# CO-HYDROLYSIS OF OIL PALM EMPTY FRUIT BUNCH FIBRES BY COMMERCIAL CELLULASE AND CRUDE XYLANASE SOURCED FROM *Aspergillus niger* USM SD2 AND *Trichoderma asperellum* USM SD4 TOWARDS BIO-ETHANOL PRODUCTION

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**by**

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| | |
|---|---|
| < | Less than |
| ± | About |
| ≤ | Less than or equal to |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| θ | Theta |
| κ | Reaction constant; kilo |
| λ | Lambda |
| μ | Micron |
| ρ | Statistical significance |
| ANOVA | Analysis of variance |
| CCD | Central composite design |
| Da | Dalton |
| FGB | First generation bioethanol |
| FPU | Filter paper enzyme unit |
| g | Gram |
| gds | Gram dried substrate |
| GHG | Green house gases |
| h | Hour |
| IU | International enzyme unit |
| Kg | Kilogram |

| | |
|---|---|
| kWh | Kilowatt hour |
| L | Litre |
| LAP | laboratory analysis protocol |
| LOF | Lack of fit; |
| M | Molarity |
| m | micro & metre |
| MEIH | Malaysian Energy Information Hub |
| min | Minute |
| ml | Millilitre |
| MPOB | Malaysian Palm Oil Board |
| MT | Metric tonnes |
| nm | Nanometre |
| NREL | National Renewable Energy Laboratory |
| $^{o}$C | Degree celcius |
| OD | Optical density |
| OPEFB | Oil palm empty fruit bunches |
| OPT | Oil palm trunk |
| pH | Potential of hydrogen |
| rpm | Revolution per min |
| RSM | Response surface methodology |
| s | Seconds |
| SEM | Scanning Electron Microscopy |
| SGB | Second generation bioethanol |
| SHcF | Separate co-hydrolysis and co-fermentation |

| | |
|---|---|
| SHF | Separate hydrolysis and fermentation |
| SScF | Simultaneous co-saccharification and co-fermentation |
| SSF | Solid State Fermentation/Simultaneous Saccharification and Fermentation |
| T | Reaction temperature |
| t | Reaction time |
| TAPPI | Technical Association of the Pulp and Paper Industry |
| USCB | United States Census Bureau |
| USDOE | United States Department of Energy |
| USEIA | United States Energy Information Agency |
| v/v | Volume/volume |
| W | Watt |
| w/v | Weight per volume |
| XRD | X-ray diffraction |

# KO- HIDROLISIS GENTIAN TANDAN BUAH KOSONG KELAPA SAWIT OLEH SELULASE KOMERSIAL DAN XILANASE MENTAH DIPEROLEH DARIPADA *Aspergillus niger* USM SD2 DAN *Trichoderma asperellum* USM SD4, ATAS PENGHASILAN BIOETANOL

## ABSTRAK

Ketidak-gunaan pecahan xilosa tandan buah kosong kelapa sawit (OPEFB) adalah faktor pengehad utama terhadap penghasilan etanol OPEFB mampan. Kajian ini menilai ko-hidrolisis berenzim gentian OPEFB melalui sinergi antara xilanase mentah yang diperoleh daripada pencilan kulat terpilih dan selulase komersial (Celluclast 1.5L) untuk menyerlahkan fermentasi cekap gula glukosa dan xilosa untuk penghasilan etanol. Kesan prarawatan-prarawatan berasid, beralkali dan auto-hidrolisis pada OPEFB untuk meningkatkan pemulihan gula boleh-fermentasi, dan ke atas prestasinya sebagai substrat untuk pengeluaran hemicellulase telah dinilai. Dengan menggunakan auto-hidrolisis sebagai prarawatan, keputusan menunjukkan bahawa terdapat keutamaan kerencaman dan kelebihan tenaga sekiranya substrat dihaluskan selepas prarawatan kimia berbanding dengan substrat dihaluskan sebelum prarawatan kimia. Namun, rawatan beralkali gentian OPEFB menunjukkan komponen holosellulosa lebih tinggi (91%) berbanding dengan rawatan berasid (77%) atau auto-hidrolisis (80%), dan telah digunakan untuk menilai ko-hidrolisis berenzim dan pengeluaran bioetanol. Dua strain kulat novel, *A. niger* USM SD2 (GenBank nos: KU882054) dan *T. asperellum* USM SD4 (GenBank nos: KU878976) yang dipencil daripada sampel tanah di sekitar sisa kelapa sawit telah dioptimumkan untuk penghasilan xilanase mentah melalui aedah sambutan permukaan (RSM). Pada keadaan optimum, pengeluaran xilanase oleh *A. niger* USM SD2 dan *T. asperellum* USM SD4 telah dipertingkatkan sebanyak 160% dan 156% (3246 IU /g dan 3,370 IU / g) masing-masing berbanding dengan aktiviti awal masing-masing adalah 1250 IU / g dan

