HYDROLYSIS OF PALM OIL MILL EFFLUENT USING ENZYMES FROM SPENT MUSHROOM SUBSTRATE OF *Pleurotus pulmonarius* (FR.) QUEL

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KUALA LUMPUR

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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HYDROLYSIS OF PALM OIL MILL EFFLUENT USING ENZYMES FROM SPENT MUSHROOM SUBSTRATE OF *Pleurotus pulmonarius* (FR.) QUEL

ABSTRACT

Mushroom cultivation and palm oil industry in Malaysia have contributed to the large volumes of lignocellulosic residues. Lignocellulolytic enzymes profiles (lignin peroxidase, laccase, xylanase, endoglucanase, and β-glucosidase) of *Pleurotus pulmonarius* (parents strains UMP001 and UMP 002, and the hybrid UMH004) during growth in sawdust substrate bags were investigated. The enzymes extracted from the spent mushroom substrate (SMS) of the strain with the highest enzymes productivity was used to hydrolyse palm oil mill effluent (POME). Optimization of parameters for POME hydrolysis to release reducing sugar was conducted using central composite design (CCD) of response surface methodology (RSM). The potential of hydrolysed POME to produce biohydrogen using acclimatized POME sludge as the inoculum was determined. The productivities of the concentrated enzymes obtained from the eleventh-week old SMS of UMH004 for lignin peroxidase, laccase, xylanase, endoglucanase and β-glucosidase were 214.1 U g^{-1}, 4.1 U g^{-1}, 2.3 U g^{-1}, 14.6 U g^{-1}, and 915.4 U g^{-1}, respectively. Enzymatic hydrolysis of POME using concentrated enzymes yielded four times higher reducing sugar (4.7 g L^{-1}) than hydrolysis using the crude enzymes, (1.1 g L^{-1}). Meanwhile, under the optimized condition using RSM (12 hours, 10% enzyme loading (v/v) at pH 5.4), 3.82 g L^{-1} of reducing sugar yield was achieved, which was consistent with the predicted reducing sugar yield, (3.76 g L^{-1}). Incubation time and enzyme loading factors had significant effects ($p < 0.05$) on the POME hydrolysis. The F-value of 38.41 from analysis of variance (ANOVA) indicated that the model could predict the optimum experimental parameters. Batch fermentation of hydrolysed POME yielded 23 times higher volume of biohydrogen when compared to non-hydrolysed raw POME.

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Keywords: Spent mushroom substrate, palm oil mill effluent, enzymatic hydrolysis, response surface methodology, biohydrogen

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HIDROLISIS EFLUEN KILANG KELAPA SAWIT MENGGUNAKAN ENZIM DARIPADA SISA SUBSTRAT CENDAWAN *Pleurotus pulmonarius* (FR.) QUEL

ABSTRAK

Penanaman cendawan dan industri minyak sawit di Malaysia telah menyumbang kepada pembuangan sisa lignoselulosa yang banyak. Profil enzim-enzim lignoselulolitik (lignin peroksidase, lakase, xilanase, endoglukanase, dan β-glukosidase) *Pleurotus pulmonarius* (Strain induk UMP001 dan UMP002, dan hybrid UMH004) sewaktu pertumbuhannya dalam bongkah habuk kayu telah dikaji. Enzim yang diekstrak daripada strain sisa substrat cendawan (SMS) dengan produktiviti enzim terbaik telah dipilih untuk hidrolisis efluen kilang minyak kelapa sawit (POME). Pengoptimuman parameter untuk hidrolisis POME menghasilkan gula penurun telah dijalankan dengan menggunakan reka bentuk komposit pusat (CCD) dalam kaedah permukaan sambutan (RSM). Potensi POME dihidrolisiskan untuk menghasilkan biohidrogen menggunakan enapcemar POME yang telah disesuaikan sebagai inokulum telah dikaji. Produktiviti enzim pekat yang diperolehi daripada SMS hibrid UMH004 pada minggu kesebelas ialah 214.1 U g^{-1} lignin peroksidase, 4.1 U g^{-1} lakase, 2.3 U g^{-1} xilanase, 14.6 U g^{-1} endoglukanase dan 915.4 U g^{-1} β-glukosidase. Proses hidrolisis berenzim POME menggunakan enzim pekat telah menghasilkan empat kali ganda lebih tinggi gula penurun (4.7 g L^{-1}) daripada hidrolisis yang menggunakan enzim mentah, (1.1 g L^{-1}). Manakala, pada keadaan optimum (12 jam, kandungan enzim 10% (v/v), pada pH 5.4), 3.82 g L^{-1} gula penurun telah terhasil, iaitu konsisten dengan penghasilan gula yang diramalkan, (3.76 g L^{-1}). Faktor masa inkubasi dan kandungan enzim memberikan keputusan yang signifikan ($p < 0.05$) ke atas hidrolisis POME. Nilai-F 38.41 daripada analisis varians (ANOVA) menunjukkan bahawa model tersebut boleh meramalkan parameter eksperimen yang optimum. Penapaian kumpulan POME yang dihidrolisiskan menghasilkan 23 kali ganda lebih banyak jumlah biohidrogen jika dibandingkan dengan POME mentah yang tidak dihidrolisiskan.

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Kata kunci: Sisa substrat cendawan, efluen kilang minyak kelapa sawit, hidrolisis berenzim, kaedah permukaan sambutan, biohidrogen

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	:	Analysis of variance
BOD	:	Biological Oxygen Demand
BSA	:	Bovine serum albumin
CCD	:	Central Composite Design
CMCase	:	Carboxymethylcellulase
CO_2	:	Carbon dioxide
COD	:	Chemical Oxygen Demand
Df	:	Degrees of freedom
DNS	:	Dinitrosalicylic acid
DOE	:	Department of Environment
g	:	gram
h	:	Hour
H_2	:	Hydrogen gas
H_2O_2	:	Hydrogen peroxide
L	:	Liter
LiP	:	Lignin peroxidase
M	:	Molar
mg	:	Milligram
Min	:	Minutes
ml	:	Milliliter
mM	:	Millimolar
MW	:	Molecular weight
Na_2CO_3	:	Sodium carbonate
NaOH	:	Sodium hydroxide

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nm	:	Nanometer
Rpm	:	Revolutions per minute
S.D.	:	Standard deviation
SMS	:	Spent mushroom substrate
U	:	Unit
U/g	:	Unit per gram
U/ml	:	Unit per milliliter
v	:	Volume
VA	:	Veratryl alcohol
w	:	Weight
µg	:	Microgram
µmol	:	Micromole

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INTRODUCTION

1.1 Background of the Study

Hydrogen production from biomass has develop into an appealing substitute to fossil fuels since it is known as the cleanest renewable energy source with zero carbon emission (Mohammadi et al., 2011). Furthermore, energy content per unit weight of hydrogen is the highest (142 kJ/g) of all known fuel (Sinha & Pandey, 2011).

Research has been conducted in biohydrogen production from lignocellulosic waste through the application of different microorganisms (Mahmod et al., 2017). In addition, production of hydrogen from wastewaters is economically and environmentally feasible since it allows waste mitigation and energy recoveries (Khanal et al., 2004).

As one of the world's leading producers of palm oil, a total of 426 mills and 51 refineries were reported in Malaysia (Chin et al., 2013; Razak et al., 2013). It was reported in 2014, about 67.28 million tonnes of palm oil mill effluent (POME) was released from 100.42 million tonnes of fresh fruit bunches (Nurul-Adela et al., 2016). Meanwhile, the oil palm plantation space has hit 5.74 million hectares in 2016 (MPOB, 2017).

POME has been classified as one of the major causes of water pollution due to the abundant discharge of wastewater with high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) concentrations (Ismail et al., 2010; Yi et al., 2010). The characteristics of POME generally varied among the days, quality of fresh fruit bunches (FFB), seasonal change and the performance of the extraction machines used during operation (Silvamany et al., 2015).

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In order to meet both domestic and global demand, the production of POME is accelerating in tones. Thus, conversion of POME into value-added products has been identified as an alternative for waste management. POME was selected as one of the lignocellulosic biomass substrates in the biofuel industry for suitable conversion into fermentable sugars. The sugars can be utilized for a higher value feedstock to produce biogas and biofuel. By converting POME into useful products, it will positively draw attention for a low-cost waste treatment and management strategy leading to sustainability of the oil palm industry (Silvamany et al., 2015).

Different pretreatments are required to unwind the lignocelluloses structure of the industrial wastes. Pretreatments with acid, alkali, microbes, and steam explosion have been conducted for delignification of the cellulose materials (Asada et al., 2011). However, the chemicals hydrolysis process has disadvantages including product separation, reactor corrosion, poor catalyst recyclability and the need for treatment of the wastes (Salam et al., 2013).

In addition, enzymes are needed to break down the holocellulose into sugars (Zahari et al., 2012). The three classes of enzymes involved in the conversion of lignin and cellulosic agro-wastes are cellulases, hemicellulases, and ligninases (Singh et al., 2003). Before or after conventional pretreatments, the enzymes would be used to reduce and replace thermochemical procedures, thus facilitate the conversion of lignocellulosic materials (Asgher et al., 2014). Furthermore, the enzymatic hydrolysis is nature-friendly and can be performed under low energy demand as compared to chemical hydrolysis (Zainan et al., 2011).

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Conventional optimization method is laborious and rigorous to handle (Rasdi et al., 2009). Thus, response surface methodology (RSM) has become an alternative since the analysis provides visual responses for the interaction among the experimental factors (Yunus et al., 2014). Furthermore, a matrix of central composite design (CCD) in RSM requires only a few numbers of experiments while producing systematic and comparable results (Tarley et al., 2009).

With arising biotechnology means, there is high opportunity to promote current enzyme source that present more advantageous enzyme characteristics just as preferable thermal stability, improved resistance to inhibitors, higher specific activities, and modified combination of various enzymes activity that escalate sugar yields at low-priced (Yang et al., 2011).

The challenge facing hydrolytic degradation of hemicellulose is the demand for the bulk of enzymes to complete the hydrolysis, urging to expensive enzyme costs (Mtui, 2009). Previous studies of POME hydrolysis have focused on using high-cost commercial enzymes to convert the organic substances in POME into monomeric sugars including Khaw and Ariff (2009); Khaleb et al. (2012); Seong et al. (2008); and Silvamany et al. (2015). Otherwise, studies on the potential enzymes from SMS for the enzymatic hydrolysis of POME has not been explored.

A low-cost and readily available source of enzymes can be obtained from the spent remnant of commercially grown edible mushroom or known as spent mushroom substrate (SMS). The mushroom industry has struggled with challenges in managing and discarding the SMS. Singh et al. (2010) reported that around 800 g of SMS is composed of every 200 g of mushroom produced. The inefficient practices of disposal and

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management of the wastes has constrained the need to study the potentials of utilizing the biomass for production of the value-added product. The SMS contains organic matter, mineral nutrients and enzymes that may be favorable for stimulating microbial degradation of hydrocarbons (Khammuang & Sarnthima, 2007).

Currently, the roles played by fungi are indispensable to human civilization. Productions of enzymes for the various industries are currently the aspect in fungal biotechnology which demonstrating high economic value. Edible mushrooms belonging to the Basidiomycetes has contributed to the annual production of about 3.2 million tonnes (Lankinen, 2004).

Genus *Pleurotus* spp. (oyster mushrooms) possessing a third place in the world production of edible mushrooms (Abdollahzadeh et al., 2007). *Pleurotus pulmonarius* (Fries) Quélet, commonly known as the grey oyster mushroom are commercially cultivated for human nutrition and largely consumed in Malaysia (Avin, 2014). Due to the reason, the SMS of *P. pulmonarius* was selected for this study.

The intention of the current study illustrates some potential strategies to lessen the environmental problem generated by the oil palm and mushroom industry. The use of the economical lignocellulosic substrate for production of enzymes by mushroom lessens the dependency on the usage of expensive commercial enzymes.

