UNIVERSITI PUTRA MALAYSIA

CONVERSION OF PADDY STRAW TO BIOETHANOL THROUGH CONSOLIDATED BIOPROCESSING USING LIGNOCELLULOLYTIC FUNGI

MONA FATIN SYAZWANEE BINTI MOHAMED GHAZALI

FS 2019 21

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By

MONA FATIN SYAZWANEE BINTI MOHAMED GHAZALI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

April 2019

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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April 2019

Chairman : Muskhazli Mustafa, PhD
Faculty : Science

Normally, paddy straw was disposed of via open burning even though it contains valuable lignocellulosic materials which can be readily converted into fermentable sugar for bioethanol production. The second-generation of bioethanol production utilizes useful lignocellulosic substrates especially cellulose for bioconversion process. However, this material is enclosed within hemicellulose and lignin matrix in the cell wall, making the accessibility of cellulose become the major problem in bioethanol production from such sources in consolidate bioprocessing (CBP). The CBP is preferable as it produces faster saccharification result, low risk of contamination and cost-effective. Nevertheless, finding an optimize condition for efficient bioethanol production in CBP is still ambiguous as a different strain of lignocellulolytic fungi has their own environment preferences. Therefore, the main aim of this study is to explore a new approach in converting paddy straw into bioethanol using only filamentous fungi throughout the entire CBP process, thus eliminating the use of yeast as a fermenter organism. In this study, the research objectives involves the pretreatment method of paddy straw, selecting the best lignocellulolytic agent for hydrolyzation, optimizing all factors influencing the bioethanol production via one-factor-at-a-time (OFAT) as well as Response Surface Methodology analysis (RSM) and evaluating the final CBP set-up.

Paddy straw sieved into three different sizes; 2 mm, 5 mm and 8 mm were prepared and underwent several physical pretreatment (autoclave, boil) and chemical pretreatment (HNO_3 and NaOH). Size five millimeter paddy straw showed the highest cellulose content (35.61%) and the percentage of cellulose content went escalated to 72.47% when pretreated with 2% (w/v) sodium hydroxide (NaOH). Pretreatment of 2% (w/v) NaOH also shown the most efficient delignification and desilication process (1.02% lignin; 5.44% ash content) compared to others. All strains of fast-growing fungi were

quantitatively assayed and the results indicate that the highest cellulases enzyme producer were *Trichoderma asperellum* B1581 (3.93 U/mL endoglucanase; 2.37 U/mL exoglucanase; 3.00 U/mL β-glucosidase; 54.87 U/mL xylanase), followed by *Aspergillus niger* B2484 (5.60 U/mL endoglucanase; 1.08 U/mL exoglucanase; 1.57 U/mL β-glucosidase; 56.85 U/mL xylanase). A further test on compatibility test revealed mutual intermingling between both *T. asperellum* B1581 and *A. niger* B2484. Six single factors that are crucial for bioethanol production were tested in one-factor-at-a-time (OFAT) analysis for both selected strains of lignocellulolytic fungi. With all factors combined, *T. asperellum* B1581 prefers 2 days of both saccharification and fermentation process at 30°C with an amount of 3% substrate level and 10% of media level. While *A. niger* B2482 prefers 3 days of saccharification, 1 day of fermentation; at 30°C with an amount of 2% substrate level and 20% of media level. The results produced by OFAT were used as the centre point in the Central Composite Design (CCD) through Response Surface Methodology (RSM) software. However, comparison between the actual and the predicted value of ethanol produced in RSM's recommended CBP set-up for both *T. asperellum* B1581 and *A. niger* B2484 showed no significant difference, thus proving the model's stability to navigate experiment. In order to test effectiveness *T. asperellum* B1581 and *A. niger* B2484 as a fungi consortium, several combination of consortia concentrations (spore/mL) were tested and the amount of ethanol was quantified. However, a single strain of *T. asperellum* B1581 (6:0) was able to match the amount of ethanol produced by consortia of *T. asperellum* B1581 and A. niger B2484 (5:1, 4:2, 3:3, 2:4 and1:5) by producing the highest total amount of ethanol (1.11 g/L). The final amount of ethanol detected by GC-FID was 1.25 g/L; which was not significantly different from the ethanol assayed spectrometrically (1.11 g/L).

