Faculty of Resource Science and Technology

Feasibility of Bioethanol Waste Stream for Production of Laccase in *Pichia pastoris*

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Feasibility of Bioethanol Waste Stream for Production of Laccase in *Pichia pastoris*

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Laccases are multipurpose enzymes that have wide biotechnological applications. The present study seeks to evaluate the feasibility of spent fermentation broth generated upon the bioethanol production from sago *hampas*, as a feedstock for production of laccases in recombinant *Pichia pastoris* GS115. Characterisation of the sago bioethanol liquid waste (SBLW) indicated glycerol as the main component, along with glucose and lactic acid. Evaluation of SBLW as a fermentation feedstock for laccase production in *P. pastoris* GS115 showed that the cell growth was generally feasible when SBLW was used as a feedstock. The activity of laccases reached the highest (0.00076 U mL^{-1} ± 3.5×10^{-5}) in fermentations that employed 40% (v/v) SBLW. This represented 73% of that obtained using the standard synthetic medium. Supplementation of 40% (v/v) SBLW with 1.0% (w/v) yeast extract (YE) yielded enhancements of 1.2-fold and 1.5-fold of biomass concentration and laccase activity, respectively. The expression of laccases was further enhanced when the 40% (v/v) SBLW and 1.0% (w/v) YE was supplemented with 2.0% (v/v) glycerol. The highest laccase activity recorded was 0.00206 U mL^{-1} ± 5.8×10^{-5}. Both of the biomass and laccase production were increased by 1.9-fold and 2.1-fold in comparison to that achieved by the standard synthetic medium. The efficacy of laccases produced by the optimised SBLW medium was evaluated in terms of the capability of the enzymes to decolourise Remazol Brilliant Blue R (RBBR) dye. The enzyme showed a decolourisation percentage of 68.6%, which represented 91% of the decolourising capability of laccases produced using standard BMMH medium. This indicates the promising efficacy of laccases produced from SBLW. In summary, this work gives an important insight into exploitation of SBLW for production of value-added products. Moreover, this work contributes to the development of recombinant laccase production using low cost and eco-friendly feedstock.

Keywords: Bioethanol spent broth, bioproducts, laccase, *Pichia pastoris*, sago

Kebolehsanaan Sisa Bioetanol untuk Penghasilan Laccase dari Pichia pastoris

ABSTRAK

Laccases adalah enzim serbaguna yang mempunyai aplikasi bioteknologi yang luas. Kajian ini bertujuan untuk menilai kebolehlaksanaan menggunakan kaldu fermentasi yang dihasilkan selepas penghasilan bioetanol dari sagu hampas sebagai bahan baku bagi produksi laccases di rekombinan Pichia pastoris *GS115. Pencirian sisa cecair bioetanol dari sagu (SBLW) menunjukkan gliserol sebagai komponen utama, bersama dengan glukosa dan asid laktik. Penilaian SBLW sebagai bahan baku fermentasi untuk pengeluaran laccase di* P. pastoris *GS115 menunjukkan bahawa pertumbuhan sel umumnya dapat dilaksanakan ketika SBLW digunakan sebagai bahan baku. Aktiviti laccases mencapai tahap tertinggi (0.00076 U mL^{-1} ± 3.5 × 10^{-5}) dalam fermentasi yang menggunakan 40% (v/v) SBLW. Ini mewakili 73% daripada yang diperoleh menggunakan media sintetik standard. Penambahan 40% (v/v) SBLW dengan 1.0% (w/v) ekstrak ragi (YE) menghasilkan peningkatan 1.2 kali ganda dan 1.5 kali ganda kepekatan biojisim dan aktiviti laccase. Ekspresi laccases diperbanyakkan lagi ketika 40% (v/v) SBLW dan YE 1.0% (w/v) ditambah dengan gliserol 2.0% (v/v). Aktiviti tertinggi laccase yang dicatatkan ialah 0.00206 U mL^{-1} ± 5.8 × 10^{-5}. Kedua-dua pengeluaran biojisim dan laccase meningkat sebanyak 1.9 kali ganda dan 2.1 kali ganda berbanding dengan yang diperoleh oleh medium sintetik standard. Keberkesanan laccases yang dihasilkan oleh medium SBLW yang dioptimumkan dinilai dari segi kemampuan enzim untuk menyahwarnakan pewarna Remazol Brilliant Blue R (RBBR). Enzim menunjukkan peratusan penyahwarnaan 68.6%, yang mewakili 91% dari kemampuan penyahwarnaan laccases yang dihasilkan menggunakan medium BMMH standard. Ini menunjukkan keberkesanan laccases yang menjanjikan yang dihasilkan dari SBLW. Ringkasnya, kajian ini memberikan gambaran penting mengenai eksploitasi SBLW*

untuk pengeluaran produk bernilai tambah. Lebih-lebih lagi, kajian ini menyumbang kepada pengembangan penghasilan laccase rekombinan menggunakan bahan makanan kos rendah dan mesra alam.