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1.2 Objectives of the Study

The objectives of this study were to:

a) profile ligninase (lignin peroxidase and laccase), hemicellulase (xylanase), and cellulase (endoglucanase and β-glucosidase), produced by SMS of *Pleurotus pulmonarius* during spawn run for twelve weeks.

b) hydrolyse POME using enzyme cocktails produced by the SMS of *P. pulmonarius*.

c) optimize conditions for enzymatic hydrolysis of POME to enhance reducing sugar yield by employing CCD of RSM.

d) compare biohydrogen production from raw and hydrolysed POME.

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LITERATURE REVIEW

2.1 Lignocellulosic Biomass

2.1.1 Composition of Lignocellulosic Biomass

Lignocellulose is a dominant complicated structural component of plants, comprises of cellulose, hemicellulose, lignin, ash and other compounds. Large amount of the cellulose, hemicellulose and lignin produced are being considered waste after were used as by-products in agriculture or forestry (Behera et al., 2014).

Cellulose is the major component of plant material and becomes the most bountiful polysaccharide on Earth (Bayer et al., 1998). It consists of unbranched, linear glucose units linked by β-1,4 glycosidic bonds and can be hydrolysed into glucose by the action of enzymes.

Hemicellulose consists of highly branched pentose and hexose in a random and amorphous structure. Hemicellulose consists of different pentose sugar such as xylan, glucuroxylan, arabinoxylan, glucomannan, and xyloglucan as compared to cellulose which contains only glucose (Dayang, 2013). Both cellulose and hemicellulose are sugar polymers and becomes a potential candidate for fermentable sugars production. Then, the sugar released can be modified into various value-added products (Ang et al., 2015; Sreenath & Jeffries, 2000).

Lignin refers to a complex three-dimensional polymer with amorphous aromatic structure linked by p-hydroxyphenylpropanaid units (Dayang, 2013). The lignin needs to be modified by chemical or biological method before hydrolysis process can occur as the cellulose and hemicellulose are hardly combined with lignin. The components and percentages of the compounds differ between plants (Prasad et al., 2007).

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2.1.2 Accessibility of Lignocellulosic Biomass

The lignocellulosic biomass can be obtained from different sources such as crop waste, municipal solid waste, forest remnants, and industrial sewage. Many studies have been reported on utilization of lignocellulosic biomass into value-added products such as rice straw (Gottumukkala et al., 2015), cassava peel (Bayitse et al., 2015), dairy manure (Shi et al., 2014) and spent mushroom compost (Fang et al., 2016).

Malaysia as one of the most substantial agricultural regions in the world, produced bounteous of lignocellulosic wastes. Researchers all over the world are progressively reported studies on the utilization of the lignocellulosic wastes because it is widespread in nature and does not compete with food crops. The wastes have also gained great attention as they are renewable, low cost, and environmentally friendly (Alvira et al., 2010).

2.2 Extracellular Enzymes in Lignocellulosic Biomass

2.2.1 Hydrolytic Enzymes

Hydrolytic enzymes present significant parts by producing easily absorbable carbon sources to fungal metabolism and growth (Wan & Li, 2012). Research has been done actively for the past three decades to enhance the economical production of the enzymes.

According to Yang et al. (2011), hydrolytic enzymes or cellulase can be defined as "mixture of enzymes that catalyse the reaction of water with cellulose to release shorter chains and ultimately soluble glucose sugar". Three main groups of cellulase are involved in the hydrolysis process, known as endoglucanase, cellobiohydrolase, and β-glucosidase. These enzymes perform synergistically to establish the efficiency of cellulose hydrolysis (Khaw & Ariff, 2009; Mun et al., 2008).

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Endoglucanases that locate on the amorphous site of cellulose chain acts by randomly break intramolecular β-1,4-glycosidic bonds. The reaction of the endoglucanases hydrolyses the cellulose chain into smaller units and increase the availability of the cellulose chain ends for catalysis of cellobiohydrolase.

Then, cellobiohydrolase reacted by cleaving at the ends of the chains to release cellobiose or also known as glucose (Gan et al., 2003). According to Nurul-Adela et al. (2016), endoglucanase can hydrolyse POME due to the ability to break and digest the POME carbohydrates.

Meanwhile, β-glucosidase is an enzyme which responsible to cleaves cellobiose to two units of glucose. The enzyme is important in cellulose hydrolysis because it removes cellobiose which inhibits the reaction of cellobiohydrolase and endoglucanases (Bayitse et al., 2015).

2.2.2 Ligninolytic enzymes

Ligninolytic enzymes comprise of three lignin-modifying enzymes which are lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase that typically found in white-rot fungi. These enzymes perform important roles in delignification of lignocellulosic biomass (Singh et al., 2010). The delignification process occurred when the enzymes degrade lignin and reduce the lignin content in the biomass.

Lignin peroxidase (LiP) was first identified from *Phanerochaete chrysosporium* during the research on the process of lignin biodegradation (Tien & Kirk, 1984). The enzyme can be used widely due to its high potential to degrade lignocellulose and other related compounds (Plácido & Capareda, 2015). Lignin peroxidase system of this fungi

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species has been recorded in several studies (Asgher et al., 2014, Harada et al., 2016, Janusz et al., 2013, and Oliveira et al., 2018).

Fungal laccases, also known as benzenediol: oxygen oxidoreductase, are blue copper-containing enzymes. It catalyses the reduction of oxygen to water followed by the oxidation of numerous substrate (Baldrian, 2006; Madhavi & Lele, 2009). Fungal laccases have been reported have high redox potential and involved in the deterioration of lignin and toxic phenol (Khammuang & Sarnthima, 2007).

The biotechnology utilization of laccase has been broadening by the establishment of laccase-mediator systems (LMS), which are capable to oxidise non-phenolic compounds (Kunamneni et al., 2007). The application of laccases has been recorded in various studies such as decolourisation of dyes (Gnanasalomi & Gnanadoss, 2013), bleaching of wood pulps (Balakshin et al., 2001), removal of various pollutants in industrial wastewater (Mayer & Staples, 2002), and food technology (Patel et al., 2016).

The potential of fungi to release high titers of ligninolytic enzymes was affected by environmental factor and fungal strains. A fungal strain that are capable to release a high concentration of suitable enzyme should be selected before optimization can be done to extract a maximum amount of the enzyme (Kunamneni et al., 2007).

2.3 Enzymatic Hydrolysis

2.3.1 Overview of Enzymatic Hydrolysis

Hydrolysis refers to a decay process when a chemical bond of the compound is desolated by a reaction with water. Cellulose hydrolysis into reducing sugar (glucose) can be defined as saccharification and takes place at economically feasible yields in the presence of a catalyst (Dayang, 2013). Fungal cellulases normally catalyse amorphous

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cellulose hydrolysis 3 to 30 times quicker than high crystalline cellulose (Arantes & Saddler, 2010).

Enzymatic hydrolysis can be described as heterogenous reactions in which insoluble cellulose is originally broken down at the solid-liquid bond over the synergistic action of endoglucanases and exoglucanases (Lynd et al., 2002). Then, liquid-phase hydrolysis of soluble intermediates, celluloligosaccharides and cellobiose take place. The reactions are catalytically cleaved to produce glucose by the action of β-glucosidase (Yang et al., 2011).

Normally, enzymatic hydrolysis is conducted at pH 4.8 and temperature between 45-50 ^{o}C. The hydrolysis has several advantages such as high glucose yield, does not produce toxic waste and corrosion problem as compared to chemical hydrolysis (Duff & Murray, 1996; Hamzah et al., 2011; Sukumaran et al., 2009).

2.3.2 Optimization of Reducing Sugar Yield

Technology for transformation of celluloses to fermentable sugars urge to be optimized and made profitable. Enzyme cocktails for a broad range of biomass feedstocks must be invented feasible at reduced costs.

Optimization of parameters is crucial because each enzyme works better when the optimal conditions support the most active shape for the enzymatic invasion towards the substrate and thus, obtain the highest yield (Sun & Cheng, 2002). The condition for optimization differs according to the source of enzymes and substrates.

Thus, optimization of multiple parameters for enzymatic hydrolysis can be achieved using RSM, which regulates optimum operation conditions by joining research designs

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with presentation by first-order or second-order polynomial equations in a consecutive experiment method (Ferreira et al., 2009).

Various factors have been identified affecting the rate of enzymatic hydrolysis such as incubation time, pH, temperature, particle size, moisture content, enzyme loading and substrate loading (Mood et al., 2013; Singh et al., 2014; Yao et al., 2007). Enzymatic hydrolysis normally requires a longer process than acid hydrolysis (Fang et al., 2010). Optimal hydrolysis time conceded enzyme to release its product sufficiently. Nevertheless, the enzyme will be denatured, and enzyme activity will be declined if incubation time reaches a certain limit.

Meanwhile, suitable pH during the hydrolysis is important for the enzyme to retain the three-dimensional configuration of the active site. Any modification of pH value might transform the ionic bonds of the enzyme, thus activating changes in the structures of the substrate and the enzymes (Hamzah et al., 2011; Mao et al., 2018).

2.4 Palm Oil Mill Effluent (POME)

2.4.1 Characteristics of POME

Palm oil wastes are among the most plentiful agricultural residues in the world (Chin et al., 2011). Together with the wastes that are generated from processing of oil palm fruits, POME has been appointed by the operators as the most expensive, harmful, and difficult waste to regulate (Madaki & Seng, 2013; Rupani et al., 2010). Raw POME formed a concentrated brownish suspension consist of 95–96% water, 0.6–0.7% oil and 4–5% total solids. POME is produced from a combination of sterilizer condensate, separator sludge and hydrocyclone wastewater in a ratio of 9:15:1, particularly (Wu et al., 2010).

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POME consist of tremendous chemical oxygen demand (COD) and biological oxygen demand (BOD) due to the hemicellulose and lignocelluloses substances (Ahmad et al., 2003; Basri et al., 2010). Thus, it could not be discharged directly into watercourses because of its high acidity.

2.4.2 Treatment of POME

There are various types of pre-treatment method have been applied with several barriers to improve biogas yield from POME. Thus, scientists have developed advance technologies to search feasible solutions for POME management (Madaki & Seng, 2013).

Typically, POME is treated in a pond system or open digestion tank which was utilized by more than 85% of the mills in Malaysia (Yunus, 2014). The mechanism was practiced broadly because of the low capital and operating tariff of the open lagoons (Sulaiman et al., 2009). But the practice requires vast land area, extended retention day up to 200 days, and emit the greenhouse gases (Ahmed et al., 2015).

Hence, production of value-added products from the wastewater could lower the treatment cost and cut down dependence on fossil fuel (Rasdi et al., 2009). POME could be a useful source for biohydrogen production and operates as a wastewater treatment system concurrently (Chin et al., 2013; Lam & Lee, 2011). The insoluble carbohydrates (cellulose, hemicellulose and starch) in POME present in a large amount as compared to the soluble carbohydrates (Ho & Tan 1983).

Pre-treatment is required before the hydrolysis process can be conducted. The pre-treated biomass will release more monomers from the complex carbohydrate polymers. The available monomers will be consumed by microbes and this will increase rate of reaction in the biogas production. Accordingly, it is crucial to hydrolyse the complex

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insoluble carbohydrates in POME before fermentation experiment or to trigger the microbial process (Nurul-Adela et al., 2016).

2.5 Mushroom Industry

2.5.1 *Pleurotus pulmonarius*

The major cultivated mushroom in Malaysia is *Pleurotus* spp., which account for 90% in comparison to other species such as *Lentinula edodes* and *Auricularia polytricha* (Saidu et al., 2011). The oyster mushrooms have been used by human cultures everywhere in the world for different benefits (Mishra et al., 2013).

Genus *Pleurotus* ranks among as one of the largest cultivated mushrooms in the world, representing 25% of total world production (Owaid et al., 2015). Meanwhile, cultivation of these mushrooms serves as a main industry in the countries of Southeast Asia (Ragunathan & Swaminathan, 2003).

Pleurotus pulmonarius (Fries) Quélet, known as *Pleurotus sajor-caju* (Fries) Singer or generally called as grey oyster mushroom is commonly cultivated commercially on enriched rubber-wood sawdust. Other than that, it can be grown on different agricultural wastes and natural resources due to its broad compatibility with the natural environment (Avin, 2014). *P. pulmonarius* has two stages in its life cycle which are the vegetative stage (mycelial growth) and the reproduction stage (fruit body development), as illustrated by typical basidiomycetes.