As a conclusion, a pretreatment of size 5 mm using 2% (w/v) NaOH had enhanced the breaking of cellulose-lignin complex, delignification, and desilication. Thus making the paddy straw becomes feasible for biofuel production. Both *T. asperellum* B1581 and *A. niger* B2484 were found to produce the highest cellulase enzyme and displayed mutual intermingling relationship suggesting the possibility of fungal consortium formation between these two species. Even though the recommended model for CBP set-up by RSM showed no significant differences between an actual and predicted value of ethanol produced, both species unable to improve the value of ethanol produced as consortia compare to single *T. asperellum* B1581 culture set-up. Thus, indicating that the potential of *T. asperellum* B1581 as single culture for bioethanol production in consolidated bioprocessing (CBP).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN BIOETANOL DARI JERAMI PADI MELALUI PENYATUAN BIOPROSES MENGGUNAKAN KULAT LIGNOSELULOLITIK

Oleh

MONA FATIN SYAZWANEE BINTI MOHAMED GHAZALI

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Pada kebiasaannya, jerami padi dilupuskan secara pembakaran terbuka walaupun ianya mengandungi bahan lignoselulosa yang dapat diubah menjadi gula fermentasi bagi penghasilan bioetanol. Bioetanol generasi kedua menggunakan substrat lignoselulosa yang penting terutamanya selulosa untuk proses biopenukaran. Namun begitu, bahan tersebut dilindungi rapi oleh hemiselulosa dan matriks lignin dalam dinding sel, lantas telah menjadi masalah utama dalam mengakses selulosa untuk pengeluaran bioetanol dari sumber tersebut menggunakan kaedah penyatuan bioproses (CBP). Keadah CBP adalah lebih sesuai digunakan kerana ia membolehkan proses pensakaridaan berlaku lebih cepat, kurang risiko pencemaran dan kos efektif. Walau bagaimanapun, pencarian keadaan yang optima bagi penghasilan bioetanol melalui CBP masih tidak jelas kerana kulat lignoselulolitik yang berlainan mempunyai pilihan persekitaran yang tersendiri. Oleh itu, tujuan utama kajian ini adalah untuk meneroka pendekatan baru dalam mengubah jerami padi menjadi bioetanol dengan hanya menggunakan kulat filamen sepanjang proses CBP, dan seterusnya mengelak penggunaan yis sebagai organisma penapaian. Oleh itu, matlamat penyelidikan merangkumi kaedah pra-rawatan untuk jerami padi, memilih agen lignoselulolitik yang terbaik untuk hidrolisis, mengoptimakan semua faktor yang mempengaruhi penghasilan bioetanol melalui satu faktor-pada-satu masa (OFAT) serta kaedah analisis tindakbalas permukaan (RSM) dan menilai tetapan akhir CBP.

Jerami padi telah ditapis menggunakan saiz yang berlainan; 2 mm, 5 mm dan 8 mm yang disediakan dan dikenakan pelbagai pra-rawatan fizikal (autoklaf, pendidihan) dan pra-rawatan kimia (HNO_3 dan NaOH). Saiz lima millimeter jerami padi telah menunjukkan kandungan selulosa tertinggi (35.61%) dan peratusan kandungan selulosa meningkat sehingga 72.47%. apabila dirawat dengan 2% (w/v) Natrium hidroksida (NaOH). Pra-rawatan 2% (w/v) NaOH juga menunjukkan proses penghapusan lignin dan desilikasi yang paling