1318 IU /g sebelum pengoptimuman. Analisis ekstra proteome menunjukkan bahawa kedua-dua enzim mentah adalah bebas selulase; tetapi telah dikuasai oleh β-1,4-xilanase dan beberapa enzim aksesori. Secara perbandingan, xilanase dari *T. asperellum* adalah lebih aktif dan menolak perencatan produk akhir (mengekalkan kira-kira 60% daripada aktivitinya pada kepekatan xilosa 50 mg/ml); manakala xilanase *A. niger* kurang aktif dan kehilangan lebih daripada 80% daripada aktivitinya pada kepekatan xilosa 50 mg/ml. Walau bagaimanapun, sebagai penanda untuk kesesuaian dalam proses yang melibatkan pengeluaran alkohol, xilanase mentah oleh *A. niger* menunjukkan aktiviti sisa yang lebih tinggi (75%) pada kepekatan 50% dan 60% masing-masing bagi etanol dan metanol berbanding dengan xilanase dari *T. asperellum* (60% aktiviti tersisa). Semasa ko-hidrolisis, suplementasi xilanase mentah masing-masing pada 250 IU : 50 FPU meningkatkan secara signifikan hidrolisis gentian OPEFB terawat, mencapai hasil teori yang lebih tinggi daripada *T. asperellum* (91.7%) atau dari *A. niger*, (91.0%) dibandingkan hasil teori (12.2% dan 7.1%) apabila enzim mentah tersebut digunakan tanpa selulase; atau 77% hasil apabila hanya selulase telah digunakan. Secara perbandingan, hasil teori etanol melalui ko-sakarifikasi dan ko-fermentasi serentak (SScF) adalah lebih baik secara signifikan daripada ko-hidrolisis dan ko-fermentasi berasingan (SHcF) ataupun kaedah fermentasi "glukosa tungga" yang konvensional. Selepas fermentasi, SScF meningkat hasil teori etanol dengan signifikan iaitu sebanyak 89% (bersama 0.33 g/g OPEFB mentah), ini adalah lebih baik daripada hasil 85% (0.31 g/g); atau 63% (0.23 g/g) masing-masing melalui SHcF atau fermentasi glukosa tinggal. Berdasarkan keputusan ini, kajian ini telah menunjukkan bahawa kira-kira 67% hasil etanol tambahan boleh dicapai dari OPEFB jika kedua-dua komponen glukosa dan xilosa di fermen melalui ko-sakaarifikasi dan ko-fermentasi serentak.

# CO-HYDROLYSIS OF OIL PALM EMPTY FRUIT BUNCH FIBRES BY COMMERCIAL CELLULASE AND CRUDE XYLANASE SOURCED FROM *Aspergillus niger* USM SD2 AND *Trichoderma asperellum* USM SD4 TOWARDS BIO-ETHANOL PRODUCTION

## ABSTRACT

Non-utilization of the xylose fractions of oil palm empty fruit bunches (OPEFB) is the main limiting factor in the production of sustainable OPEFB ethanol. This study assessed the co-enzymatic hydrolysis of OPEFB fibers through a synergy between crude hemicellulases sourced from selected fungal isolates and a commercial cellulase (Celluclast 1.5L) to enhance efficient fermentation of its glucose and xylose sugars for ethanol production. The effects of acid, alkaline and auto-hydrolysis pre-treatments on OPEFB to enhance the recovery of the fermentable sugars, and on its performance as a substrate for hemicellulase production were assessed. By using auto-hydrolysis, results showed that there were preferential compositional and energy advantages of substrate-refining post chemical pre-treatment over substrate-refining prior to chemical pre-treatments. However, alkaline-treatment of OPEFB fibers showed higher holocellulose component (91%) compared to acid (77%) or auto-hydrolysis (80%) treatments and was used to assess co-enzymatic hydrolysis and bio-ethanol production. Two novel fungal strains, *A. niger* USM SD2 (GenBank nos: KU882054) and *T. asperellum* USM SD4 (GenBank nos: KU878976) isolated from soil samples around oil palm wastes were optimized for crude xylanase production *via* solid state fermentation of OPEFB by response surface methodology (RSM). At optimum conditions, xylanase production by *A. niger* USM SD2 and *T. asperellum* USM SD4 were enhanced by 160% and 156% (3246 IU/g and 3,370 IU/g) respectively relative to their respective initial activities (1250 IU/g and 1318 IU/g) prior to optimization. Extra-proteome analyses showed that both crude

enzymes were cellulase-free, but were dominated by β-1,4-xylanase and a few accessory enzymes. Comparatively, xylanase from *T. asperellum* was more active and resisted end-product inhibition (retaining about 60% of its activity at 50 mg/ml xylose concentration); while that from *A. niger* was less active and lost more than 80% of its activities at 50 mg/ml xylose concentration. However, as a marker for suitability in processes involving alcohol production, crude xylanase by *A. niger* showed higher residual activities (75%) at 50% and 60% respective concentrations of ethanol and methanol compared to that from *T. asperellum* (60% residual activities). During co-hydrolysis, supplementation of respective crude xylanases at 250 IU : 50 FPU significantly enhanced the hydrolysis of treated OPEFB fiber, achieving higher theoretical yields from *T. asperellum* (91.7%) and *A. niger* (91.0%) compared to the theoretical yields (12.2% and 7.1%) when respective crudes were used without cellulase, or the 77% yield when only the cellulase was used. Comparatively, the theoretical ethanol yield *via* simultaneous co-saccharification and co-fermentation (SScF) was significantly better than separate co-hydrolysis and co-fermentations (SHcF) or the conventional "single glucose" fermentation methods. After fermentation, SScF significantly enhanced theoretical ethanol yield by 89% ( the equivalent of 0.33 g/g raw OPEFB) which was better than 85% yield (0.31 g/g); or 63% yield (0.23 g/g) respectively *via* SHcF or single glucose fermentation. Based on these results, this study has shown that about 67% additional ethanol yield could be achieved from OPEFB if both its glucose and xylose components were fermented *via* simultaneous co-saccharification and co-fermentation.

# CHAPTER 1 INTRODUCTION

## 1.1 General Background

Global attention towards renewable and environmentally friendly energy sources has resurged recently due to the geometric depletion of world fossil fuel reserves and the resultant environmental concerns such as environmental pollutions and global warming (Haq et al., 2015). Presently, a large chunk of the global economy is directly dependent on the availability of fuel. This is because, fuel scarcity leads to a hike in fuel price, affect the cost of transportation of goods and services which will in turn cause serious inflation and a possible economic recession. On the other hand, emission of greenhouse gases, in addition to its health implications (especially when carbon monoxide is emitted due to incomplete combustion) leads to incessant changes in the world climate with a devastating ecological effect not limited to flooding, but a consequent loss of lives and properties as a result of the ecological disaster. In view of these challenges, several alternative energy sources with negligible environmental impacts have been identified as a possible substitute to meet up the world energy demands. Chief among these *via*ble potential sources is bio-ethanol which is currently the most investigated environmentally friendly alternative energy sources in the automobile industry. This is due to its verifiable competitive advantages in terms of renewability and lesser environmental impacts compared to gasoline.