During the vegetative stage, the mycelia secrete enzymes that deteriorate celluloses and lignin. The deteriorated compounds are absorbed by the hyphae for the mycelial growth. Jasmine (2012) stated that temperature and light are the important environmental factors that initiate fruit body development.

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During the reproductive stage, the fruit body development generally lasted for two to three days, followed by a resting phase of about ten days before the next cycle of the fruiting stage. *P. pulmonarius* generally exhibited five to six cycles before the mushroom bags were deposited as wastes.

The mushroom has gained great popularity for artificial cultivation because of its fast mycelia growth, huge saprophytic colonization strength, simple and low-priced cultivation techniques (Sheikh et al., 2010). The study on *P. pulmonarius* has been done broadly due to its ability in secreting a range of ligninolytic and cellulolytic enzymes such as laccase, xylanase and cellulases (Massadeh & Modallal, 2007).

2.5.2 Disposal of Spent Mushroom Substrate

The advance in the mushroom industry is leading to the generation of the SMS, or also known as a by-product released after the harvest of edible mushroom fruit bodies (Ahlawat & Sagar, 2007; Shitole et al., 2014; Wu et al., 2013). The current disposal practice of SMS in Malaysia is either burning or dumping in the nearby area (Figure 2.1).

Figure 2.1: A mass of SMS bags was disposed and burned at a local mushroom farm.

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The burning of the SMS has caused air pollution because of smoke emission and its adverse effect on the environment (Singh, 2008). Meanwhile, the common practice of disposal to landfill is becoming inefficient due to the increasing cost and shortage of land in city areas.

Expenditure of transportation and logistics will be high if the expansion of new landfill is required (Yunus, 2014). Other than that, SMS was normally disposed by burying or composting with animal manure (Rasib et al., 2015). Alternatives method of employing these agricultural residues are required to mitigate the environmental pollution issues related with ongoing disposal method, such as open burning and soil incorporation (Zhang et al., 2002).

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2.5.3 Uses of Spent Mushroom Substrate

The potentials of using spent mushroom substrate for generation of value-added products have been studied such as for the decolorisation of chemically different dyes (Singh et al., 2010), production bulk enzymes (Singh et al., 2003), utilization of energy feedstock, landfilling and crop production (Williams et al., 2001).

SMS have been intended as suited substances to enhance the soil quality in the contaminated sites due to its organic matter component and macronutrients (Medina et al., 2012). The spent substrate of *P. pulmonarius* was able to treat wastewater polluted by organochlorine pesticides due to the ligninolytic enzymes secreted in the SMS (Juárez et al., 2011).

A proper and effective technique is required to manage the valuable SMS so that its usage can be optimized (Jamaludin et al., 2012). Enzymatic hydrolysis has been identified as one of the techniques to convert mushroom wastes into sugars. It has some benefits, such as substrate specificity, low energy consumption, and environmental security (Irawati et al., 2012). The effort was matching with the National Agro Food Policy (2011-2020) which focuses on increasing mushroom production and to transform the agro waste into valuable products (Bakar et al., 2012).

2.6 Biohydrogen

Biohydrogen is hydrogen gas, H_2 produced from agricultural and industrial wastes by the biological method. Researchers are actively analysing the microbial hydrogen production due to its high energy yield, cost-effective, environmentally friendly, sustainability and able to treats waste (Lin et al., 2018; Mohammadi et al., 2011; Taifor et al., 2017). In fact, water was produced as the only product during the combustion of

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hydrogen (Sompong et al., 2007). Thus, it may be a significant substitute to fossil fuel due to plenty of renewable resources available.

Biohydrogen is normally released via one of four practices: biophotolysis of water by green algae, indirect biophotolysis by cyanobacteria, photofermentation by photosynthetic bacteria, and dark fermentation by strict or facultative anaerobic bacteria (Guo et al., 2010).

However, dark fermentation becomes the most favorable method because it can be processed in condition without light and oxygen, produces higher cell growth, low capital cost, provides chances for improvement such as optimizing system conditions and immobilizing microorganisms (Seelert et al., 2015).

There are several factors affecting biohydrogen production such as specificity and concentration of substrate, the structure of reactor, hydraulic retention time (HRT), organic loading rate (OLR), pH, temperature, oxidation-reduction potential and nutritional demand (Mu et al., 2006).

2.6.1 Biohydrogen Production from POME

Fermentative bioenergy production from POME has a large potential for the environmental research and energy in Malaysia, especially when combined with the wastewater treatment system (Takriff, 2015).

POME contains high concentration of lipid, minerals, carbohydrates, protein, and nitrogenous compounds. Thus, POME has been considered as a suitable substrate for biohydrogen production (Sompong et al., 2007).

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Many studies have been done on the biohydrogen production from POME involving different experimental conditions such as pre-treatment of POME using *Clostridium butyricum* (Kamal et al., 2012), application of granular sludge system and fixed film reactor (Mohammadi et al., 2014), and two-stage thermophilic and mesophilic fermentation condition (Krishnan et al., 2016).

According to Lam and Lee (2011), biohydrogen yield from POME is the highest compared to other wastes and wastewaters further emphasized the potential to derive biohydrogen from POME. The application of a low-cost pool of enzymes prior to anaerobic digestion of POME could enhance the rate of biohydrogen process and biohydrogen yield (Garritano et al., 2017).

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MATERIALS AND METHODS

3.1 Enzymes Profiling

3.1.1 Collection and Storage of Spent Mushroom Bags

SMS of three different *P. pulmonarius* strains (UMP001, UMP002, and UMH004) were collected from Nas Agro Farm, Sepang, Selangor, Malaysia. Five bags were collected for each strain at weekly intervals from inoculation date over a period of twelfth weeks (Figure 3.1). The bags were operated directly or after storage in a freezer at a temperature -20 ^{o}C for one week. For the experiment of profiling, the bags were collected over a duration of twelve weeks at weekly intervals from inoculation date.

Figure 3.1: The SMS bags at NAS Agro Farm, Sepang, Selangor.

3.1.2 Preparation of Enzyme Extracts

The contents of five bags were broken up by hand and mixed meticulously (Figure 3.2). Three replicate samples were taken from this mixture for the extraction and enzymes analysis. A total of 100 ml tap water (pH 4.0) was poured to 10 g of the mushroom substrate in a shake flask (250 ml). The mixture was incubated in a shaking incubator

(DAIHAN Labtech Co., Singapore) at speed 150 rpm, for one hour at 4 ºC (Singh et al., 2003). Then, the mixture was grounded for one minute using a Waring blender.

Figure 3.2: Five bags for each strain of SMS were collected and mixed up in a container before extraction.

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The solids were divided by centrifugation at 9000 rpm for 20 minutes and the crude enzymes in the form of supernatant containing fungal enzymes were obtained (Singh et al., 2011). The supernatant of the enzymes was kept in 1.5 ml microcentrifuge tubes at -20 ºC for 24 hours before enzyme assays. The method for preparation of crude enzymes was applied throughout this study unless otherwise stated.

3.1.3 Enzyme Assays

The assays of lignin peroxidase, laccase, xylanase, endoglucanase, β-glucosidase, and total soluble protein of SMS was carried out at weekly intervals for twelfth weeks from the time of inoculation. Every enzyme activity was expressed in international units (U), defined as the quantity of enzyme required to release one µmol product per min.

The enzyme productivity was recorded based on unit obtained per gram of substrate (U g^{-1}), under the assay condition, unless otherwise stated. The enzyme assays were performed in triplicate and measured by UV spectrophotometer (Shimadzu spectrophotometer UV-160A, Japan).

Lignin peroxidase activity was determined by recording the increase in absorbance at 310 nm because of the oxidation of 2 mM veratryl alcohol to veratraldehyde (Have et al., 1998). The reaction was started by addition of H_2O_2 at a last concentration of 0.5 mM. The standard used was 3, 4 dimethoxybenzaldehyde (veratraldehyde).

Laccase activity was determined by the increase in absorbance at 525 nm due to the formation of tetramethoxy-azo-bis methylenequinone from the reaction of laccase with syringaldazine (Harkin & Obst, 1973; Leonowicz & Grzywnowicz, 1981). Syringaldazine of 0.1 mM in 50% ethanol (w/v) was used as the substrate.

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Xylanase activity was determined using 1% (w/v) suspension of xylan from oat spelts in 50 mM sodium citrate buffer pH 4.8 (Bailey et al., 1992). Xylose was used as a standard. The concentration of reducing sugars was measured by dinitrosalicylic acid (DNS) method of Miller (1959) at wavelength λ = 575 nm.

Endoglucanase activity was determined by using 1% sodium salt of carboxymethyl cellulose as the substrate (Kim et al., 1992). The DNS method was used to measure the amount of reducing sugars released with glucose as standard at 575 nm (Miller, 1959).

Measurement of β-glucosidase activity was completed according to the method by Kim et al. (1992). The substrate used was 0.5 mM p-nitrophenyl-β-D-glucopyranoside in 50 mM sodium citrate buffer, pH 4.8. The amount of p-nitrophenyl liberated was determined spectrophotometrically at λ = 400 nm with p-nitrophenol as the standard.

The extracellular soluble protein was measured using the Bradford dye-binding technique with crystalline bovine serum albumin, BSA as standard (Bradford, 1976). The mechanism is based on the reaction between the Coomassie Brilliant Blue G-250 reagent and proteins in the solution and measured by recording the absorbance of the solution at wavelength λ=595 nm.

3.1.4 Statistical Analysis

One-way ANOVA was used to investigate the means of results. The significance of the difference between means was resolved by Duncan's multiple range test at 95% least significant difference ($p < 0.05$).

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3.2 Enzymatic Hydrolysis of POME

3.2.1 Preparation of POME as substrate

The palm oil mill effluent (POME) was collected from Jugra Palm Oil Mill, Banting, Selangor, after acidification process at cooling pond (Figure 3.3). The temperature of the collected POME was 60 ºC. The pre-settled POME was preserved at 4 ºC in cold room to reduce and minimize the degradation of the effluent from microbial reaction before analysis.

Then, the POME was characterized and analyzed. The pH of the pre-settled POME was 5.00, with chemical oxygen demand (COD) of 27000-28000 mg/L. The total nitrogen and protein recorded were 0.11% and 0.70%, respectively. Whereas, the concentration of the reducing sugar in POME was recorded at 0.77 g/L. Meanwhile, POME sludge was collected from anaerobic pond of the same palm oil mill and used as inoculum (Zainal et al., 2018).

Figure 3.3: The POME was collected from Jugra Palm Oil Mill, Banting, Selangor.

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3.2.2 Preparation of Concentrated Enzymes

Five bags of selected SMS were collected from a second batch and processed. Contents of the five bags were crushed and mixed meticulously. Three replicate samples were taken from the mixture for the extraction and enzymes assays. The crude enzymes were concentrated using a freeze dryer (Christ ALPHA 1-4 LDplus, Germany). The enzyme powder was placed in a sealed container and stored at 4 ºC fridge before further hydrolysis.

The concentration of concentrated enzymes at 100 mg/ml was fixed and used for enzymatic hydrolysis of POME. Meanwhile, the remaining supernatant of crude enzymes was stored in 15 ml centrifuge tubes in a freezer (-20 ºC) before enzyme assays and hydrolysis of POME.

3.2.3 Hydrolysis of POME

The selected concentrated enzymes and crude enzymes were used to treat POME in hydrolysis experiment. The control experiment, without enzyme added into the POME, was conducted simultaneously. The hydrolysis was carried out on the POME in Erlenmeyer flask (250 ml), according to a method modified from Nurul-Adela et al., (2016). The mixture consists of 10% (v/v) enzyme loading (10 mL enzyme per 100 mL of POME). The pH of POME was adjusted to 4.8 using sodium hydroxide (0.1 M). The sample was incubated at 50 ºC, 150 rpm, for 12 hours in a rotary incubator shaker according to a method modified from (Silvamany et al., 2015).