berkesan (1.02% lignin; 5.44% kandungan abu) berbanding dengan yang lain. Semua jenis kulat yang mempunyai kadar pertumbuhan yang cepat telah diuji secara kuantitatif dan hasilnya telah menunjukkan bahawa pengeluar enzim selulase yang tertinggi adalah *Trichoderma asperellum* B1581 (3.93 U/mL endoglucanase; 2.37 U/mL exoglucanase; 3.00 U/mL β-glucosidase; 54.87 U/mL xylanase), diikuti oleh *Aspergillus niger* B2484 (5.60 U/mL endoglucanase; 1.08 U/mL exoglucanase; 1.57 U/mL β-glucosidase; 56.85 U/mL xylanase). Ujian lanjut mengenai keserasian antara dua jenis kulat yang berbeza telah menunjukkan tiada persaingan antara kedua-dua pencilan *T. asperellum* B1581 dan *A. niger* B2484. Enam faktor telah diuji dalam analisis satu faktor-pada-satu masa-(OFAT) untuk kedua-dua jenis kulat lignoselulolitik yang terpilih. Dengan menggabungkan semua faktor, *T. asperellum* B1581 memberi respon yang optima pada hari kedua bagi kedua-dua proses sakarifikasi dan penapaian pada suhu 30°C dengan jumlah paras substrat 3% dan 10% tahap media. Sementara *A. niger* B2482 memberikan respon yang optimum pada hari ketiga proses sakarifikasi, 1 hari penapaian; pada suhu 30°C dengan jumlah substrat 2% dan 20% tahap media. Keputusan yang dihasilkan oleh OFAT telah digunakan sebagai titik tengah dalam Rekabentuk Komposit Sentral (CCD) melalui kaedah analisis tindakbalas permukaan (RSM). Walau bagaimanapun perbandingan antara nilai sebenar dan nilai ramalan etanol yang diberikan oleh perisian RSM untuk *T. asperellum* B1581 dan *A. niger* B2484 tidak menunjukkan perbezaan yang nyata, dan telah membuktikan kestabilan model untuk digunapakai bagi tujuan eksperimen. Untuk menguji keberkesanan *T. asperellum* B1581 dan *A. niger* B2484 sebagai konsortium kulat, beberapa kombinasi kepekatan konsortia (spora/mL) telah diuji dan jumlah penghasilan etanol ditentukan. Namun, *T. asperellum* B1581 (6:0) dapat menandingi jumlah etanol yang dihasilkan oleh konsortium *T. asperellum* B1581 dan *A. niger* B2484 (5:1, 4:2, 3:3, 2:4 dan 1:5) dengan penghasilan jumlah etanol yang tertinggi (1.11 g/L). Jumlah akhir etanol yang dikesan oleh GC-FID adalah 1.25 g/L; di mana nilainya tidak jauh berbeza dengan nilai etanol yang diuji secara spektrometri (1.11 g/L).

Sebagai kesimpulan, pra-rawatan padi dengan saiz 5 mm menggunakan 2% (w/v) NaOH telah meningkatkan pemecahan kompleks selulosa-lignin, penghakisan lapisan lignin dan desilikasi. Oleh itu menjadikan jerami padi layak dan sesuai digunakan untuk penghasilan bioetanol. Kedua-dua *T. asperellum* B1581 dan *A. niger* B2484 menunjukkan keupayaan dalam menghasilkan enzim selulase tertinggi dan mempunyai hubungan percampuran yang baik untuk pembentukan konsortium kulat antara dua spesies ini. Walaupun model yang disyorkan untuk proses CBP oleh RSM tidak menunjukkan perbezaan yang nyata antara nilai sebenar etanol dan ramalan yang dihasilkan, kedua-dua spesies tidak dapat meningkatkan nilai etanol yang dihasilkan secara konsortia berbanding dengan tetapan kultur tunggal *T. asperellum* B1581. Oleh itu, penggunaan kulat *T. asperellum* B1581 secara tunggal mempunyai potensi untuk digunakan bagi pengeluaran bioetanol menerusi proses CBP.

ACKNOWLEDGEMENTS

All praises to Allah S.W.T for giving me a lifetime opportunity to pursue my dreams, an absolute strength to face all the hardships and guide me throughout the journey. May Allah S.W.T continue to bless us all in this life and hereafter.

I would like to express my gratitude to my parents, Hj. Mohamed Ghazali B Mohamed Yousoff and Hjh. Fawziah Bt Md Noor, whom has been my enthusiastic cheerleader, along with their continuous support, duaa's and endless love that has become pillars to my success. Thank you for offering me comfort and always been there for me when I was feeling down. I am truly blessed to have parents that totally understand my passion and always believe in me.

I also wish to thank my elder bothers, Dr. Farid Ezanee, Mohamed Firdauz Mukhzanee, sister in law, Mazwani Wan Ibrahim and nephews, Daniel Mukhreez and Danish Mikhael for bringing me joy, provided me with moral support, love and courage through high and low. I am so lucky and grateful to have such a wonderful family by my side throughout my journey.