Bio-ethanol is a form of ethanol produced by the action of certain microorganisms on simple sugars especially glucose. Ethanol, an alcohol family, is a chemical compound with an age-long use in human history. It is a major constituent of alcoholic beverages and has been identified for several medical and industrial applications. In the automobile industry, ethanol is used in car engines as a blend (E85) or sole source of ignition.

Bioethanol is currently being produced in commercial quantities for automobile use in the USA and Brazil (Dinita et al., 2011)

Currently, most of the ethanol produced from renewable resources comes from sugarcane and some starchy grains which are considered first generation sources (USDOE, 2016). Although developed, the long-term *via*bility of first generation bioethanol faces sustainability question. This is because, production of these raw materials requires significantly large hectares of cultivatable land leading thus to a corresponding hike in food prices and an ultimate food insecurity and inflation due to competition (Dinita et al., 2011). On the other hand, the second generation or lignocellulosic bio-ethanol is considered a sustainable energy alternative. Lignocellulosic biomass used as feedstock for production are not only immensely available all over the world but are most often mainly agricultural wastes from crops such as wheat, corn, sugarcane and oil palm tree (Tye et al., 2016). Hence, the question and concern about food security do not arise.

Despite its tremendous potentials in terms of meeting energy needs and providing environmental benefits, lignocellulosic bio-ethanol is yet considered a commercially non-*via*ble or a cost-intensive alternative energy source (Alvira et al., 2010). This is due to the nature of most of the available lignocellulose related technologies in the world which impact directly on the final energy requirement and processing costs of the whole production process (Kuhad et al., 2011). Presently, to produce one gallon of lignocellulosic ethanol presently costs about $1.5 dollars (or $63 per barrel), an amount greater than the cost per barrel of crude petroleum ($40-$45). Due to this cost difference, bio-ethanol is currently being sold at a retail price higher ($3.07/gallon) than gasoline ($1.91/gallon) in the U.S. (USDOE, 2016; USEIA, 2016). It is with the view to abate this growing concern that recent researches into lignocellulosic bio-ethanol are hence

channeled towards making the process cost-effective (Raman and Gnansounou, 2014; Mattam et al., 2016; Rajendran et al., 2016).

Depending on the source, lignocellulosic biomass consists principally of different percentage of cellulose homopolymer of repeated glucose units, a complex hemicellulose consisting mainly of xylose sugar, lignin and some other less important components. Even though all these major components have been identified and used for the production of several industrial products such as ethanol (Sun and Cheng, 2002) and biogas (Taherzadeh and Karimi, 2008) from cellulose, xylose (Rahman et al., 2007) and xylitol (Albuquerque et al., 2014) from hemicellulose, and lignosulfonate (Tan et al., 2013) and vanillin (Verman et al., 2016) from lignin; only the glucose monomeric units are considered as important substrate for bioethanol production in most previous studies (Tye et al., 2016). Conversely, as highlighted in recent reviews (Alvira et al. 2010; Tye et al., 2016), the lignin and hemicellulose fractions were often identified as stumbling blocks against a successful lignocellulosic bioethanol production. However, for a sustainable cost-efficient lignocellulosic ethanol, both the glucose and xylose components of lignocellulosic biomass must utilized for fermentation. This is inline with the sugestion by Kuhad et al. (2011) who have reported that a cost efficient lignocellulosic ethanol is dependent on the utilization of the two major sugar components, the xylose and glucose. In view of this, the use of the hemicellulosic fractions of lignocellulosic biomass is now the focus of intense researches in the bio-ethanol research industry (Chandel et al., 2011; Kuhad et al., 2011).

Review of previous literature has shown that non-utilization of the xylose fraction of lignocellulosic biomass stemmed from the inability of common yeast, *Saccharomyces cerevisiae,* to readily ferment pentose sugars for bioethanol production. Hence, according to Chandel et al. (2011) and Kuhad et al. (2011), the conventional process for hemicelluloses utilization is the separate fermentation of xylose-containing liquor after

acid or autohydrolysis by a competent microorganism. However, this method, as observed by Hong et al. (2013) and Lim and Lee (2013), is nevertheless mitigated by several factors which include, self degeneration of xylose fraction, generation of fermentation inhibitors and cost-intensiveness of the whole process due to the cost of neutralizing the usually acidic fermentation liquor. Moreover, several other methods, according to Shen and Wyman (2011), have been proposed to enhance xylose utilization from lignocellulosic substrates; yet achieving efficiency in lignocellulosic ethanol using both the glucose and xylose components is an area of intense research in the bioethanol industry. This study hopes to use non-conventional processes to enhance xylose utilization using oil palm empty fruit bunches (OPEFB) as a representative substrate.

The OPEFB is a lignocellulosic waste generated from oil palm tree (*Elaeis guineensis*) which is largely cultivated in Indonesia, Malaysia, and some other southeast Asian countries (Yano et al., 2009). Lim and Lee reported that Malaysia is the world's second-largest exporter of palm oil products after Indonesia (Lim and Lee, 2012). In 2016, the annual report by the Malaysia Palm Oil Board (MPOB) for the 2015 planting year showed that Malaysia cultivated 5.64 Ha of land and produced 29.67 million MT of oil palm products from 98.34 million MT of fresh fruit bunches (MPOB, 2016). Due to this large cultivation, huge oil palm residues are generated as waste such that OPEFB alone was reported to climax far beyond 22 million MT in 2015 (MPOB, 2016). OPEFB is a sugar-rich biomass with great potentials for bioethanol production. It contains about 28-30 % hemicellulose in addition to main cellulose component (50-60%) and relatively small lignin (17-19%) fractions (Goh et al., 2010). Quite a lot of studies have been reported on the use of OPEFB as a substrate for bio-ethanol production (Sudiyani and Hermiati, 2010; Millati et al., 2010; Tan et al., 2013 and Duangwang et al., 2016), but the various process technologies employed in those studies are yet inefficient, based on a techno-economic evaluation as was also observed by Do and Lim (2016).