After 12 hours, samples were withdrawn and centrifuged at 3500 rpm for 10 minutes to discard the residual solids remaining after hydrolysis (Mun et al., 2008). Reducing sugar liberated in the supernatant were determined by DNS method of Miller (1959). A glucose standard graph having a linear regression of $y = 0.4748x - 0.1117$, $R^2 = 0.9709$,

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was employed to calculate the reducing sugar concentration. All the tests were conducted in triplicate.

3.2.4 Optimization of Enzymatic Hydrolysis

The optimization of hydrolysis variables was carried out by employing a CCD of RSM based on Zainal et al. (2017). Three significant factors were identified as incubation time (A), enzyme loading (B) and pH (C). The actual and coded values of each factor is recorded in Table 3.1.

Table 3.1: Actual and coded values of factors for optimizing POME hydrolysis.

Factors	Component	Level		
		-1	0	1
A	Incubation time (hour)	0	12	24
B	Enzyme loading	0	5	10
C	pH	4.8	5.4	6.0

Figure 3.4 shows the factors and levels considered in this study. The concentration of reducing sugar (g/L) as the response, was determined by the DNS method of Miller (1959). Design Expert Software (Stat-Ease Inc., Minneapolis, USA, Version 6.0.7) was used for the study design, data analysis and graphical analysis of the data.

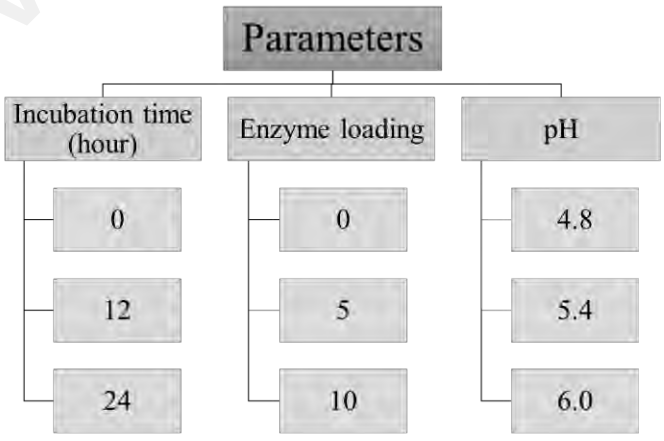

Figure 3.4: Experimental design to study reducing sugar yield.

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A total of 20 experiments were performed to optimize the experiment parameters. The concentrated enzymes were inoculated into the POME in the rotary shaker under various conditions of pH value and incubation time. The design matrix for the CCD was displayed in Table 3.2. ANOVA was used to examine the significance of linear and quadratic effects of the three variables.

Table 3.2: The matrix design of CCD.

Run	Factors		
	A: Time (h)	B: Enzyme loading (%, v/v)	C: pH
1	12	5	5.4
2	12	5	5.4
3	12	5	5.4
4	24	10	6.0
5	0	0	4.8
6	24	5	5.4
7	12	5	4.8
8	0	10	4.8
9	12	5	6.0
10	0	5	5.4
11	0	10	6.0
12	12	5	5.4
13	24	0	4.8
14	12	10	5.4
15	12	5	5.4
16	12	5	5.4
17	0	0	6.0
18	24	0	6.0
19	24	10	4.8
20	12	0	5.4

The three-dimensional surface plots were applied to display the effects of the independent variables on the response. The fit of the model was calculated by the result of R^2 and adjusted R^2 coefficient. The statistical significance of the second-order model equation was measured by a significant F-value and an insignificant lack-of-fit F-value.

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The Design Expert software was used to validate the model optimum value of chosen variables acquired by resolving regression equation. The anticipated optimum value was affirmed by the experiment using the preferred optimum values of the three variables.

3.3 Biohydrogen Production from POME Hydrolysate

3.3.1 Batch Fermentation of Biohydrogen

POME hydrolysate from the optimized condition was used for biohydrogen production. The control experiment, without enzyme added into the POME, was conducted simultaneously. Batch hydrogen fermentation was operated in 156-mL serum bottles, with a working volume of 100 ml (Choi & Ahn, 2015) as presented in Figure 3.5.

Figure 3.5: The batch fermentation of biohydrogen using an incubator shaker.

The enriched acclimatized POME sludge was used for hydrogen fermentation using raw POME (control) and hydrolysed POME as substrate at mesophilic condition (37 ºC), with initial pH of 5.5 and being shaken at 150 rpm for 24 hours. The fermentation was started by adding 20% (v/v) POME sludge to 80% (v/v) POME medium (Khaleb et al.,

2012). The anaerobic condition was set by sparging nitrogen gas for 5 minutes during the beginning of the experiment (Zainal et al., 2018).

3.3.2 Statistical Analysis

The amount of biogas composition was analyzed by a gas chromatograph (Perkin Elmer, AutoSystem Gas Chromatograph, 600 series LINK) prepared with a thermal conductivity (TCD) and a pack GC column Supelco, with 40/80 carboxen 1000, MR2924D, 10’ x 18’ (Zainal et al., 2018). The liberated reducing sugar was analyzed by DNS method of Miller (1959).

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RESULTS AND DISCUSSIONS

4.1 Enzymes Profiling

4.1.1 Lignin Peroxidase Productivity (LiP)

A gradual increase in lignin peroxidase productivity at first week (18.8 U g^{-1}) to fifth week (24.6 U g^{-1}) was observed in UMH004 (Figure 4.1). Then, the productivity decreased significantly ($p < 0.05$) by the sixth week (15.9 U g^{-1}). The productivity of lignin peroxidase increased at the seventh week and peaked significantly at eighth week (22.0 U g^{-1}), which then decreased until the twelfth week (12.7 U g^{-1}).

Comparable to UMH004, a gradual increase was observed in the productivity of lignin peroxidase for UMP001 from the first week (12.7 U g^{-1}) to third week (22.0 U g^{-1}). The lignin peroxidase productivity for UMP001 then fluctuated between the fourth week to ninth week and exhibited a significant peak at tenth week (64.4 U g^{-1}). The productivity of lignin peroxidase then decreased until the twelfth week (46.3 U g^{-1}).

There was a high productivity of lignin peroxidase at first week for UMP002 (36.7 U g^{-1}) which further decreased significantly until the third week (19.0 U g^{-1}). The productivity of lignin peroxidase then increased significantly up to a fifth week (60.1 U g^{-1}) but decreased gradually to ninth week (27.6 U g^{-1}). After that, the productivity of lignin peroxidase increased significantly from the tenth week (42.4 U g^{-1}) continued to peak at the eleventh week (82.9 U g^{-1}) which was the highest as compared to other weeks for UMP002.

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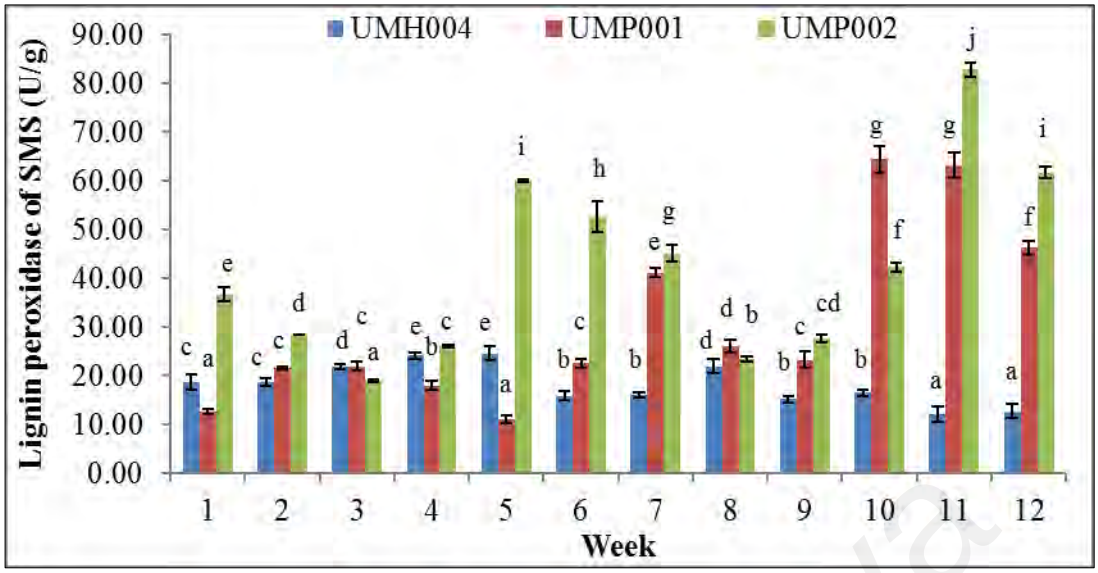

Figure 4.1: The productivity of LiP from first week to twelfth week. Different alphabet indicates there is a significant difference between the values ($p < 0.05$).

A gradual increment pattern in lignin peroxidase productivity from the first week to the fifth week for UMH004, first week to the third week for UMP001, and from the third week to the fifth week for UMP002, may be indicating an active lignin degradation by the growing fungal mycelium. The trend of the enzyme profiles agrees with Jasmine (2012), who reported that lignin degradation is correlated with the vegetative stage of fungal growth.

A decrease in the lignin peroxidase productivity by the sixth week for UMH004, the fourth week for UMP001, and the sixth week for UMP002 were observed which apparently indicated that maybe the lignin barrier has been degraded and the growing fungal mycelium has access to the cellulosic component of the wood for carbon and energy source (Singh, 2008). The decrement pattern of lignin peroxidase also probably indicating the mushroom has formed fruit bodies. Isikhuemhen and Mikiashvilli (2009) explained that the enzyme activities of peroxidases, laccase and endoglucanase were lower after mushroom fruiting since mushroom production affected enzyme activities differently.

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The second peak of lignin peroxidase by the eighth week for UMH004, tenth week for UMP001, and eleventh week for UMP002, are probably due to the production of veratryl alcohol (VA). VA is known as a secondary metabolite of white-rot fungi and performs as cofactor for the lignin peroxidase in lignin degradation (Kulshreshtha et al., 2013)., The secretion of lignin peroxidase is usually induced when the mycelial growth was low in nutrient levels (Singh et al., 2003).

4.1.2 Laccase Productivity

The laccase productivity ranged from 0.3 U g^{-1} to 6.6 U g^{-1} for UMH004 (Figure 4.2). The highest productivity of laccase was recorded at third week (6.6 U g^{-1}). Then, the productivity decreased significantly ($p < 0.05$) at the fourth week (2.2 U g^{-1}) and increased significantly at the fifth week (6.2 U g^{-1}). The productivity decreased significantly at the sixth week (0.3 U g^{-1}), before fluctuated up to the twelfth week (1.3 U g^{-1}).

The laccase productivity in UMP001 was high at first week (3.3 U g^{-1}) but significantly decreased until the fourth week (0.5 U g^{-1}). A significantly gradual increase was recorded from the fifth week and peaked up to 16.3 U g^{-1} at the seventh week. The productivity then decreased significantly by the ninth week (4.2 U g^{-1}) before peaked up to 10.2 U g^{-1} at the eleventh week. At the twelfth week, the laccase productivity decreased significantly (5.8 U g^{-1}).

The laccase productivity in UMP002 was high at first week (9.4 U g^{-1}) but decreased significantly by the second week (3.6 U g^{-1}), then increased significantly at third week (5.8 U g^{-1}) and continued to reach the peak at fifth week (16.9 U g^{-1}). The laccase productivities from the sixth week to the twelfth week were lower than productivity in the fifth week.