A very special gratitude dedicated to my chairman of supervisory committee, Associate Professor Dr. Muskhazli Mustafa, for his guidance, patience, wisdom, advice and support in assisting me to complete my study and produce a better quality of research. He has helped me to understand and explore more about research field, developed new technical and soft skills, inspire my creativity, encourage my critical thinking, building my confidence and shaped me into a better person. I am so grateful to have a very motivating supervisor like him and could not have imagined having a better supervisor for my PhD project. I would also like to wish my gratitude towards my other committee members; Associate Professor Dr. Nur Ain Izzati Mohd Zainudin for providing me with several identified fungi samples from Mycology Laboratory and guides me in mycology; and Associate Professor Dr. Nor Azwady Abdul Aziz, for providing me support, advice and guidance in statistical analysis. May Allah S.W.T continue to reward all of you for your good deeds.

My immense gratitude to the Ministry of Higher Education Malaysia (MOHE) for providing me with financial support under MyBrain15- MyPhD programme throughout my entire study period. To the Universiti Putra Malaysia, thank you for the Geran Putra – Inisiatif Putra Siswazah (GP-IPS/2016/9485600) which has become an immense help towards minimizing our financial stress during the study. I wish to thank all Plant Systematic and Microbes Laboratory members especially Pn. Azleen Ahmad Adli and Pn. Farah Kamaludin for their assistance throughout the length of my projects.

Special thanks to Pn. Norlinawati Abd Halim from Microbiology Laboratory, Faculty of Food Science and Technology for allowing me to used their equipment throughout my study. Lastly, I would like to thank Mr. Hamezan Muhammad@Ahmad from Chromatography Laboratory, Faculty of Food Science and Technology, for his advice and guidance in operating gas-chromatography (GC-FID) during sample analysis.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	Acid-detergent fiber
ADL	Acid-detergent lignin
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CAZYmes	Carbohydrate active enzymes
CBH	Cellobiohydrolase
CBP	Consolidated bioprocessing
CCD	Central composite design
CMC	Carboxymethyl-cellulose
DP	Degree of polymerization
EI	Enzymatic index
FAO	Food and Agriculture Organization
g	gram
GC-FID	Gas chromatography – flame ionization detector
GHG	Greenhouse gases
h	Hours
HSD	Honestly significant difference
IU	International Unit
kWh	kilowatt hour
kPa	kilopascal
L	Litre
M	Molar
mm	millimeters
mL	milliliters

min	minutes
OFAT	One-factor-at-a-time
pH	potential of Hydrogen
ppb	Parts per billion
PPO	Polyphenol oxidase
psi	pounds per square inch
RSM	Response surface methodology
RPM	Rotation per minute
SD	Standard deviation
SEM	Scanning electron microscope
U/mL	Units per millilitre
w	weight
v	volume
3D	3-dimensional

CHAPTER 1

INTRODUCTION

1.1 Background of the study

In December 2017, the Food and Agriculture Organization (FAO) has elevated its estimation of global rice production in the year 2017 by 2.9 million tonnes to 759.6 million tonnes (503.9 million tonnes, milled basis) and China remains the largest paddy producer in the Asia region (FAO, 2018). Rice is one of the most favorite staple food for Malaysian, and with the implementation of National Agrofood Policy 2011-2020 (NAP 4), the local rice production has been increased to ensure country's stock of rice is sufficient to meet future demands as the population of Malaysian increases over the years (Rajamoorthy, Abdul Rahim and Munusamy, 2015). About 23% of overall paddy weight will generate a by-product known as paddy straw, which is frequently disposed off by open burning to clear the fields for the next cycle of rice planting (Kaur and Phutela, 2018). Paddy straw are also refered as an agricultural waste comprising of the dry stalks of crops and usually collected after harvesting period (Bakker, Elbersen, Poppens and Lesschen, 2013). The disposal of paddy straw through open burning was carried out to eliminate the sources of rat infestation, insect pests and to prevent rice diseases (Rosmiza, Davies, Rosniza Aznie, Mazdi and Jabil, 2014). Burning of biomass material releases wide-range of gases such as carbon monoxide (CO), carbon dioxide (CO_2), methane (CH_4), aldehyde, organic acid and inorganic elements, volatile and semi-volatile organic compounds, and particulate matter (PM); affecting people lives nearby (Yadav and Devi, 2018).