## 1.2 Justification/Problem Statement and Significance

To make the lignocellulosic ethanol production process more competitive and cost-efficient with higher bioethanol yield, there is a need to ensure a complete hydrolysis and fermentation of both the cellulose and hemicellulose fractions of biomass sugars for ethanol production. However, like most other lignocellulosic biomass—sugarcane bagasse (Chandel et al., 2012); seaweed (Tan and Lee, 2014), oil palm trunk (Prawitwong et al., 2012) and reed, (Lu et al., 2012), most available reports on lignocellulosic bio-ethanol from OPEFB (Han et al., 2011; Sudiyani et al., 2013; Zhu et al., 2014) have focused mainly on the cellulosic fraction neglecting the otherwise equally essential hemicellulosic fraction. On the other hand, the utilization of OPEFB hemicellulosic fractions will lead to a minimum 15% increase in ethanol yield based on hypothetical data from earlier reports (Yano et al., 2009; Goh et al., 2010).

Comparative analysis of previous studies showed that OPEFB fibre is composed on the average of 54% cellulose, 28% hemicellulose and about 17% lignin (Goh et al., 2010; Tye et al., 2016). Therefore, assuming based on data available from earlier studies as reported by Goh et al.(2010), that a 70% total sugar recovery and 75% fermentation efficiency were achieved during hydrolysis and fermentation stages respectively, only 145 g of ethanol (or 14.5%) could be achieved per 1 kg of raw OPEFB by the conventional approach of non-xylose utilization. If, on the other hand, the hemicelluloses fraction was hydrolyzed and fermented, by the above hydrolytic and fermentation assumptions, an approximate 0.22 kg of ethanol yield (i.e. 22.2 %) per kg of raw OPEB could be attained. This amount to an additional 53.1% of the previous ethanol yield without xylose utilization.

In cases of the use of OPEFB for bioethanol production, a systematic review of previous literature (Table 2.6) has revealed that non-utilization of its xylose fraction for ethanol production is not restrictively caused by the identified fermentation problem but a

consequence of several lapses in all the integrated technological processes leading to bioethanol production. Other identified major lapses include; non-use of proper pre-treatment techniques that retains the hemicellulosic fraction as much as its cellulose counterpart during the removal of the recalcitrant lignin; poor hydrolytic yield due to the type of enzyme and conditions of hydrolysis used (Yano et al., 2009; Hamzah et al., 2011), and most importantly, inefficient fermentation technique that failed to ensure complete utilization of the xylose component of the hydrolysed products during fermentation as shown in earlier reports . For example, sulphite pretreatment by Tan et al. (2013) caused a significant loss of xylan component of the treated OPEFB from 19.3-2.1% after pretreatment; inefficient hydrolytic conditions repored by Sudiyani et al., (2013) reduced the hydrolytic yield per kg of OPEFB by about 33%, while the incomplete or non-utilization of the xylose fraction of OPEFB which characterised the various reports by Han et al. (2011); Millati et al. (2011); Piarpuzán et al. (2011); Zainudin et al.(2012); Tan et al. (2013) and Duangwang et al. (2016) has reduced the eventual ethanol yields based on total sugar contents of raw OPEFB to the range between 8 and 20%.

Selection of proper pre-treatment type and conditions is a factor towards a successful xylose utilization and ultimately efficient lignocellulosic ethanol production. This is because, findings by each of Cardona and Sánchez (2007); Schmer et al. (2008) and Conde-Mejía et al. (2012) have shown that each type of pre-treatment under selected treatment conditions displayed different destructive potential on their respective target substrates and has been shown to require some energy input that may not be compensated by the eventual ethanol yield at the end of fermentation. For instance, even though an alkaline pre-treatment has been shown to preserve the hemicelluloses fraction of lignocellulosic fibre, a severe pre-treatment condition such as, high chemical concentration or temperature will definitely negatively impact on the xylose fraction of the biomass during treatment as was noticed in earlier work (Piarpuzán et al., 2011; Choi

et al., 2013; Dahnum et al., 2015). Based on this premise, effects of three types of chemical pre-treatment methods on the overall OPEFB sugar components will be investigated in this study through a less energy-requiring approach of substrate treatment.

Similarly, the rate and type of enzymatic hydrolysis have been identified as a major rate-limiting step in bio-ethanol production from lignocellulosic substrates. Improving the rate of hydrolysis by enzymatic enhancement through optimized co-enzyme hydrolysis using both cellulase and hemicellulase enzymes will not only enhance the hydrolytic product yield but will also, according to Olofsson et al. (2008) and Kuhad et al. (2011) greatly reduce the processing cost of bioethanol production through any of the known fermentation methods. Therefore, the development of low-cost and effective hemicellulase enzymes for hemicellulose hydrolysis is considered panacea to effective xylose fermentation. This is due to its comparative advantage to enhance the release of xylose sugar during co-hydrolysis with cellulase, over the conventional acid hydrolysis method. Acid hydrolysis is characterized by the generation of large fermentation inhibitors and loss of a chunk of xylose sugar due to degeneration (Chandel et al., 2011; Howard et al., 2003). More importantly, on-site enzyme production using same feedstock respectively for both enzyme and ethanol production has been proposed (Zhu et al., 2014) as an efficient means to reduce the challenges posed by feedstock availability in addition to reducing overall processing cost and logistics for efficient bioethanol production. In this study, hemicellulase enzymes will be produced onsite by solid state fermentation of OPEFB using some selected soil fungal species isolated from oil palm waste dumping sites. Moreover, the production and characterization of respective hemicellulase will be determined based on the assay of xylanase enzyme which is the most important and most abundant component enzyme of hemicellulase complex. Finally, respective enzymes will be characterized and compared on the basis of their respective abilities to enhance the activity of commercial cellulase towards complete hydrolysis of treated OPEFB biomass

through a synergistic effect of co-enzyme hydrolysis (i.e. co-hydrolysis system) for onward bioethanol production.