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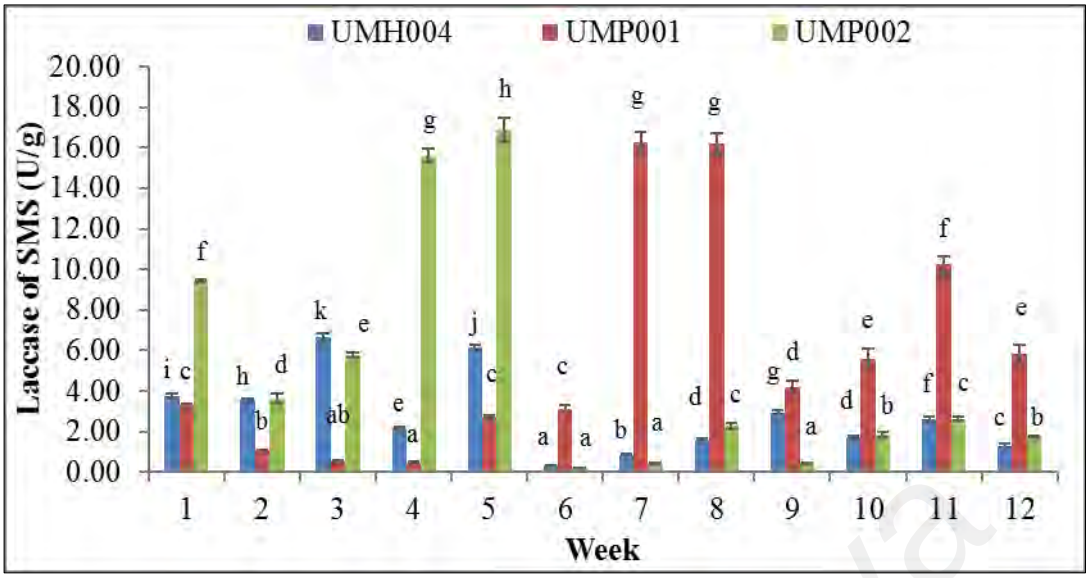

Figure 4.2: The laccase productivity from first week to twelfth week. Different alphabet indicates there is a significant difference between the values ($p < 0.05$).

Laccase is the most common enzyme in SMS from *Pleurotus sajor-caju* (Kumaran et al., 1997). Peak in laccase productivity by the third week, fifth week, ninth and the eleventh week for UMH004, seventh week and eleventh week for UMP001, fifth week and the eleventh week for UMP002, maybe indicating the vegetative stage of mycelia growth. This condition agrees with Fen et al. (2014), which reported that laccase is expected to involve in fruit bodies development and stress defense.

Meanwhile, the reduction in laccase production after the mentioned weeks probably coincides with the fruiting stage. Laccase accounts for two percent of the mycelial protein. Fruit bodies development may need additional nitrogenous substance beyond that produced by the mycelium. Thus, extracellular protein as well as laccase may be deteriorated, and the products of the deterioration may be assimilated for sporophore development (Wood, 1980).

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High laccase productivity (9.4 U g^{-1}) was observed during the first week (seventh day) for UMP002. The result is consistent with a study by Vikineswary et al. (2006) which exhibited a high laccase productivity (7.6 U g^{-1}) on the eleventh day during fermentation of *Pycnoporus sanguineus* on sago hampas. The productivity of laccase at the eleventh week (2.6 U g^{-1}) are consistent with a study recorded by Singh et al. (2003), which reported the laccase productivity of 2.6 U g^{-1} of SMS during the third month of spawn run.

4.1.3 Xylanase Productivity

The xylanase productivity ranged from 0.1 U g^{-1} to 1.4 U g^{-1} for UMH004 (Figure 4.3). The productivity at first week was 0.2 U g^{-1} but decreased significantly ($p < 0.05$) by the second week (0.1 U g^{-1}). The productivity increased significantly at the third week (0.5 U g^{-1}) and the fourth week (0.7 U g^{-1}). Then, the productivity fluctuated between the fifth week (0.4 U g^{-1}) until the ninth week (0.7 U g^{-1}). The productivity peaked significantly by the tenth week (1.4 U g^{-1}), before decreased significantly to eleventh (0.9 U g^{-1}) and twelfth week (0.4 U g^{-1}).

Meanwhile, the productivity of xylanase in UMP001 fluctuated between the first week (0.6 U g^{-1}) to fifth week (0.7 U g^{-1}), then increased significantly at the (ninth week (0.3 U g^{-1}) and peaked significantly at the tenth week (1.6 U g^{-1}), which was greater than productivity at the other weeks. Meanwhile, the productivity of xylanase in UMP002 was 0.2 U g^{-1} during the first week and increased significantly to 0.6 U g^{-1} in the third week. The productivity then decreased at the fourth week (0.4 U g^{-1}), fluctuated and peaked significantly by the eighth week (3.6 U g^{-1}). Then, the productivity decreased significantly by the ninth week (0.6 U g^{-1}) before fluctuated to the twelfth week (2.4 U g^{-1}).

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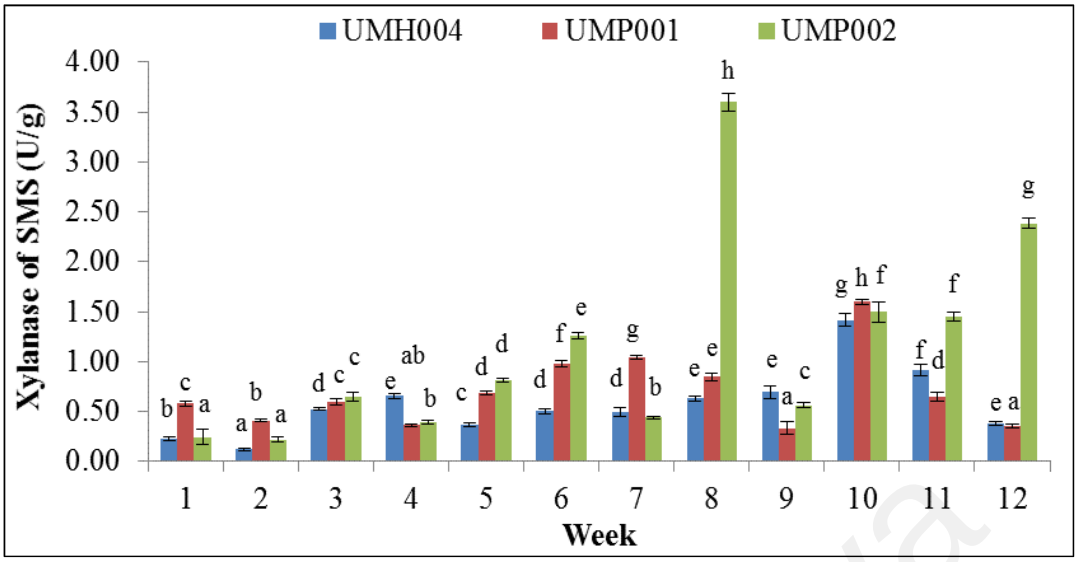

Figure 4.3: The xylanase productivity from first week to twelfth week. Different alphabet indicates there is a significant difference between the values ($p < 0.05$).

An increase in xylanase productivity by the third week for UMH004, fifth week for UMP001 and UMP002, might be due to a greater availability in hemicellulose component of the cell wall to the xylanolytic enzymes after removal of lignin (Singh, 2008).

Meanwhile, the increment in xylanase productivity by the fourth and the tenth week for UMH004, seventh week for UMP001, and eighth week for UMP002, are probably due to the formation of mushroom fruit bodies. The increment also coincided with the decrease in laccase production by specified weeks. Terashita et al. (1998) reported that xylanase activity peaked at the end of vegetative mycelial growth phase and decreased toward the completion of mycelial maturation.

A similar pattern has been reported during the degradation of *Eucalyptus grandis* wood chips by *Ceriporiopsis subvermispora* by which the peak in xylanase activity was observed only after the discharge of a considerable amount of lignin from wood cell walls (Ferraz et al., 2003). A decline in xylanase productivity by the fifth week and eleventh week for UMH004; fourth, ninth, and eleventh week for UMP001; fourth, seventh, and

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ninth week for UMP002, were recorded which probably due to the catabolite repression by the released simple sugars from cellulose or hemicelluloses (Haltrich & Steiner, 1994).

4.1.4 Endoglucanase Productivity

The productivity of endoglucanase ranged from 2.2 U g^{-1} to 3.4 U g^{-1} for UMH004 (Figure 4.4). A comparable productivity of 2.2 U g^{-1} was recorded during the first week, second week, and seventh week. Meanwhile, a high endoglucanase productivity was recorded at the tenth week (2.9 U g^{-1}). But the productivity increased significantly ($p < 0.05$) by the eleventh week (3.4 U g^{-1}), and then decreased significantly at the twelfth week (2.5 U g^{-1}).

The productivity of endoglucanase in UMP001 ranged from 2.2 U g^{-1} to 3.1 U g^{-1}. The productivity of 2.2 U g^{-1} was recorded at the first week and eighth week, while the highest endoglucanase productivity was recorded at the tenth week (3.1 U g^{-1}). The productivity of endoglucanase for UMP002 during the first week was comparable to UMH004 and UMP001 which was 2.2 U g^{-1}. After that, the productivity decreased and fluctuated until it peaked significantly at the tenth week (4.7 U g^{-1}). Then, the productivity decreased significantly by the twelfth week (2.7 U g^{-1}).

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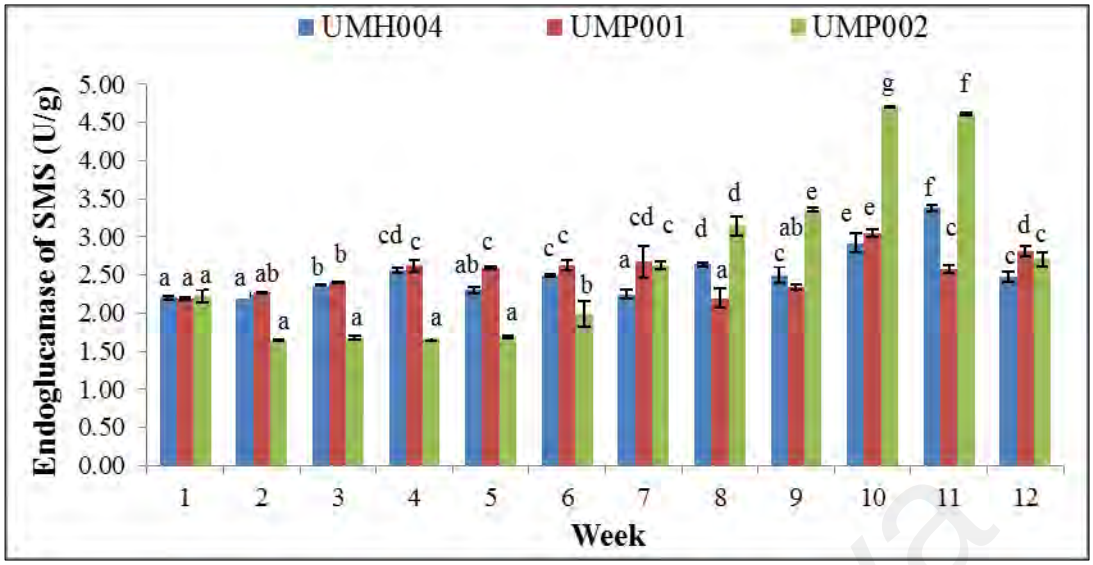

Figure 4.4: The endoglucanase productivity from first week to twelfth week. Different alphabet indicates there is a significant difference between the values ($p < 0.05$).

The peaks in the endoglucanase productivity were observed at the fourth, sixth, eighth and eleventh week for UMH004; tenth and twelfth week for UMP001; seventh and tenth week for UMP002, which might be coincided with the fruiting stage of the mushroom. The fruiting stage of UMH004 starts after four weeks and lasts for two to three days. Meanwhile, the fruiting stage for UMP001 and UMP002 starts after seven or eight weeks from the date of inoculation. After that, the mushroom bags are closed for ten days for the mycelium to grow before reopening the bags for fruiting.

Meanwhile, the decrement in endoglucanase productivity are probably due to mushroom fruit bodies has been harvested at the previous week. Mata and Savoie (1998) reported that reported that there was a decrease in the endoglucanase and β-glucosidase activity after harvesting of fruit bodies of *Lentinula edodes*. The endoglucanase productivities at the eighth week for UMH004 (2.6 U g^{-1}), and UMP001 (2.2 U g^{-1}) are consistent with the endoglucanase productivity of 1.98 U g^{-1} at the eighth week from the time of inoculation in *P. sajor-caju* (Singh et al., 2003).

