the co-operation, a helping hand and kind friendship during the study. Jazaakumullahu khairan kathira (May Allah reward you all with great abundance).

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LIST OF ABBREVIATIONS

A.niger	*Aspergillus niger*
A.terreus	*Aspergillus terreus*
ANOVA	Analysis of variance
BET	Brunauer–Emmett–Teller
BLAST	Basic Local Alignment Search Tool
CCD	Central composite design
CMC	Carboxymethyl cellulose
cP	Centipoise
CrI	Crystallinity index
DES	Deep Eutectic Solvent
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
DOE	Department of Environment
EG	Choline chloride: Ethylene glycol (1:2)
FCCCD	Face Centered Central Composite Design
FTIR	Fourier transform infrared spectroscopy
$FeSO_4.7H_2O$	Ferrous Sulphate Heptahydrate
GLY	Choline chloride: Glycerol (1:2)
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
ILs	Ionic liquids
ITS	Internal transcribed spacer
K_s	Kinetic constant
LOI	Lateral order index
MA	Choline chloride: Malonic acid (1:2)

MCC	Microcrystalline of cellulose
$MgSO_4.7H_2O$	Magnesium Sulphate Heptahydrate
MEA	Malt extract agar
NCBI	National Center for Biotechnology Information
OFAT	one-factor-at-a-time
RH	Rice husk
RSM	Response surface methodology
SD	Standard deviation
SEM	Scanning electron microscopy
TGA	Thermal Gravitational Analysis
U/ml	One unit is the amount of enzyme activity which will catalyse 1 micromole of the substrate per minute under standard conditions
UniMAP	Universiti Malaysia Perlis
XRD	X-ray Diffraction

KAJIAN MENGENAI SISTEM GENERASI KETIGA CECAIR IONIK – SELULASE HALOFILIK UNTUK HIDROLISIS LIGNOSELULOSA

ABSTRAK

Penukaran biojisim lignoselulosa menjadi produk bernilai tambah memerlukan tiga proses iaitu pra-rawatan, hidrolisis dan juga penukaran gula ringkas menjadi produk akhir. Faktor yang mengehadkan ketiga-tiga proses tersebut terletak pada proses pra-rawatan. Kaedah pra-rawatan konvensional biasanya menggunakan asid kuat atau alkali yang yang mengundang masalah pencemaran. Kaedah pra-rawatan terkini menggunakan generasi ketiga Cecair Ionik (ILs) yang juga dikenali sebagai Pelarut "Deep Eutectic" (DESs) yang dikenalpasti sebagai mesra alam dan kos efektif. Walau bagaimanapun, kelemahan utama kaedah pra-rawatan ini ialah tidak serasi sepenuhnya dengan enzim yang diperlukan dalam proses hidrolisis. Isu keserasian telah ditangani dalam kajian ini dengan menghasilkan enzim yang toleran dengan garam (enzim halofilik) dan juga penyediaan pelarut DES yang mempunyai kelikatan yang rendah dan tahan haba. Enzim tersebut dihasilkan dari kulat halofilik yang baharu dipencilkan dan dikenalpasti sebagai *Aspergillus terreus* UniMAP AA-6 sementara DESs telah disintesis dan dipilih menggunakan choline klorida sebagai penerima ikatan hidrogen dan asid karboksilik dan alkohol poliol yang terpilih sebagai penderma ikatan hidrogen. Keserasian antara selulase halofilik dan DESs dinilai dengan memantau kestabilan selulase halofilik terhadap kepekatan ILs komersial dan DESs. Kesesuaian sistem DES-selulase untuk hidrolisis lignoselulosa dinilai berdasarkan pengeluaran glukosa, penggunaan tenaga dan prestasi kinetik. Daripada kajian didapati, selulase halophilic menunjukkan kestabilan yang lebih tinggi terhadap 10 % (v/v) ILs dan juga stabil dan mengekalkan 90 % daripada aktiviti asalnya terhadap 10 % (v/v) DESs. Sistem DESs-sellulase mempamerkan peningkatan peratusan glukosa yang lebih tinggi dan penggunaan tenaga yang lebih rendah berbanding dengan sistem alkali cair manakala dari segi prestasi kinetik, DES mempamerkan prestasi kinetik baik yang mencerminkan keupayaan DESs untuk bertindak sebagai media untuk hidrolisis yang mana ini sangat baik untuk aplikasi di dalam sistem DES-selulase. Akhir sekali, berkaitan dengan aspek pengeluaran glukosa, sekam padi yang dirawat dengan sistem DESs-selulase halofilik menunjukkan setanding dengan kaedah ILs yang biasa diaplikasikan dalam pra-rawatan tetapi dengan faedah tambahan dari segi aspek kos dan tidak mencemarkan alam sekitar. Penemuan ini telah membuka laluan yang lebih baik dan pendekatan untuk "*in situ* saccharification" yang menggunakan lignoselulosa yang telah dirawat oleh DESs.