As earlier identified, the type of fermentation employed determines the efficiency or otherwise of lignocellulosic ethanol system. Most previous studies on OPEFB did not utilize its xylose fraction during fermentation primarily based on the type of pre-treatment, the fermenting microorganism and the type of fermentation. Besides, few reports that considered xylose utilization were characterized by poor theoretical ethanol yield. Nonetheless, successful fermentation of all component sugars is a precursor for efficient lignocellulosic ethanol production. By the provision of the findings in various earlier reports (Table 2.6), this review showed that there has been no published work on co-fermentation of both the xylose and glucose sugars from OPEFB towards improved bioethanol production. Therefore, this study is aimed at improving ethanol yield from OPEFB by ensuring complete utilization of all the structural sugar components using a non-conventional fermentation approach as will be further elucidated. This study will compare ethanol yields of two fermentation methods, single fermentation and co-fermentation by a co-culture system of glucose and xylose fermenters. Under each method, yields from the use of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) will be respectively compared.

## 1.3 Objectives

As a function of the research scope, objectives of this study are hereunder highlighted as follows:

i. To investigate the effects of three pre-treatment methods (alkaline, acidic and auto-hydrolysis) on OPEFB biomass for the recovery of fermentable sugars during substrate hydrolysis

ii. To evaluate the effects and suitability of two chemo-mechanical pre-treatment approaches on the pre-treatment energy requirement and saccharification efficiency on OPEFB biomass.

iii. To isolate and screen potential xylanase-producing fungal strains for xylanase production, and subsequently investigate the utilization of alkali, acid and auto-hydrolysis pretreated OPEFB biomass as a substrate for xylanase production by the selected potential isolates.

iv. To optimize (using RSM) the cultural conditions of potential soil fungal isolate(s) for crude xylanase production , and assess the xylanase efficiencies in enhancing the co-hydrolysis of treated OPEFB biomass through a synergistic effect with commercial cellulase towards high recovery of fermentable sugars.

v. To assess and compare the efficiency of two fermentation techniques, the conventional single glucose fermentation, and selected co-sugar fermentation approaches, on overall ethanol yield *via* the evaluation of respective carbon-carbon (C-C) balance in relation to the initially untreated OPEFB substrate.

## 1.4 Main Research Scope and Idea

The overall idea of this research is to enhance efficient ethanol yield from OPEFB biomass by maximizing the utilization of both the cellulose and hemicellulose components of the raw material using improved methods. Hence, this study was focused on improving all the integrative processes from pre-treatment through enzymatic hydrolysis to fermentation, that lead to successful utilization of both the glucose and xylose fractions of OPEFB for bioethanol production with greater attention on enzyme production and fermentation stages.

Essentially, the effectiveness of pre-treatment and or substrate hydrolysis were expressed in this study as percentage sugar recovery based on initial total sugar or holocellulose

content of original untreated biomass. This method is projected as a better approach than the conventional yield expression *via* theoretical sugar concentration of the treated biomass which undoubtedly is a biased evaluation approach for the assessment treatment and hydrolytic efficiencies. On the other hand, expression of yield by the initial total sugar contents will take the respective percentage sugar loss or un-hydrolysed sugar during pre-treatment into consideration. Additionally, the efficiency of the whole production process will be assessed by evaluating the ethanol yield based on carbon-carbon (C-C) balance or theoretical ethanol concentration of untreated OPEFB which is rarely found in the earlier literature that reported ethanol production from OPEFB biomass.

# CHAPTER 2 LITERATURE REVIEW

## 2.1 Introduction

The continuous depletion and the attendant environmental problems due to the utilization of fossil fuel have necessitated the shift of global attention towards renewable energy sources. Based on its geometric rate, the world population is estimated to increase from the current 7.3 billion to about 9 billion by 2030 (USCB, 2016). On the other hand, oil production worldwide is expected to decline from its current 25 billion barrels to about 5 billion barrels by 2050 (Dinita et al., 2011), a situation that may consequently lead to energy crises. Hence the future energy demands and security will no doubt be a key determinant factor in regional and geopolitical economics. Given this reality, nations, organisations, industrialists and policy makers all over the world are now investing in alternative, renewable and sustainable sources of energy.

Bioethanol is one of the major renewable sources employed as a sustainable replacement, especially in the automobile sector. This is because unlike gasoline, ethanol is an oxygenated fuel (around 35% oxygen) with high octane value like that of petroleum fuels. Besides, ethanol runs combustion engines at higher compression ratios to provide superior performance (Wheals et al., 1999). Currently, bioethanol is produced in commercial quantities and is used in countries such as India, Brazil, and the United States as the sole source of ignition or as a blend (E85 or E90 meaning 15% or 10% ethanol added to gasoline) to power car engines (Dinita et al., 2011). In those countries, bioethanol is currently produced from first generation sources (starch and sugar-based feedstocks such as corn and sugarcane) raising, therefore, the question of long-term sustainability of these sources (USDOE, 2016). Continuous use of first generation sources will definitely lead to problems such as food insecurity and economic nuisance like inflation due to natural competition between the two end products: food and biofuel. On

the other hand, bioethanol from lignocellulosic sources addresses the challenges of long term sustainability which characterized the first generation sources. This is because lignocellulosic substrates are non-food materials which are abundant, renewable, largely available all year round and are generated in million tonnes around the world mainly as agricultural wastes (Dinita et al., 2011). Based on these characteristics, lignocellulosic ethanol could be adjudged as the most promising alternative energy sources which could readily address the energy security section of the sustainable development goals (Dahnum et al., 2015).