Kata kunci: *Bioproduk, cecair sisa bioetanol, laccase,* Pichia pastoris*, sagu*

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LIST OF ABBREVIATIONS

ABTS	2,2’-azinobis-(3-ethylbenzthiazoline 6-sulfonic acid)
BMGH	Buffered minimal glycerol histidine medium
BMMH	Buffered minimal methanol histidine medium
BOD	Biochemical oxygen demand
CO_2	Carbon dioxide
cm	Centimeter
COD	Chemical oxygen demand
Cu	Copper atom
°C	Degree celcius
DCW	Dry cell weight
$g_{dcw}\ L^{-1}$	Dry cell weight in gram per liter
g	Gram
$g\ L^{-1}$	Gram per liter
HPLC	High Performance Liquid Chromatography
H	Hour
HMF	Hydroxymethylfurfural
$IU\ g^{-1}$	International units per gram
$IU\ mL^{-1}$	International units per milliliter

L	Liter
µL	Microliter
µmol	Micromole
µ (h^{-1})	Micro per hour
mg L^{-1}	Milligram per liter
mL	Milliliter
mL min^{-1}	Milliliter per minute
mm	Millimeter
mM	Millimolar
min	Minutes
M	Molar
Nm	Nanometer
n	Number of variable
OD	Optical density
h^{-1}	Per hour
min^{-1}	Per minute
M^{-1} cm^{-1}	Per molar per centimeter
%	Percentage
PDA	Potato dextrose agar

RBBR	Remazol Brilliant Blue R
rpm	Revolution per minute
SBLW	Sago bioethanol liquid waste
SHH	Sago *hampas* hydrolysate
SSF	Solid state fermentation
TOC	Total organic compound
U	Unit
U g^{-1}	Unit per gram
U mL^{-1}	Unit per milliliter
U L^{-1}	Unit per liter
v/v	Volume per volume
w/v	Weight per volume
YE	Yeast extract
YMB	Yeast malt broth
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose

CHAPTER 1

INTRODUCTION

1.1 Introduction

Exploitation of agricultural waste as alternative feedstocks for bioethanol production has gained increasing interests among biorefineries. One of the potential agricultural wastes in Malaysia is sago fibre, which composed of approximately 50% to 60% of residual starch along with other lignocellulosic components (Mohammad et al., 2020). The use of hydrolysate of sago fibre as a substrate for producing bioethanol has been reported by Awang-Adeni and co-workers (Mohammad et al., 2020).

Given a far-sighted context, with the increasing demand for bioethanol at the industrial level in the future, it is estimated that the production of the waste stream following bioethanol distillation will also increase. Direct disposal of the waste stream may lead to serious environmental pollutions due to the high organic content, dissolved solids and other toxic compounds (Espana-Gamboa et al., 2011). Therefore, there is an emerging need to investigate the fate and potential of the waste stream generated following the bioethanol production.

Research in Brazil, India and Europe have shown the feasibility of exploiting vinasse, the glycerol-based stillage discharged after the industrial production of bioethanol using molasses derived either from starch crops (wheat and cassava), sugar crops (beet and sugarcane) or cellulosic material (harvesting crop residues and wood) (Christofoletti et al., 2013), as feedstocks for production of several products such as polyhydroxyalkanoates (Bhattacharyya et al., 2012; Pramanik et al., 2012) volatile fatty acids and biohydrogen (Sydney, 2013), xylitol (Salgado et al., 2010) and transaminases (Suhaili et al., 2019).

Similar potential could also be envisaged in the case of the stillage generated from the bioethanol production using sago *hampas* as a substrate. To date, the exploitation of the waste stream generated following the production of bioethanol from sago fibre are poorly explored. Thus, a fundamental investigation into properties and applications of the waste stream will shed some light on its rational fate and direction.