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4.1.5 β-glucosidase Productivity

There was a gradual increase in the productivity of β-glucosidase from the first week (76.2 U g^{-1}) onwards to the fourth week (197.8 U g^{-1}) for UMH004 as recorded in Figure 4.5. Then, the productivity decreased by the fifth week (124.6 U g^{-1}), followed by an increased by the following week and peaked significantly ($p < 0.05$) at the seventh week (210.8 U g^{-1}). Then, the productivity decreased significantly at the eighth week (78.7 U g^{-1}) and fluctuated until the twelfth week (104.6 U g^{-1}).

The productivity of β-glucosidase was not different in UMP001 between the first week (118.6 U g^{-1}) and the second week (118.3 U g^{-1}). Then, the productivity increased significantly by the fourth week (382.8 U g^{-1}). The β-glucosidase productivity fluctuated between fifth week to the eleventh week and peaked significantly during the twelfth week (828.1 U g^{-1}).

Whereas, a high β-glucosidase productivity of UMP002 was recorded at the first week (148.7 U g^{-1}), which further decreased continuously to fourth week (111.7 U g^{-1}). The productivity then peaked significantly to 372.2 U g^{-1} by the seventh week, which was the highest than productivity at other weeks for UMP002.

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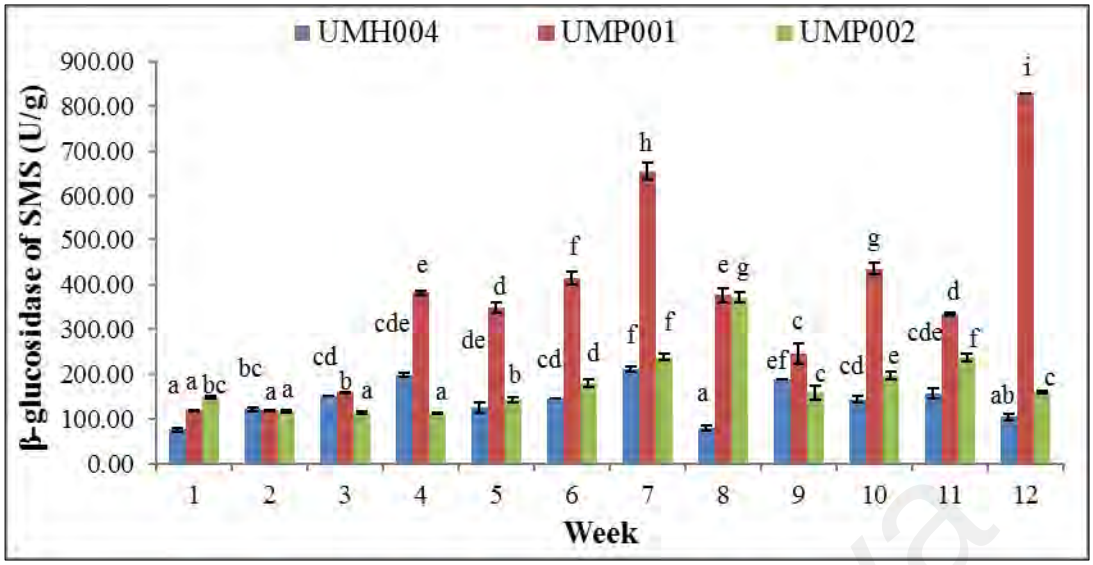

Figure 4.5: The β-glucosidase productivity from first week to twelfth week. Different alphabet indicates there is a significant difference between the values ($p < 0.05$).

The peak in β-glucosidase productivity was detected at the fourth week for UMH004; twelfth week for UMP001; and sixth week for UMP002, occurred might be due to a decrease in laccase production. The coordination of laccase and cellulase enzyme activities during distinct stages of growth and fruit body formation has been recorded in several white rot fungi (Singh, 2008). Lechner and Papinutti (2006), reported that endoglucanase and β-glucosidase activity were at peaked after a decrease in laccase activity in *Lentinus tigrinus*. The productivity of β-glucosidase in this study at the eighth week (78.7 U g^{-1}) is consistent with the β-glucosidase productivity at the eighth week (83.2 U g^{-1}) in a study on SMS of *P. sajor-caju* by Singh et al. (2003).

Ligninolytic enzymes productivities from the current work might be recognized quite low as compared to a study by Singh et al. (2003). Enzymes source to degrade POME can be obtained from a mixture of SMS of *P. pulmonarius* with other strain of SMS. A study described by Wu et al. (2013), proved that SMS of *Flammulina velutipes* might be used as a cheaper carbon source to treat wastes. However, a detail study to determine the feasibility of a mixed culture system for ligninolytic and hydrolytic enzymes is required.

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The concentration of protein for the SMS from first week to twelfth week was recorded in Table 4.1.

Table 4.1: The protein concentration of *P. pulmonarius* over the period of twelve weeks.

Week	Protein concentration (mg ml^{-1})		
	UMH004	**UMP001**	**UMP002**
1	0.05	0.02	0.05
2	0.04	0.08	0.06
3	0.12	0.03	0.02
4	0.13	0.02	0.15
5	0.27	0.12	0.12
6	0.21	0.14	0.14
7	0.08	0.27	0.20
8	0.21	0.26	0.19
9	0.20	0.24	0.18
10	0.22	0.14	0.15
11	0.36	0.15	0.16
12	0.16	0.14	0.08

The vegetative stage for strains UMP001 and UMP002 requires about eight weeks for complete colonization of the substrate. Whereas, the strain UMH004 requires about four weeks for complete colonization before starts fruiting. This condition agrees with Avin (2014), who reported that both UMP001 and UMP002 exhibited a longer period than the UMH004 strain for the formation of fruit bodies and day to harvest. Furthermore, Avin et al. (2012) reported that the strain UMH004 produce higher yield, improve resistance to disease and increase productivity compared to strains UMP001 and UMP002.

Therefore, the strain UMH004 was selected for further experiments of enzyme extraction and enzymatic hydrolysis of POME. The spent mushroom bags of UMH004 are usually discarded between twelve to sixteen weeks due to a very low yield of fruiting bodies. It was recorded there were high titers of endoglucanase and β-glucosidase in the eleventh-week old bags. Thus, the eleventh-week old bags of UMH004 can be a reliable source for hydrolytic and ligninolytic enzymes.

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4.1 Hydrolysis of POME

4.1.1 Enzymes Productivity of SMS for Enzymatic Hydrolysis

Eleventh week-old bags of SMS from UMH004 were selected from a second batch. The productivity of the crude enzymes and concentrated enzymes were recorded as shown in Table 4.2.

Table 4.2: Enzymes productivities of eleventh week-old SMS of UMH004.

Enzyme	Productivity (U g^{-1})	
	Crude enzymes	Concentrated enzymes
Lignin peroxidase	90.2 ± 3.7	214.1 ± 1.3
Laccase	2.4 ± 0.0	4.1 ± 0.0
Xylanase	1.1 ± 0.1	2.3 ± 0.1
Endoglucanase	3.3 ± 0.3	14.6 ± 2.6
β-glucosidase	731.1 ± 8.7	915.4 ± 52.1

The lignin peroxidase productivity of the concentrated enzyme (214.1 U g^{-1}) was 2.4-fold greater than the productivity of the crude enzyme (90.2 U g^{-1}). The laccase productivity of the concentrated enzyme (4.1 U g^{-1}) was 1.8-fold greater than laccase productivity of the crude enzyme (2.4 U g^{-1}).

Meanwhile, the xylanase productivity (2.3 U g^{-1}) of the concentrated enzyme was 2.1-fold higher than the xylanase productivity of the crude enzyme (1.1 U g^{-1}). The endoglucanase productivity of the concentrated enzyme (14.6 U g^{-1}) was 4.4-fold higher than the productivity of the crude enzymes (3.3 U g^{-1}). The β-glucosidase productivity of the concentrated enzyme was 1.3-fold greater than the productivity of the crude enzyme.

4.1.2 Preliminary Hydrolysis of POME

POME contains a large volume of insoluble suspended solids and organic matters. Therefore, hydrolysing the POME substrate degrade the carbohydrate into simpler sugar to raise the biohydrogen production rate.

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Reducing sugar concentration in POME after hydrolysis with concentrated enzymes was six-folds higher than reducing sugar concentration in raw POME. Whereas, reducing sugar yield after hydrolysis with crude enzymes was one-fold higher than reducing sugar in raw POME. Meanwhile, reducing sugar concentration after hydrolysis using concentrated enzymes was four-folds higher than reducing sugar yield when using crude enzymes (Table 4.3).

Table 4.3: Reducing sugar yield after 12 hours hydrolysis.

POME samples	Reducing sugar (g L^{-1})
Hydrolysed POME by concentrated enzymes	4.72 ± 0.08
Hydrolysed POME by crude enzymes	1.07 ± 0.11
Raw POME (Control)	0.79 ± 0.04

A lower reducing sugar yield using crude enzymes cocktail revealed that it is impractical to utilize the crude enzymes for enzymatic hydrolysis of POME. The yield of reducing sugar in the present study was lower than reducing sugar produced from hydrolysis of POME solid in a study recorded by Mun et al. (2008). The authors recorded a total of 22.8 g L^{-1} and 16.8 g L^{-1} of reducing sugar when the POME solid was hydrolysed at 12 hours using crude cellulase and commercial cellulase enzymes respectively. Substrate concentration may affect POME hydrolysis depending on the accessible enzyme active sites (Nurul-Adela et al., 2016).

Zhu et al. (2008) explained that structural characteristic of substrates, solids amount, enzyme concentration, and hydrolysis time greatly affected the rate and duration of enzymatic hydrolysis of lignocellulosic material. Meanwhile, Yao et al. (2007) reported that high enzyme loading is required to reach a high level of cellulose conversion into glucose. Thus, the crude enzymes were concentrated for the optimization experiment.

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Pandiyan et al. (2014) recorded enzymatic hydrolysis of alkaline pretreated *Parthenium* sp. utilizing cellulase enzyme cocktail released a total reducing sugar of 85.8%. In another report, the hydrolysis of oil palm trunk by crude enzymes from local fungus *Aspergillus fumigatus*, released 13.15 g L^{-1} of the reducing sugar (Ang et al., 2015).

4.1.3 Optimization of Hydrolysis using RSM

CCD of RSM establish a mathematical correlation model among significant factors for reducing sugar yield from enzymatic hydrolysis of POME (Azaliza et al., 2009). The matrix of CCD and the experimental outcomes for optimization of enzymatic hydrolysis of POME were demonstrated in Table 4.4.

Table 4.4: Experimental result for reducing sugar optimization.

Run	Factors			Response
	A: Time (h)	B: Enzyme loading (%, v/v)	C: pH	Reducing sugar (g L^{-1})
1	12	5	5.4	2.91
2	12	5	5.4	2.92
3	12	5	5.4	2.90
4	24	10	6.0	3.44
5	0	0	4.8	0.90
6	24	5	5.4	2.56
7	12	5	4.8	2.79
8	0	10	4.8	2.90
9	12	5	6.0	2.63
10	0	5	5.4	1.66
11	0	10	6.0	2.49
12	12	5	5.4	2.86
13	24	0	4.8	0.97
14	12	10	5.4	3.82
15	12	5	5.4	2.89
16	12	5	5.4	2.55
17	0	0	6.0	0.90
18	24	0	6.0	0.95
19	24	10	4.8	3.24
20	12	0	5.4	1.16

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The highest reducing sugar yield achieved during the optimization experiment (3.82 g L^{-1}) was lower than reducing sugar obtained in the preliminary experiment (4.72 g L^{-1}). This action might be due to application of different pH of POME. Ferreira et al. (2009) explained that yield of reducing sugar is affected by the change of chemical content in biomass and various conditions of pretreatment and enzymatic hydrolysis technique.

Responses collected from the experiments were analysed using ANOVA as indicated in Table 4.5. The model F-value of 38.41 indicated that the model was significant and there was only a 0.01% chance that the model F-value will exist due to noise. The "P-value" for the model was < 0.0001 while the P value for model terms A, B, and A^2 were less than 0.0500 indicated the significant variables.