Due to the nature, structure and arrangement of its various polymeric components, successful ethanol production from lignocellulosic biomass is shown to require several integrated processes from initial stage of biomass preparation through hydrolysis to the final fermentation stage. However, the associated cost implication of these processes and other logistics currently make lignocellulosic bio-ethanol a commercially non-*via*ble alternative; notwithstanding its remarkable potentials earlier highlighted. This is because, each of the processing steps impacts directly on the total energy requirement and processing costs of the whole production process (Kuhad et al., 2011). With the view to address this concern, studies on lignocellulosic bio-ethanol are hence channeled towards improving all the technological processes to attain high efficiency, cost-effectiveness, and sustainability (Raman and Gnansounou, 2014; Mattam et al., 2016; Rajendran et al., 2016).

Based on the foregoing, this review is aimed to address each of the leading integrative process technologies, in the light of its ***concept, prospect*** **and** ***limitations***, and ***current strategic improvements*** aimed towards achieving efficient and sustainable lignocellulosic ethanol production. In this review all the factors which are often separately addressed and are hitherto scattered all over literature will be galvanised together in order to avail intending researchers the problem of perusing through several articles before

having a grasp of the overall idea of lignocellulosic ethanol, in a bid to develop research questions.

## 2.2 Factors Affecting The Efficiency of Lignocellulosic Ethanol Production

In the bioethanol industry, the two main sugar monomers, glucose, and xylose are the important substrates for efficient ethanol production. However, in lignocellulosic biomass, these sugars are locked up in the complex structure of the lignocellulosic biomass which, due to its recalcitrance, prevents biomass saccharification and subsequent utilization of the sugar monomers for ethanol production. Consequently, a conventional lignocellulosic bioethanol production involves a four-step approach: biomass pre-treatment to disrupt the lignin-hemicellulose barrier complex by removing one of the polymers; hydrolysis of the complex polysaccharides to simple fermentable sugars *via* chemical or enzymatic approach; fermentation of glucose and/or xylose monomers to ethanol by competent microorganism, and lastly, collection of ethanol by fractional distillation. A schematic of the process of ethanol production from lignocellulosic materials is represented in Fig. 2.1.

However, due to the cost and logistics of these integrative but necessary processes, lignocellulosic ethanol is reported based on techno-economic evaluations (Do et al., 2015; Rajendran et al., 2016) as cost intensive. The United States' Department of Energy has also reported that ethanol production from agricultural feedstock is although desirable, yet it is currently a cost-intensive alternative (McAloon et al., 2000; USDOE, 2016).