One of the industrially relevant enzymes laccases, were used as the model case throughout this study. Laccases are polyphenol oxidases that are widely used as bioremediators for treating wastewater generated from paper and pulp, petrochemical and textile industries (Chandra & Chowdhary, 2015), bioremediation of xenobiotics and as biosensors in medical diagnostics (El-Batal et al., 2015). The demand for laccases for industrial purposes has been rapidly increased, hence necessitates the development of recombinant laccase production. Previously, a laccase producing strain, *P. pastoris* GS115 was established by Sing (2017). The laccase gene from the fungus *Marasmius cladophyllus* UMAS MS8 was expressed heterologously in *P. pastoris* GS115 with the aim of getting an increased level of laccase enzyme expression as compared with the native fungus *M. cladophyllus* (Sing, 2017). Nonetheless, development of strategies for optimal and inexpensive production of laccases by *P. pastoris* GS115 is still under-examined since the strain is relatively new.

Previous studies of laccase production were based on a synthetic medium, Buffered Methanol-Complex Medium (BMMH) (Maestre-Reyna et al., 2015), which is fossil-based and often deemed as expensive. Hence, there is a need for developing a cost-effective and sustainable strategy for producing laccases in *P. pastoris* GS115. Then, the efficacy of laccases produced in *P. pastoris* GS115 was evaluated in terms of the capability of the enzymes to decolourise Remazol Brilliant Blue R (RBBR). RBBR dye is an example of an

anthraquinone dye, which has a structural resemblance to some polycyclic aromatic hydrocarbons (Bohacz, 2020). The decolourisation of RBBR dye by ligninolytic enzymes of white rot fungi such as laccases have been widely reported (Adak et al., 2016; Syafiuddin & Fulazzaky, 2021), and the RBBR dye was decolourised the fastest as compared to the other dyes (Sing et al., 2013; Noman et al., 2020).

The main aim of this study is to explore the feasibility of liquid waste generated after the production of bioethanol from sago *hampas*, as a fermentation feedstock for recombinant laccase production in *P. pastoris* GS115. The specific objectives of this research are:

i. To characterise the composition of liquid waste generated upon the production of bioethanol from sago *hampas* with particular emphasis on its fermentable sugars and inhibitory compounds;
ii. To investigate the feasibility of sago bioethanol liquid waste as a fermentation feedstock for producing recombinant laccases in *P. pastoris* GS115; and
iii. To optimise the production of recombinant laccases in *P. pastoris* GS115 using sago bioethanol liquid waste (SBLW) as a feedstock.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioethanol

Bioethanol is an alcohol produced by microbial fermentation. According to Cripwell et al. (2020), bioethanol is commonly produced from sugary and starchy crops such as corn and sugarcane. It is clear and colourless liquid, biodegradable and has low toxicity. Due to the huge demand worldwide, the global production of bioethanol is rapidly increasing. The world bioethanol production was increased about 23% from 90 billion L in 2012, to 117 billion L in 2014 and is expected to reach approximately 158 billion L by 2023 (Toor et al., 2020).

According to Lisin (2020) more than 70% of the ethanol production in the world is produced by Brazil and the United States, which are known as two major ethanol producers. Both countries have started mass production of bioethanol derived from sugarcane and corn since early 1970s. There is a rising emphasis in the production of bioethanol from lignocellulosic biomass for the past few years. More cellulosic ethanol will be eventually produced from various types of feedstocks, but intensive research are needed to reduce the cost production of ethanol to make them more economical (Chandel et al., 2019). The bioethanol production can be minimised if renewable substrates are utilised. Thus, the use of biomass as substrates for bioethanol production is seen as an interesting option.

Some developing countries are able to reduce the notable amount of oil imported and replace their existing fossil-based fuel by producing bioethanol (Yusoff et al., 2021). Bioethanol is widely known as an alternative energy source with high potential in automotive applications since the natural energy resources such as fossil fuel, petroleum and coal are

estimated to deplete within the next 40 - 50 years (Abbas et al., 2020). Besides, bioethanol result in improved vehicle performance, reduce the consumption of the fossil fuels, improve air quality and less toxicity (Du et al., 2016).

2.1.1 Applications of bioethanol

Bioethanol is widely used as liquid biofuel in power automobiles in which the application is about 75% of the total bioethanol produced in the world. It is currently used as the major additive for gasoline in automobile industries, and these combination helps to reduce the hydrocarbon and carbon monoxide emissions from vehicles (Jhang et al., 2020). The combination of ethanol and gasoline can be at any concentration up to 100% ethanol. This has vastly minimised the consumption of petroleum fuel. Air pollution can also be reduced too as bioethanol is generally CO_2 neutral. Moreover, bioethanol is also used in other sectors such as in the chemical, transportation and pharmaceuticals industry. Table 2.1 outlines the summary of applications of bioethanol in different sectors.