Table 4.5: ANOVA for quadratic response surface model from the hydrolysis of POME.

Source	Sum of square	DF	Mean square	F value	Prob > F
Model	15.81	9	1.76	38.41	< 0.0001
A	0.53	1	0.53	11.67	0.0066
B	12.12	1	12.12	265.09	< 0.0001
C	0.02	1	0.02	0.33	0.5769
A^2	1.01	1	1.01	21.98	0.0009
B^2	0.14	1	0.14	3.03	0.1122
C^2	5.682E-005	1	5.682E-005	1.243E-003	0.9726
AB	0.17	1	0.17	3.74	0.0818
AC	0.04	1	0.04	0.95	0.3523
BC	4.513E-003	1	4.513E-003	0.10	0.7599
Residual	0.46	10	0.05	-	-
Lack of fit	0.36	5	0.07	3.49	0.0983
Pure error	0.10	5	0.02	-	-
Total	16.26	19	-	-	-

*Standard deviation: 0.21, Mean: 2.37, Coefficient of variation: 9.02, Prediction error sum of squares: 3.48, Adjusted R-squared: 0.9466, Predicted R-squared: 0.7858, Adequate precision: 19.74

The coefficient of determination (R^2) of the model was 0.9719, conveys model adequacy and signifies that the model is applicable and able to be accepted. The regression equation of the reduced quadratic model for the response was represented in

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Equation 4.1. The equation shows a valid association among the parameters studied, and data acquired appeared to best fit the second-order polynomial as distinguished to another polynomial models.

$$Y\ (g\ L^{-1}) = 2.79 + 0.23A + 1.10B - 0.039C - 0.60A^2 - 0.22B^2 - 4.545E\text{-}003C^2 + 0.15AB + 0.074AC - 0.024BC \quad (4.1)$$

Where the coded variable Y represents the concentration of reducing sugar (g L^{-1}), while A, B, and C represent time, enzyme loading and pH respectively. It consists of 1 offset term, 3 linear terms, 3 quadratic terms and 3 interactions. The simultaneous interactions of the variables on the response were analysed from three-dimensional contour plots.

Figure 4.6 displayed a three-dimensional contour plot of the model for reducing sugar yield, as a function of incubation time (A) and enzyme loading (B) at pH 4.8. An increase in reducing sugar concentration could be acquired with increasing incubation time until 12 hours while increasing enzyme loading from 0 to 10% (v/v). This might be because of delignification and decrystallization of substances in POME and the increase in enzymes catalytic activities. As time prolonged, the enzyme active sites might have reduced, and the release of inhibitors will lower the hydrolysis rate (Gao et al., 2014).

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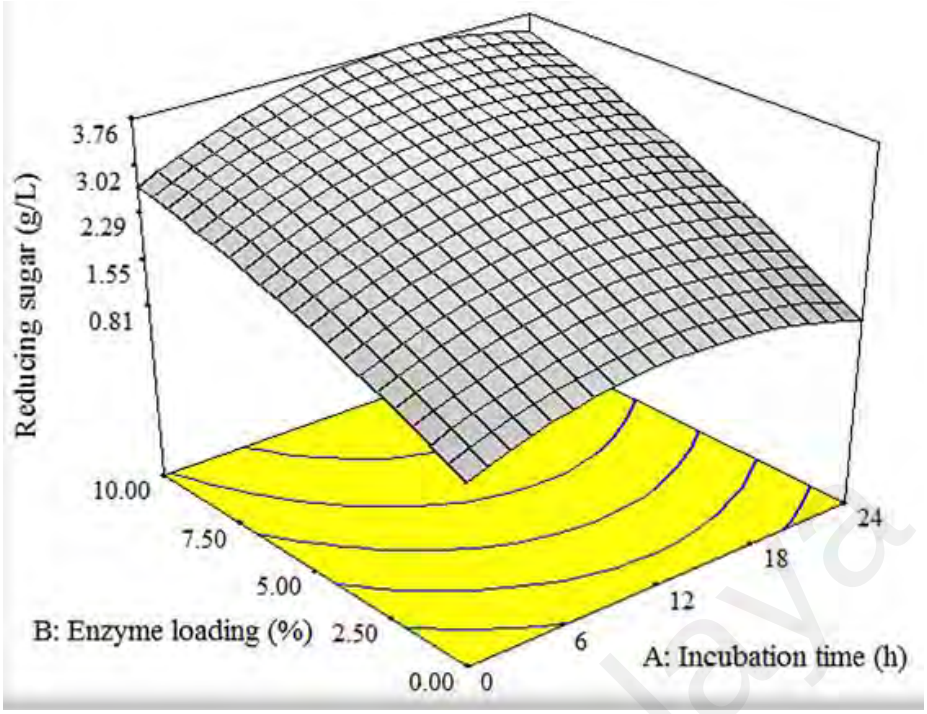

Figure 4.6: Response surface plot of reducing sugar yield from the effect of incubation time and enzyme loading at pH 4.8.

A high reducing sugar yield of 3.76 g L^{-1} maybe acquired with 10% (v/v) of enzyme loading and incubation time for more than 12 hours. However, a further increase in incubation time more than 18 hours would result in a small decrease in reducing sugar yield. Khaw and Ariff (2009) suggested that an increase in saccharification rate and sugar yield was found proportional to the increasing enzyme concentration.

At 10% enzyme loading, and between 12 to 18 hours incubation time, the yield of reducing sugar declined to 3.72 g L^{-1} and 3.68 g L^{-1} as pH was increased to 5.4 and 6.0 respectively (Figure 4.7 and Figure 4.8). Thus, different in initial pH of the reaction mixture showed small effects on the total reducing sugar yield. The condition might be because of adaptability of cellulase enzyme complex for the small range pH value (Pandiyan et al., 2014).

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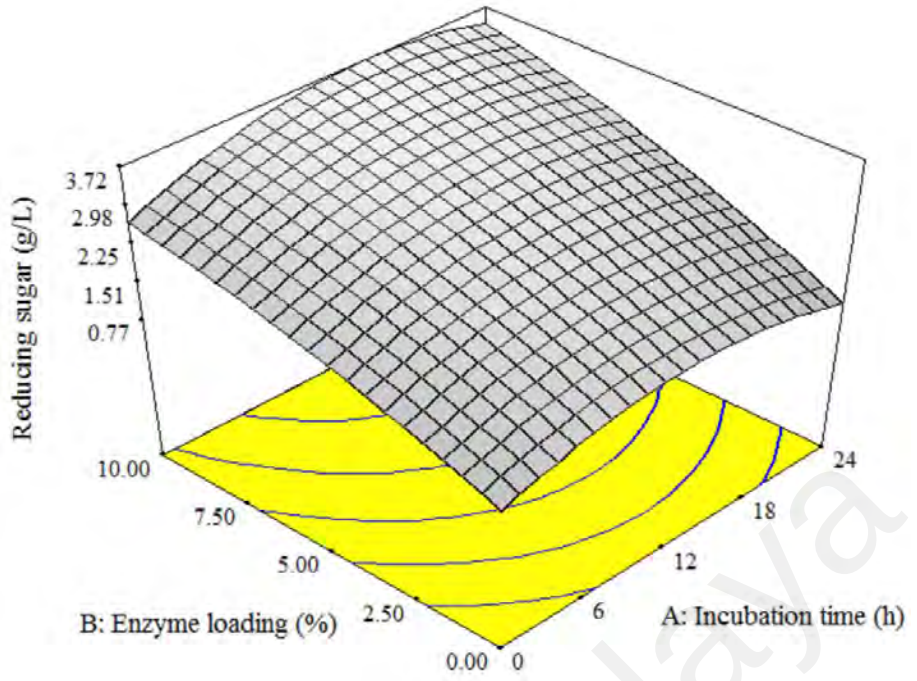

Figure 4.7: Response surface plot of reducing sugar yield from the effect of incubation time and enzyme loading at pH 5.4.

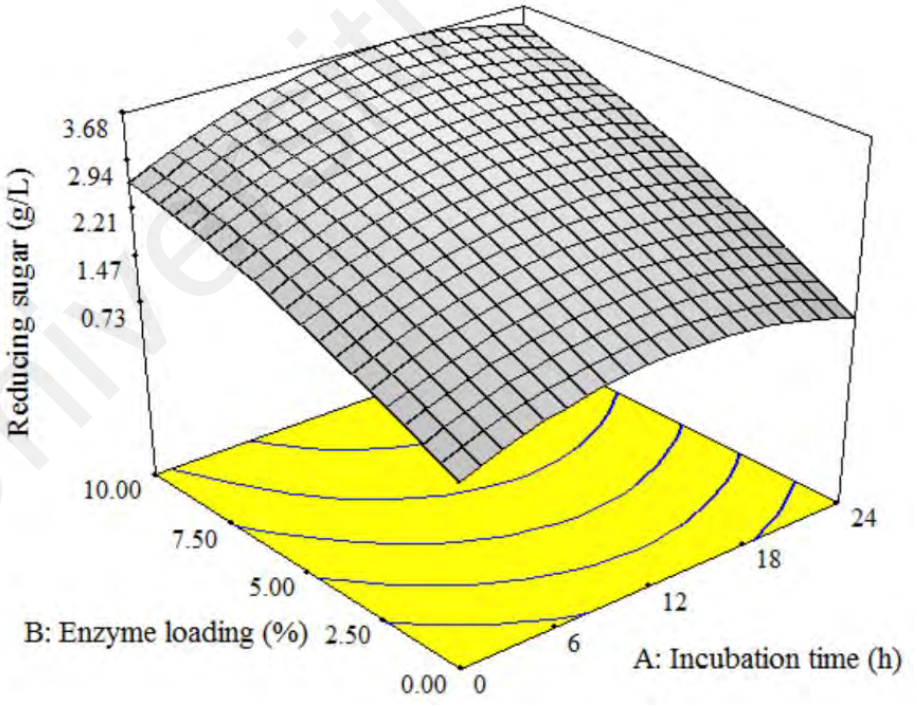

Figure 4.8: Response surface plot of reducing sugar yield from the effect of incubation time and enzyme loading at pH 6.0.

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A highest reducing sugar yield of 3.76 g L^{-1} was anticipated with 10% enzyme loading (v/v) and pH of 4.8 for 15 hours incubation time. At the optimized condition, the enzymatic hydrolysis of POME produced 3.75 g L^{-1} of total reducing sugar, which was consistent with the predicted value (Figure 4.9). Therefore, the model used was suitable to anticipate the optimal levels of the experimental parameters.

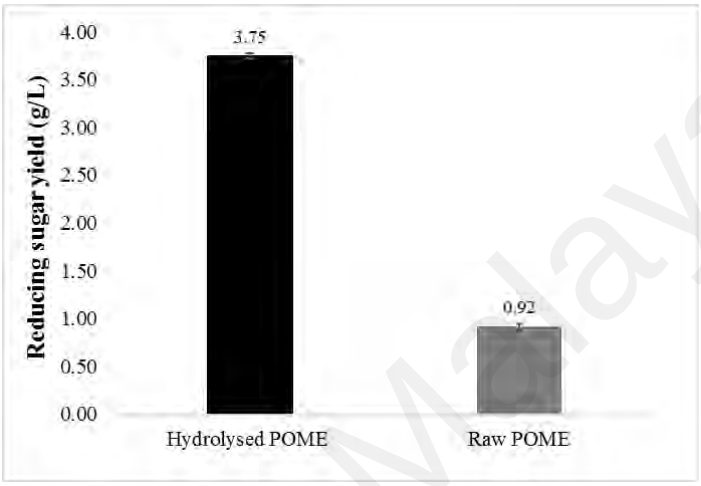

Figure 4.9: Reducing sugar yield in a verification experiment at the optimum condition.

Figure 4.10 showed a schematic diagram for the breakdown of lignocellulosic biomass from the POME using enzymes from the SMS of *P. pulmonarius*.