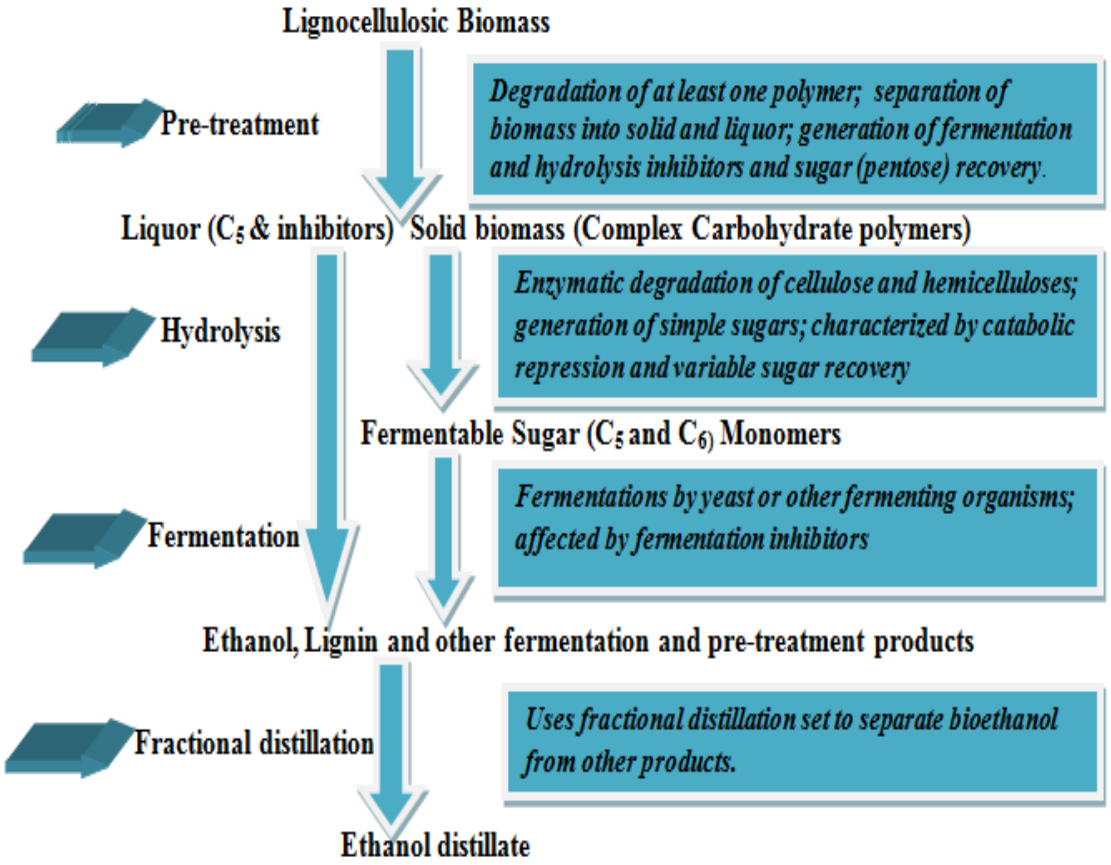


Figure 2.1: Schematic view of lignocellulosic ethanol production

Attaining cost efficiency is the hallmark of recent researches into lignocellulosic ethanol. In spite of its potential as a sustainable alternative energy source, lignocellulosic ethanol is yet to be deployed on full-scale commercialization. This is due to the cost of process logistics and technological problems associated with all the integrative processes - from feedstock supply to sugar fermentation- leading to ethanol production from lignocellulosic biomass (Kuhad et al., 2011; Paulova et al., 2015). A study of existing literature has shown that the present lag of lignocellulosic ethanol processes is hinged on so many factors which may be directly or indirectly related to the lignocellulosic bioconversion processes. These factors could be broadly categorised as either *process-independent* or *process-dependent* factors and each will be discussed in the light of their respective concepts and how they separately affect the efficiency of lignocellulosic ethanol production.

### 2.2.1 Process-independent or external factors

Process-independent factors affect the properties and possible availability of lignocellulosic biomass for bioconversion to ethanol. These factors may be directly related to the biomass (intrinsic) or from some external sources or condition (extrinsic).

a. ***Intrinsic factors***

i. *Types of lignocellulosic biomass used for bioethanol*

Lignocellulosic substrates are important due primarily to their structural sugar components (the cellulose and hemicellulose). Biomass type and structural sugar components are regarded as intrinsic process-independent factors affecting lignocellulosic ethanol production. It is predicted that biomass with a higher ratio of cellulose gives higher conversion and efficient glucose recovery than those of lower cellulose contents (Goh et *al.,* 2010). Hence, the percentage composition of these sugars relative to lignin component in a particular biomass, no doubt, determines its eventual applicability or otherwise for bioethanol production. Biomass with holocellulose content between 70 and 80% are considered potential substrates; while a high lignin-containing biomass (30 – 50%) are often considered not too suitable for lignocellulosic ethanol production. This is because the presence of lignin, which is naturally recalcitrant to enzymatic attack, determines the ease of access to the structural sugars of biomass (Umikalsom et al., 1997a). Meanwhile, the ease of enzyme access to sugars (in other words, percentage lignin component) depends greatly on the types, properties and source of biomass. Lignocellulosic substrates are generally divided into two broad categories [wood and non-wood] each of which is further divided into subdivisions based on the type and properties of respective biomass (Tye et al., 2016):

Wooden lignocellulosic biomass, classified as hard or softwood are potential substrates for lignocellulosic ethanol production. The potential of these substrates is due

to their cellulosic composition between 50 and 60% (Sun and Cheng, 2002; Dinita et al., 2011) relative to lignin contents. They are however differentiated based on the components of their respective hemicellulose and lignin. Hardwood contains xylan as the major hemicellulose backbone while softwood consists of mannan (Kuhad et al., 2011). Similarly, the lignin composition of softwood (25-35%) biomass is more than hardwood (18-25) (Sun and Cheng, 2002) making the former less desirable for ethanol production due to its higher recalcitrance.

Conversely, the use of wood sources for bioethanol generation is affected by the high energy requirement for size reduction prior to treatment. Sun and Cheng (2002) have reported that 130 kWh/ton is needed to reduce a tonne of selected hardwood to 1.6mm compared to 7.5 kWh/ton required to reduce corn straw to the same size. Similarly, inefficient hydrolysis due to the high viscosity of wooden biomass (Gaona et al., 2015) and a shortage of feedstock due to deforestation is a common impediment against wood ethanol. More importantly, the accumulation of greenhouse gas and the corresponding global warming due to wood logging and deforestation is now the most recent principal reason why the use of wood for ethanol generation is discouraged (Hamelinck et al., 2005).

In contrast, non-wood biomass generally consists of lower cellulose compared to wood sources. It addresses the sustainability questions of wooden feedstock and is now the subject of intense research in the lignocellulosic ethanol industry. Non-wood sources have widespread abundance and the cost of their procurement is relatively cheap (Dinita et al., 2011). They are often characterized by low lignin contents and are structurally loose compared to wood biomass; hence processing cost and energy is comparatively low (Sun and Cheng, 2002; Tye et al., 2016).There are several types of non-wood biomass identified based on their respective sources as native plants e.g. Switchgrass, non-wood plant fibres e.g. bast fibers, agricultural residues e.g. corn stover, oil palm field and

processing wastes e.g. (oil palm frond and oil palm fresh fruit bunches), municipal wastes e.g. newspaper print and wastes of paper and pulp mill.

Of all these feedstocks, agricultural residues are widely used. This is due to their non-competitiveness with food, large cultivation and abundance. Various lignocellulosic biomass such as various cereal straws (Gu et al., 2013; Kumar et al., 2013; Yang et al., 2013), sugarcane bagasse (Zhang et al., 2013), oil palm biomass (Hong et al., 2013) and others, which are abundantly available have been used for bioethanol production. In Malaysia, wastes from oil palm tree are largely available and are far richer sources of both cellulose and hemicelluloses. Goh et al. (2010) reported that the amount of cellulose-hemicellulose content of oil palm tree is far more than many other agricultural wastes, several forest residues, and municipal organic wastes.

ii. *Other biomass properties*

Meanwhile, other biomass properties such as porosity and cell wall thickness have been described and reported to affect the successful use of lignocellulosic biomass for bioethanol production. Chandra et al. (2007) reported that substrate size in relation to enzymes' could cause pore-trapping of cellulase if the biomass internal area of lignocellulosic biomass is much larger than its external area. Also, the plant stems of woody tissue, grass cuticle and tree bark due to their characteristic waxy barrier, impedes enzymes accessibility (Alvira et al., 2010).