Paddy straw is normally being utilised as materials for cattle feed, fuel for residential cooking, thatching for rural houses, gasification, power generation, mulching material, and paper mills (Roy and Kaur, 2015). The compositions of paddy straw are 32.15% cellulose, 28% hemicellulose and 19.6% lignin; which contains major constituents of lignocellulosic materials (Shawky, Mahmoud, Ghazy, Asker and Ibrahim, 2011). Ideally, only cellulose and hemicellulose have the ability to be converted into fermentable sugars (Moiser *et al*., 2005). These lignocellulosic materials (wood and agricultural crops residue) are promising feedstock for generating variety of great products such as bioethanol (Yoswathana, Phuriphipat, Treyawutthiwat and Eshtiaghi, 2010).

Biofuels are renewable and sustainable energy resources which offer an alternative solution to our conventional fuel sources dilemma as well as an effort to put a halt to climate change (Hidayat, Rochmadi, Wijaya and Budiman, 2016). Up to June 2017, the Malaysia Automotive Association (MAA) has released the total number of Malaysian vehicles on the roads standing at 28,181,203 units (MAA, 2017). With the advancements in the transportation

industry in Malaysia, the demands of energy have increased over the years and urged scientist to develop new renewable energy source to replace the ordinary conventional fuel. Research on renewable energy has gained supports from Malaysian government with the implementation of 5-Fuel Policy (2001) under the 8th Malaysia Plan, in which targeted to achieve 5% mixture of RE, but, only manage to achieve 1.8% in the following year (Loh and Choo, 2013).

The development of first-generation bioethanol based on food crops suffers from the criticism due to competition between food supply and bioethanol development; causing a sudden increase in food prices (Naik, Goud, Rout and Dalai, 2010). The weakness of first-generation bioethanol has highlighted the need to develop second-generation bioethanol based on lignocellulosic agro waste (Sims, Mabee, Saddler and Taylor, 2010). The second generation bioethanol develops from woody biomass are more energy efficient, flexible in terms of their feedstock and not competing with the human food resources (Havlik *et al*., 2011). Currently, the most progressive technologies in bioethanol production are focusing on transforming lignocellulosic feedstock into transportation energy (Bakker *et al*., 2013). Theoretically, paddy straw can generate up to 205 billion liter ethanol across the globe, from a single biomass feedstock with only 5% of total consumption (Belal, 2013).

In order to develop bioethanol efficiently and cost-effective from cellulosic feedstock, the degradation of biomass by energetic cellulases and fermentation by dynamic fermentative microorganism are basically important (Takano and Hoshino, 2012). In this case, the lignocellulolytic fungi are the best choice. There are many species of lignocellulolytic fungi such as *Aspergillus* sp., *Trichoderma* sp., *Fusarium* sp. and *Neurospora* sp. (Ferreira, Mahboubi, Lennartsson and Taherzadeh, 2016). These natural occurring fungi may have several benefits with their fermentability in producing bioethanol over the standard baker's yeast *Saccharomyces cerevisiae* (Okamoto, Nitta, Maekawa and Yanase, 2011). Most of the lignocellulolytic fungi secrete extracellular and hydrolytic enzymes that work as a biocatalyst for lignin and cellulosic materials degradations (Mtui, 2012). These enzymes comprise of cellulolytic enzymes (e.g cellulase) and ligninolytic enzymes (e.g lignin peroxidase, manganese peroxidase and laccase) (Manavalan, Manavalan and Heese, 2015). Cellulase is a family of at least 3 groups of enzymes; endo-(1,4)-β-D-glucanase (EC 3.2.1.4), exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21) (Kuhad, Gupta and Singh, 2011a). Researchers have developed strong interests in the production of cellulases as of their utilizations in industries of alcohol fermentation, brewing, pulp and paper industry as well as textile industry (Nasr, Badawi, Mona, Demerdash and Barakat, 2015). Besides, cellulose and hemicellulose content can be enzymatically degraded into simple sugars by cellulases and hemicellulases (Berlowska *et al*., 2016).