STUDIES ON THE THIRD GENERATION OF IONIC LIQUIDS – HALOPHILIC CELLULASE SYSTEM FOR LIGNOCELLULOSE HYDROLYSIS

ABSTRACT

The conversion of lignocellulosic biomass into value-added products requires pre-treatment, hydrolysis (saccharification) and the conversion of simple sugar into end products. The limiting factor of these three processes lies in the pre-treatment steps. Conventional pre-treatment methods normally use strong acids or alkali which pose environmental problems. Recently pre-treatment using the third generation of Ionic Liquids (ILs), also known as Deep Eutectic Solvents (DESs) has been considered green and cost-effective. However, the main drawback of DESs pre-treatment is that it is not fully compatible with the enzyme required in saccharification. This compatibility issue was addressed in the present study by producing a salt tolerant enzyme (halophilic enzyme) and formulating low viscosity, thermal stable DES. The halophilic enzyme was produced from a newly isolated halophilic fungus, identified as *Aspergillus terreus* UniMAP AA-6; DESs were synthesized and screened using choline chloride as a hydrogen bond acceptor and selected carboxylic acids and polyol alcohols as hydrogen bond donors. The compatibility between halophilic cellulase and DESs was evaluated by monitoring the stability of halophilic cellulase in the presence of various concentrations of commercial ILs and DESs. The applicability of the DES-cellulase system for lignocelluloses hydrolysis was evaluated based on glucose production, energy consumption and kinetic performance. It was found that halophilic cellulase showed higher stability in the presence of 10% (v/v) ILs and also was stable and retained 90 % of its original activity in the presence of 10% (v/v) DESs. The DESs-Cellulase system exhibited higher glucose percentage enhancement and lower energy consumption as compared to diluted alkali system, while in terms of kinetic performance, DES exhibited good kinetic performance, which reflects the ability of DESs to serve as good saccharification media for the DESs-cellulase system. Finally, with regard to glucose production, rice husk treated with DESs-halophilic cellulase system were as good as the typical IL pre-treatment method but with extra benefits in terms of cost and environmental aspects. These findings have demonstrated a better approach for the *in situ* saccharification of DESs pre-treated lignocelluloses.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Lignocellulosic biomass is one of the most abundant biopolymer and renewable energy resources for the production of biofuel and other bio-products (Pande & Bhaskarwar, 2012; Tadesse & Luque, 2011). The major components of lignocellulosic biomass are polymeric carbohydrates which consist of cellulose (30-50 wt %), hemicelluloses (15-35 wt %) and lignin (10-30 wt %) (Kumar, Barrett, Delwiche, & Stroeve, 2009; Lynd, Weimer, van Zyl, & Pretorius, 2002). Cellulose represents the largest part of lignocellulose which can be hydrolysed to generate monomeric sugars units. These sugars can be converted via the bio-processing approach to a wide range of value-added chemicals.

In general, the conversion of lignocellulosic biomass to desired products consists of three major steps: 1) Lignocellulose is pre-treated to bring the sugar polymers into a form suitable for hydrolysis, 2) The enzymatic hydrolysis of cellulose to fermentable sugar and 3) The fermentation of simple sugar into desired products (C.-Z. Liu, Wang, Stiles, & Guo, 2012). However, the physicochemical and compositional features of lignocelluloses limit their enzymatic hydrolysis process. Structural complexity a major barrier for green and cost effective technology for processing lignocellulosic materials to sugars and other bio-products (Vancov, Alston, Brown, & McIntosh, 2012). Thus, the pre-treatment process of lignocelluloses is a crucial step in this conversion.