b. ***Extrinsic factors***

Extrinsic factors indirectly affect lignocellulosic ethanol production based on their respective effects mainly on biomass availability. Seasonal availability of source crops; low crop yield due to unpredictability of weather; biomass degeneration due to poor storage and potential competing uses of the generated lignocellulosic wastes have all been identified as important factors which affect biomass availability for onward bioconversion

to ethanol (Banerjee et al., 2010) . Of all these factors, potential competing use of biomass for other equally important economic products is the major factor affecting the future sustainability of lignocellulosic ethanol. This is more so that, compared with ethanol, some of the other end-uses require less laborious and environmentally friendly techniques to attain final product (Banerjee et al., 2010). Lignocellulosic wastes are now used for soil conditioning (Tye et al., 2016); domestic fuel and cattle fodder (Banerjee et al., 2010); pulp and paper making (Leh et al., 2008); binderless board (Hashim et al., 2016) and others. Thus, the future sustainability of lignocellulosic ethanol depends on how beneficial and how acceptable these alternative products are to the larger society compared with ethanol.

### 2.2.2 Process-dependent factors

Each stage of lignocellulosic bioprocessing is characterized by certain process conditions which usually are inimical to the success of the ethanol production process. Process-dependent factors are various process conditions which have direct impacts on lignocellulosic ethanol production. These conditions circumvent the various process logistics and process technologies leading to successful lignocellulosic ethanol. Eventhough the convenional way to evaluate the efficiency of lignocellulosic ethanol production is by the net cost of the whole production process and the eventual carbon-carbon balance after fermentation (Millati et al., 2011), these process conditions which are domiciled at the successive stages of lignocellulosic ethanol production process are essentially foremost to determine the success or otherwise of the entire production process.

a. ***Process logistics***

Process logistics refer to factors which although directly affect production but are superfluous to the production process in terms of design or technology. These factors are mostly related to the cost implication of lignocellulosic ethanol at all stages of the

production process. All the processes leading to successful lignocellulosic ethanol production attract some specific cost which, most often, are independent of previous or subsequent stages (Do et al., 2015). The cost of the procurement of commercial enzymes; cost and energy requirements of pre-treatment; personnel costs; nutrient supplementation costs; harvesting, pre-processing, storage and transportation costs for biomass delivery to the refinery and other logistics have all been implicated (Allen et al., 1998; Sokhansanj and Hess, 2009; Conde-Mejía et al., 2012) to summarily impact on lignocellulosic ethanol as, presently, an economically non-*via*ble alternative. Not-too-long ago, the United State's Department of Energy (USDOE) had reported the cost implication of ethanol production from corn starch and lignocellulosic substrates (McAloon et al., 2000). By its report, one gallon of lignocellulosic ethanol amounted to $1.5 dollars (or $63 per barrel) far higher than the present cost per barrel of crude petroleum ($28-$30). Due to this cost difference, bio-ethanol is currently being sold in the U.S. at a retail price higher ($3.07/gallon) than gasoline ($1.914/gallon) (USDOE, 2016; USEIA, 2016). In addition, other petroleum products like diesel (12 gallons/barrel crude) clearly project gasoline as a cheaper energy source but bio-ethanol as a cost-intensive alternative.

b. ***Process technologies***

Process technologies refer to integrative factors with direct impact on the lignocellulosic ethanol system. There factors are numerous and the magnitude of their respective effects are reflected at each step of lignocellulosic ethanol production. A comprehensive critique of these factors is presented under section 2.3.

## 2.3 Process Technologies Affecting Lignocellulosic Ethanol Production

### 2.3.1 Substrate pre-treatment

The first step of lignocellulosic bio-processing into ethanol is the disruption of the complex arrangement of its major structural polymers; cellulose; hemicellulose and lignin. This process is referred to as substrate pre-treatment. Biomass pre-treatment is

defined, according to Garrote et al. (1999), as a kind of destructive strategies which involves the degradation of at least one of the complex lignocellulosic polymers that prevents enzyme hydrolysis of cellulose or eventual fermentation of its sugar monomers into ethanol. Generally, lignocellulosic polymers are recalcitrant due to the complex arrangement of its respective polymers, thus preventing enzymatic hydrolysis of cellulose or eventual fermentation of its sugar monomers into ethanol. Therefore, the main aim of pre-treatment is to enhance enzymatic accessibility and digestibility of lignocellulosic biomass through degradation of physical and chemical barriers; disruption of cellulose crystallinity and increasing the total surface area for subsequent hydrolysis (Alvira et al., 2010). Biomass pre-treatment is regarded as the major economic bottleneck against efficient lignocellulosic ethanol process. This is due to the characteristics loss of structural sugars, generation of inhibitors and high cost and energy demand usually of the downstream processes like waste water treatment, pentose recovery, and size reduction. Hence, future acceptability of any form of pre-treatment will depend on its affordability at a reduced cost, to enhance biomass hydrolysability with minimum enzyme dose with shorter time for bioconversion; non-generation of fermentation inhibitors and its preservation of biomass with minimum loss during pre-treatment (Sun and Cheng, 2002; Yang and Wyman, 2008; Alvira et al., 2010).

a. ***Types of pre-treatment***

Several methods of pre-treatment have been reported (Alvira et al., 2010; Kuhad et al., 2011). Based on the respective treatment mechanisms and energy consumption, pre-treatment can be broadly categorised into three; physical e.g. milling treatment (Yano et al., 2009); chemical e.g. auto-hydrolysis (Garrote et al., 1999), dilute acid and alkali pre-treatment (Rahman et al., 2007; Kuhad et al., 2011; Tye et al., 2012) and biological e.g. the use of white-rot fungi for delignification (Conde-Mejía et al., 2012; Perez et al., 2002). Two or more of these pre-treatment types can also be used together as a single