There are 4 significant processes for robust lignocellulosic biomass production which depend on the variations in saccharification or fermentation conditions: separate hydrolysis and fermentation (SHF), simultaneous saccharification and

fermentation (SSF), consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF) (Parisutham, Kim, and Lee, 2014). The consolidated bioprocessing, or CBP, converts lignocellulosic material into desired products in a single step without adding exogenous enzymes and this process certainly has been a subject of research interest in recent years (Olson, McBride, Shaw and Lynd, 2012). A combination of enzyme secretion, saccharification, and fermentation process in the same bioreactor has been known for economical manufacturing of bioethanol by evading the high expenses on investment, feedstock as well as the equipment for microbial enzyme production (Hasunuma *et al*., 2013). The principal of cost reduction in CBP derives from either: (1) fermentative organism secretes essential cellulolytic enzymes for degradation of biomass or (2) cellulolytic organism which has the ability to ferment and thus eliminating the need for a separate enzyme production step (Linger and Darzins, 2013).

1.2 Problem statement

Even though bioethanol may induce various benefits that lead towards minimizing the environmental impact, more research is required especially in fermentation technology modification, preparation of raw materials and for economical bioethanol production (Bhatia, Johri and Ahmad, 2012). Paddy straw is a good material for the development of bioethanol but the presence of high ash and silica content in the feedstock has limited the bioconversion process to occur efficiently (Ibrahim, 2012). Unlike the first-generation of bioethanol, the second-generation utilizes the lignocellulosic substrates known as cellulose which is enclosed within hemicellulose and lignin matrix in the cell wall, making the accessibility of cellulose become the major problem in bioethanol production from such sources (Wi, Choi, Kim, Kim and Bae, 2013).

The mechanism of bioconversion process involved the production of sugars from biomass which requires new biotechnological improvements in order to ensure their efficiency enhancement and also economically applicable. Although some fungal strains are exclusively known as lignocellulolytic and thermostable, most of these fungal strains failed to secrete satisfactory amounts of cellulolytic enzymes that are mandatory for a productive cellulose conversion into desired product (Dashtban, Schraft and Qin, 2009). Choosing an appropriate cellulase for saccharification process is extremely challenging as each material have structural difference and difference of enzymatic activities in industrial cellulase reagents (Takano and Hoshino, 2018).

Besides selecting a productive strain, designing a suitable culture condition is also crucial to improve the efficiency of ethanol production systematically by either adding or eliminating components from the formulation, which resulted in a more stabilized and reproducible culture conditions (Dong, Zhao, Ma and Zhang, 2012). The amount of cellulase production seems to rely upon several factors such as incubation period, pH, temperature, carbon, nitrogen sources and cations (Gautam *et al*., 2011).

The consolidated bioprocessing (CBP), in which enzyme secretion, break down of substrate, and fermentation process is achieved in one-step using either single microorganism or a group of compatible microorganisms and also known as the most economically attractive method for bioconversion of lignocellulosic biomass into bioethanol (Olson *et al*., 2012; Ho *et al*., 2012; Wang *et al*., 2015a). However, the most difficult step in CBP is the selection of an appropriate microorganism or microbial consortium that secretes suitable hydrolytic enzymes corresponding to the lignocellulosic material, and produce ethanol (Paulova, Patakova, Branska, Rychtera and Melzoch, 2015).

1.3 Objectives of the study

The idea of this study is to utilize pretreated paddy straw as bioethanol material using consolidated bioprocessing (CBP) approach with the help from lignocellulolytic fungi. Therefore, the main aim of the study is to explore the use of lignocellulolytic fungi in consolidated bioprocessing (CBP) for an efficient conversion of paddy straw into bioethanol. The outline of the research approach is shown in Figure 1.1 and the design of the study was based on the following specific objectives to address the foregoing issues as stated in 1.2:

i. To determine the optimized pretreatment method for paddy straw in removing silica, delignification and enhanced the accessibility towards cellulosic content
ii. To determine the best lignocellulolytic agent for hydrolyzation of lignocellulosic materials of paddy straw.
iii. To determine the factors that influences the optimization of bioethanol production via one-factor-at-a-time (OFAT) analysis and optimizing the physico-chemical parameters using Response Surface Methodology analysis (RSM).
iv. To evaluate the efficiency of the final CBP set-up using either single microbe or consortium microbes to improve the amount of ethanol yield.