Typically, the pre-treatment of lignocelluloses is classified into physical, chemical, physico-chemical and biological pre-treatments. Different pre-treatment methods adopted different strategies and mechanism for the deconstruction of highly recalcitrant lignocellulosic biomass structures. However, conventional pre-treatment methods have some environmental issues and drawbacks. These include the use of hazardous chemicals and the emission of hazardous byproducts from conventional biomass pre-treatment methods. In addition, some of the methods need to be operated in severe conditions and have a high energy demand like high pressures, temperatures, acidic and alkaline conditions. This can make the pre-treatments environmentally detrimental and expensive (Mäki-Arvela, Anugwom, Virtanen, Sjöholm, & Mikkola, 2010). Consequently, more efficient and environmentally benign pre-treatments methods are deemed necessary.

The use of Ionic liquids (ILs) for the pre-treatment of lignocelluloses has increased over the past few years (Badgujar & Bhanage, 2014; Brandt, Grasvik, Hallett, & Welton, 2013; Tadesse & Luque, 2011). ILs are salts that are emitted in a liquid form at room temperature. ILs offer several unique beneficial properties such as recyclability (X. Wang, Li, Cao, & Tang, 2011), non flammability (V. V. Singh, Boopathi, Ganesan, Singh, & Vijayaraghavan, 2010), non-corrosive (Hermanutz, Gähr, Uerdingen, Meister, & Kosan, 2008), high thermal stability (Domańska & Bogel-Łukasik, 2005) and non-volatility i.e., low vapour pressure (Brennecke & Maginn, 2001). Due to these features and properties, ILs are considered as green solvents. In addition, ILs are also known as "Designer Solvents" because they consist of anion and cation, whereby these features enable ILs ion properties and chemistry to be designed and tailored for desired applications (Plechkova & Seddon, 2008; Sheldon, 2005).

The dissolving power of ionic liquids has been typically attributed to the interaction between the charged species of ILs and hydroxyl groups on the cellulose via strong hydrogen-bonding (Brandt et al., 2013). A possible dissolution mechanism suggests that ionic liquids compete with lignocellulosic components for hydrogen bonding, thus disrupting its three dimensions network. Tadesse and Luque (2011) explained these hydrogen bonds are broken upon interaction of the cellulose-OH and ILs, which in turn rendered more opened cellulose molecular chains. This interaction ultimately leads to cellulose dissolution.

Since the pioneering work on the excellent role of ionic liquids (ILs) for the dissolution of cellulose (Swatloski, Spear, Holbrey, & Rogers, 2002), extensive research has been done on the application of ILs for lignocellulosic biomass pre-treatment (Hou, Smith, Li, & Zong, 2012; Tadesse & Luque, 2011). Nevertheless, IL technologies for industrial scale applications still have drawbacks and limitations in terms of environmental and economical factors. Several ILs are toxics because of their release in the soil and water may threaten its inhabitants (Shamsuri & Abdullah, 2010). Decomposition of ILs like [Bmim]BF_4 and [Bmim]PF_6 in the presence of water can cause severe water pollution when exposed to the environment due to the formation of hydrofluoric and phosphoric acid (Zhu et al., 2009). Another environmental problem associated with ILs is that of poor biodegradability. Most synthesized ILs are not readily biodegradable. They persist in the environment and may lead to environment damage (Morrissey et al., 2009). In addition, most ILs are highly viscous at room temperature and are difficult to purify (Kunz et al., 2011). These factors make ILs processes expensive, which in turn affects the overall cost of large scale applications. Therefore, environmental and economical factors need to be considered for ionic liquids to be more feasible for industrial applications.

Mäki-Arvela et al. (2010) have stated several requirements that have to be considered prior to choosing ionic liquid for the pre-treatment process. The ionic liquids should be made of renewable and natural components which are non-toxic, non-odorous, inexpensive and biodegradable.

In short, as an alternative to conventional ILs, Abbott, Capper, Davies, Rasheed, and Tambyrajah (2003) introduced a third generation of ILs known as Deep Eutectic Solvents (DESs). This third generation of ILs have similar properties to conventional ILs but with additional advantages which have come up as a promising alternative for conventional ILs. They introduced new types of solvents which are formed by a combination of urea as hydrogen bond donor and choline chloride as quaternary ammonium salt. The combination of these materials produced a eutectic mixture that was liquid at ambient temperature. Hydrogen-bonding interactions between these components are the main cause of the deep freezing-point depression of the mixture. Moreover, DESs are also called Low-Transition-Temperature Mixture (LTTMs) by several authors (María Francisco, van den Bruinhorst, & Kroon, 2013) because most of the DESs showed low glass transitions temperature instead of melting points.