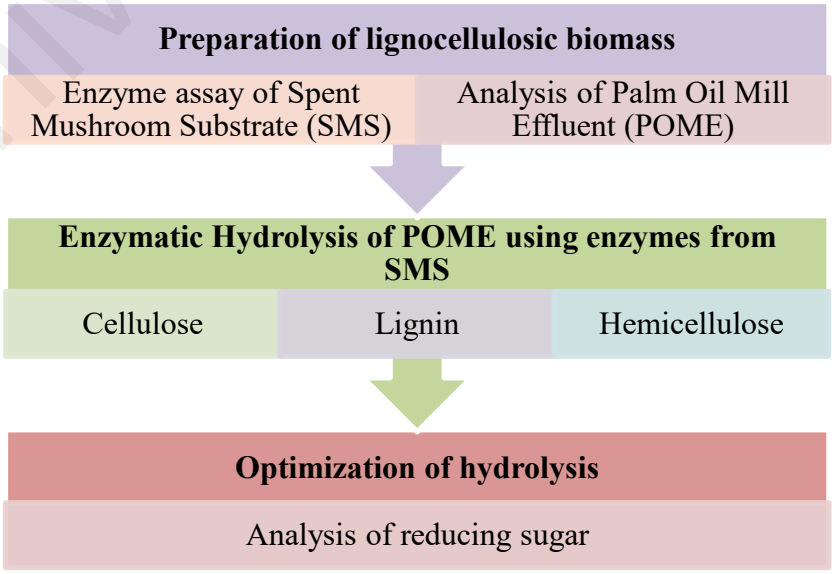

Figure 4.10: Schematic diagram for hydrolysis of POME and optimization using RSM.

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4.2 Batch Biohydrogen Fermentation

Biohydrogen cumulative volume from hydrolysed POME was 23-folds higher than hydrogen cumulative volume obtained in raw POME after 24 hours incubation (Figure 4.11).

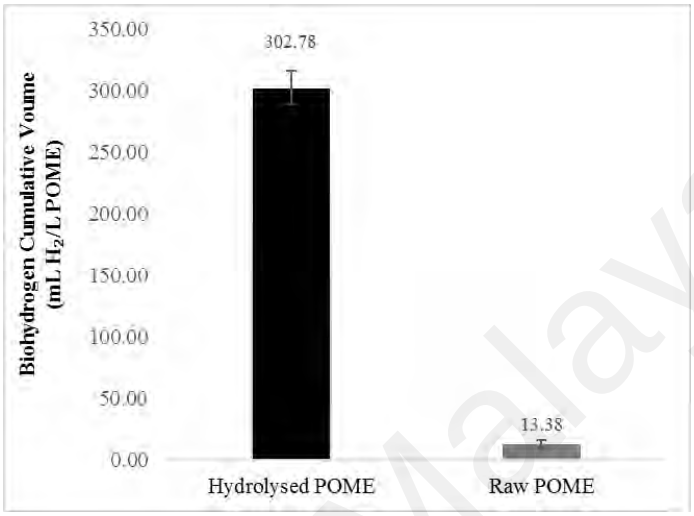

Figure 4.11: Biohydrogen cumulative volume after 24 hours of fermentation.

Similarly, the biohydrogen volume recorded in hydrolysed POME was 23-folds higher than biohydrogen produced by raw POME (Figure 4.12). A lower biohydrogen yield from the raw POME as compared to hydrolysed POME might be because of the high soluble metabolite accumulation available in the raw POME.

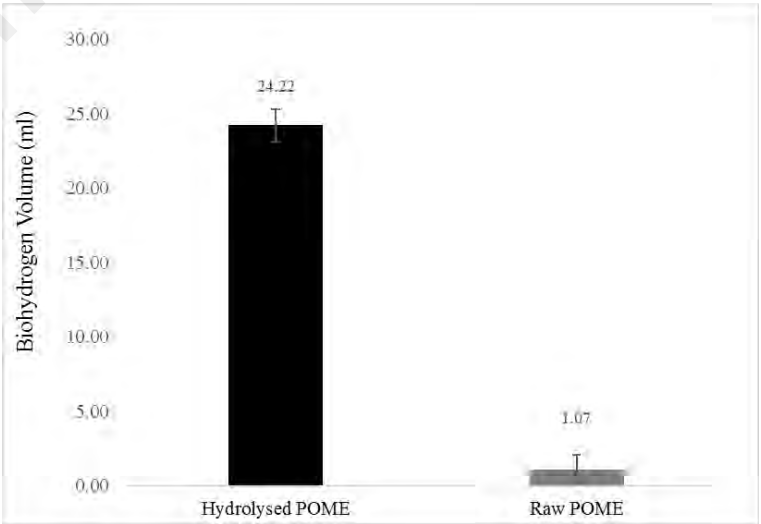

Figure 4.12: Volume of biohydrogen after 24 hours of fermentation.

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Lee et al. (2002) explained that hydrogen and soluble metabolites were produced during acidogenesis process. Thus, a high concentration of acidic metabolites possibly prevents cell growth by invading the cell membrane, results in a low hydrogen yield (Chong et al., 2009).

Fermentative hydrogen yield was affected by several factors including pH, temperature, substrate concentration and amount of inoculum feed (Norfadilah et al., 2016). Meanwhile, the biohydrogen output, biogas volume, variation of organic acids released, and specific hydrogen production rate were affected by medium pH value of the reaction condition (Kapdan & Kargi, 2006). In addition, most researchers reported the highest hydrogen yield is in the range between pH 5.0 to 6.0 (Fang & Liu, 2002).

The changes in POME composition after fermentation were recorded in Table 4.6. The reduction in reducing sugar yield from 3.75 g L^{-1} to 2.51 g L^{-1} in this study indicated that hydrogen producers might have consumed the sugar for their growth and other metabolites production (Rasdi et al., 2012).

Table 4.6: POME composition after 24 hours of fermentation.

POME sample	Hydrolysed POME	Raw POME
Reducing sugar (g L^{-1})	2.51 ± 0.08	0.57 ± 0.03
Final pH	5.41 ± 0.01	5.49 ± 0.01
Percentage of biohydrogen (%)	83.52 ± 2.37	36.35 ± 8.17
Percentage of CO_2 (%)	16.48 ± 2.37	63.65 ± 8.17
H_2 production rate (ml H_2/h)	1.01 ± 0.05	0.04 ± 0.01

The percentage of biohydrogen released from hydrolysed POME was 2.2-folds higher than the raw POME. Meanwhile, the percentage of carbon dioxide in the raw POME was 3.9-folds higher than the hydrolysed POME. As stated by Fang and Liu (2002), hydrogen content ensued an opposite trend of carbon dioxide at pH value ranged from 4.0 to 7.0.

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The biohydrogen production in the current study (302.78 mL H_2. L^{-1} POME) was lower than biohydrogen production in a study by Khaleb et al. (2012), which was 1439 mL H_2. L^{-1} POME was recorded (Table 4.7).

Table 4.7: Comparison of yield after batch biohydrogen fermentation.

Treatment of POME	Temp. (°C)	pH	Biohydrogen cumulative volume (mL H_2. L^{-1} POME)	Biohydrogen productivity (mL H_2. L^{-1} POME. h^{-1})	Reference
Enzymatic hydrolysis	37	6.0	1439.00	87.19	Khaleb et al., 2012
Enzymatic hydrolysis	35	6.5	134.74	8.42	Garritano et al., 2017
Acid-heat treatment	37	7.0	4304.00	NA	Kamal et al., 2012
Enzymatic hydrolysis	37	5.5	302.78	12.62	Present work

*NA: Not available

The difference might be due to higher enzymes activities from the commercial enzymes that was used during initial POME hydrolysis. This condition led to more sugar degradation and a higher biohydrogen production.

Meanwhile, biohydrogen production in this study was 2.2-fold higher than the one recorded by Garritano et al. (2017). The difference might be due to a higher initial pH (6.5) was applied during the fermentation, thus hydrogen producers could not adapt to the starting environment and may have been inhibited to produce hydrogen (Khanal et al., 2004).

Whereas, Fang and Liu (2002) described that hydrogen yield hit the optimum value at pH 5.5. This condition agrees with a study by Chong et al. (2009), that released a maximum hydrogen yield of 3195 ml H_2. L^{-1} POME at pH 5.5. Production of biohydrogen in the current study was 14-folds lower than hydrogen production in a study by Kamal et al. (2012), since the POME used was initially pre-treated using acid and heat which are

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hazardous, poisonous, and risky, despite the lignocelluloses could be hydrolysed effectively.

Mahmod et al. (2017) recorded 0.18 mmol $L^{-1}.h^{-1}$ of biohydrogen from the batch fermentation of acid-pretreated POME, which was 97% higher than raw POME. The result showed that POME pretreated with dilute acid is efficient for biohydrogen production. However, the use of acids for the pretreatment of POME is expensive and unreasonable for a larger scale hydrogen production.

Moreover, the acid-heat pretreatment method might have formed inhibitory compounds such as furfural. Therefore, biological enzymatic hydrolysis could be an alternative for degradation of the lignocellulosic materials since it is environmentally friendly.

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RECOMMENDATION AND CONCLUSION

5.1 Recommendation

Regardless of the various researches for enzymes uses from the mushrooms, little studies have been done for the commercial yield of the enzymes. The result of this study perhaps be a basis to enlarge the research opportunity in utilizing the SMS to treat another palm oil wastes and lignocellulose wastes, concurrently suggest an effective approach of utilizing waste from the developing mushroom industry to treat POME wastewater.

Thus, enzyme purification is required to fully understand the catalytic properties and differences in the enzyme composition. The purification of LiP, laccase, xylanase, endoglucanase and β-glucosidase shall be implemented and the catalytic properties of the enzymes from SMS of *P. pulmonarius* should be be distinguished to enzymes from SMS of other white rot fungi.

Another work to show that the crude enzymes extract is rigid for industrial application, heat-tolerance and pH-tolerance of each of the enzyme activities should be measured. Determination of the optimum temperature and pH, half-life time at various temperature and pH should be evaluated.

The promising potential of enzymes from the SMS of *P. pulmonarius* should be carried out to treat the SMS itself and other biowastes such as corn straw, sugarcane baggase, wheat straw, cotton stalk and oil palm empty fruit bunches.

Lastly, it is appropriate if the experiment for biohydrogen production is presented as time course and the soluble metabolites released after the fermentation is analysed.

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5.2 Conclusion

The study of using enzymes from SMS for treatment of palm oil wastes is certainly limited. However, hydrolyzing of POME using the SMS from a local mushroom farm is possible in this study. The weekly profiles showed that high titers of enzymes were present in *P. pulmonarius* bags.

Further, high productivities of concentrated enzymes extract were acquired from the second batch of eleventh week-old UMH004 bags. High titers of the concentrated enzymes LiP (214.1 U g^{-1}) and β-glucosidase (915.4 U g^{-1}) indicated that the SMS possibly be a good source of ligninolytic and hydrolytic enzymes.

In conclusion, the hydrolysis of POME and production of the fermentable sugars were carried out using the concentrated enzyme cocktail. Reducing sugar yield was optimized by employing CCD of RSM, according to the three parameters which were incubation time, enzyme loading and pH. The results showed that POME hydrolysis was significantly influenced by incubation time and enzyme loading ($p < 0.05$).

Meanwhile, the pH exhibited a minimum effect on the response ($p > 0.05$). The highest reducing sugar concentration (3.76 g L^{-1}) was accomplished at 12 hours incubation time with 10% enzyme loading (v/v).

It can be concluded that the fermentation of biohydrogen from the hydrolysed POME resulted in 302.78 ml H_2. L^{-1} POME, 23-folds higher than the raw POME (13.38 78 ml H_2. L^{-1} POME).

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Meanwhile, the rate of biohydrogen production after 24 hours of fermentation released 1.01 ± 0.05 ml H_2 per hour from the hydrolysed POME, 25 times higher than raw POME which was 0.04 ± 0.01 ml H_2 per hour.

Thus, the use of crude enzymes extracted from biowaste such as SMS to treat the wastewater is much cheaper compared with refined enzymes. SMS and POME may be valuable resources to produce value-added products and favorable option to the current treatment and dumping system.

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