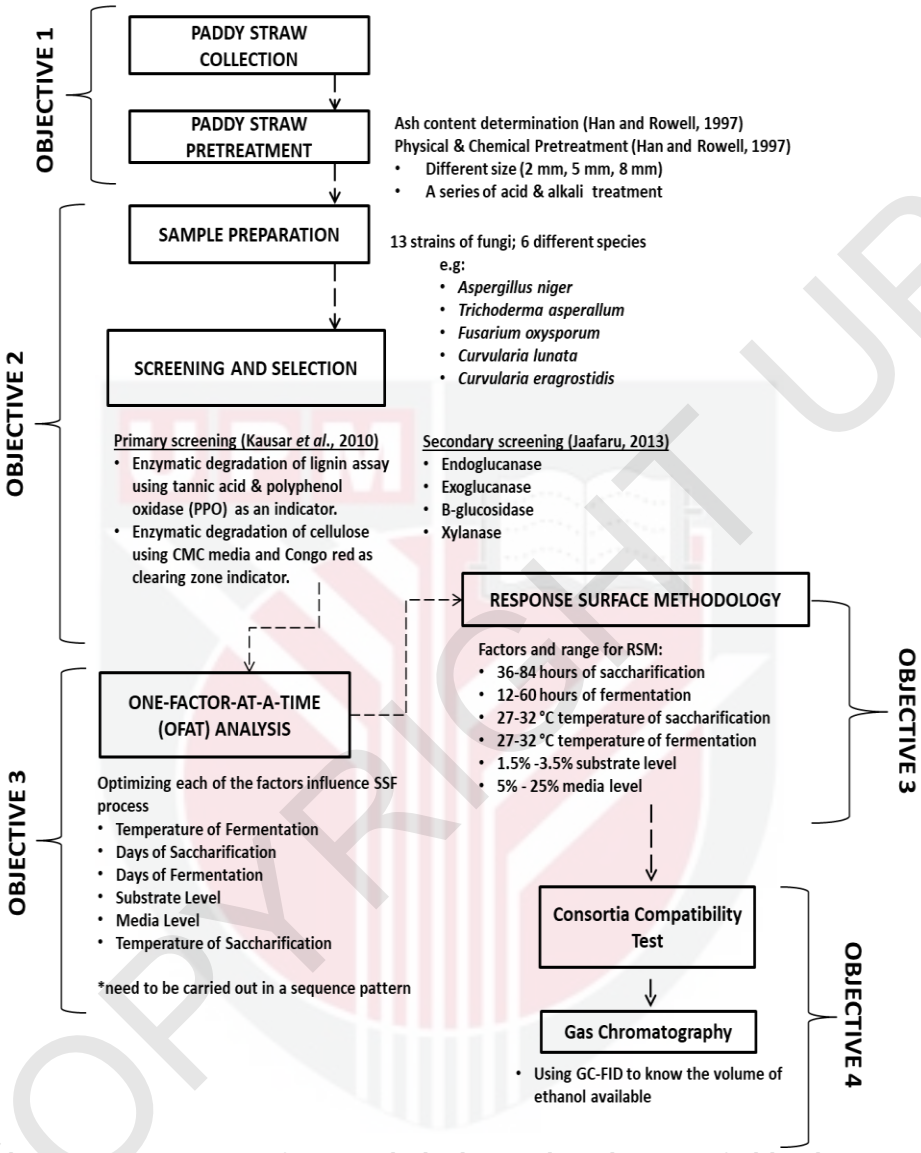

Figure 1.1: Summary of research design and attainment of objectives

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FAO Rice Market Monitor, April 2018, Volume XXI - Issue No. 1

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LIST OF PUBLICATIONS

Mona Fatin Syazwanee, M.G., Nurul Shaziera, A.G., Nur Ain Izzati, M.Z., Nor Azwady, A.A. and Muskhazli, M. (2018). Improvement of delignification, desilication and cellulosic content availability in paddy straw via physico-chemical pretreatments. *Annual Research & Review in Biology*, 26 (6), 1-11. doi: 10.9734/ARRB/2018/40947

Mona Fatin Syazwanee, M.G., Nur Ain Izzati, M.Z., Nor Azwady, A.A. and Muskhazli, M. Screening of lignocellulolytic fungi for hydrolyzation of lignocellulosic materials in paddy straw for bioethanol production. *Malaysian Journal of Microbiology*, 15 (4), pp. xxx-xxx. doi: http://dx.doi.org/10.21161/mjm.180250

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