Similar to conventional ILs, DESs exhibit low volatility and high thermal stability. In addition, similar to conventional ILs, DESs are known as designer solvents which consist of hydrogen bond donor and quaternary ammonium salt. These properties allow DES components to be designed and tailor-made for various applications. However, unlike most conventional ILs, DESs are made from natural components which are biodegradable, non-toxic and cheap. In addition, they are very easy to prepare by simply mixing two components, thus avoiding all the problems of purification and waste disposal generally associated with ILs (María Francisco et al., 2013; Q. Zhang, Vigier, Royer, & Jérôme, 2012).

Due to the advantages of DESs as green solvents and their versatility as designer solvents, DESs can be widely applied in such applications as organic synthesis (Azizi & Manocheri, 2012), separations (H. Zhao, Baker, & Holmes, 2011), catalysis (B. Singh, Lobo, & Shankarling, 2011) and electrochemistry (Abbott, Capper, McKenzie, & Ryder, 2007).

Inspired by the potential advantages brought by DES in various fields, DESs are used in lignocellulosic biomass processing (Maria Francisco, van den Bruinhorst, & Kroon, 2012; Q.-P. Liu, Hou, Li, & Zong, 2012; Q. Zhang, Benoit, De Oliveira Vigier, Barrault, & Jérôme, 2012). However, most of the current publications on the application of DESs in biomass processing mainly discuss the capability and role of DESs in lignocellulose solubilisation and decrystallization, neglecting the suitability and compatibility of DESs for the subsequent hydrolysis/saccharification process.

Similar to ILs, DESs components consist of salt, which is incompatible with the subsequent enzymatic process which uses an ordinary cellulase enzyme. The charged groups on the enzyme surface will interact with ionic groups in the solution to cause deformation of the enzyme molecular structure. Due to this behaviour, cellulases have been reported to become inactivate in the presence of low ILs concentration (Sayantan Bose, Armstrong, & Petrich, 2010; M. B. Turner et al., 2003). Therefore, a washing process is required to remove residual ILs after the pre-treatment process. Large scale washing requires extra energy, thus incurring a greater processing cost (Engel, Mladenov, Wulfhorst, Jager, & Spiess, 2010; T. Zhang et al., 2011). In addition, the cleaning protocol is lengthy and burdensome. To avoid the extensive clean-up process, it is critical to develop a compatible IL-cellulase systems in which the IL is able to process lignocelluloses and at the same time cellulases still remain stable and highly active in the presence of ILs. This system can be exploited and projected for the *in situ*

saccharifcation of lignocelluloses pre-treated DESs. This idea was introduced by Kamiya et al. (2008), whereby the pre-treatment and saccharification process is performed in a single-pot, as a result skipping expensive cellulose regeneration and ILs residual washing steps.

This idea can be effectively utilized if cellulases tolerant to DESs are used. In the light of this discussion, it is important to produce cellulases which are compatible with the saline condition of DESs. The enzyme can be produced from halophilic microorganisms (salt tolerant microbes) relying on their capability to secrete enzymes which are active in a high saline environment (Oren, 2010).

Therefore, the design and preparation of cheaper, non-toxic, enzyme-compatible ILs which are capable of lignocelluloses pre-treatment is considered to be one of the major challenges in ILs-biomass processing industries (Badgujar & Bhanage, 2014; L. Li et al., 2012). Apart from that, generally, most DESs exhibit high viscosity, which is not preferable in industry as it is difficult to handle, require additional force to pump in the solvent and thus affect the operating cost in industrial applications (Q. Zhang, K. D. O. Vigier, et al., 2012). Additionally, harsh conditions such as high temperature may affect the DES stability (Badgujar & Bhanage, 2014). Hence, the design of low viscosity and thermally stable DESs is an important feature that needs to be considered when selecting DESs as a solvent for lignocelluloses pre-treatment. Furthermore, glucose production enhancement, energy consumption and kinetic performance of the DES-cellulase system are important parameters when considering the evaluation of the DES-halophilic cellulase system in hydrolyzing simple cellulose and complex cellulose.