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INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA  
بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

PROCESS OPTIMIZATION ON PRODUCTION  
OF LIGNIN PEROXIDASE OF SEWAGE  
TREATMENT PLANT SLUDGE IN A STIRRED  
TANK BIOREACTOR AND ITS  
BIODEGRADATION OF SYNTHETIC  
INDUSTRIAL DYES

BY

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## ABSTRACT

The increasing volume of sewage sludge produced and the total managing cost every year has been one of the major environmental issues in Malaysia. Bioconversion of sewage treatment plant (STP) sludge by liquid state bioconversion (LSB) is proposed to solve these problems through the recovery of products such as lignin peroxidase (LiP) enzyme. A lab-scale study was carried out to produce LiP enzyme by white-rot fungus *Phanerochaete chrysosporium* using STP sludge as a major substrate. The experiments were conducted in two liter stirred tank reactor (STR). The optimization of aeration and agitation rates was done using full-factorial design (FFD). Using the statistical analysis, the optimum aeration and agitation rates determined were 2.0 vvm and 200 rpm respectively with maximum production of 225 UL<sup>-1</sup> in 3 days of fermentation. The validation experiment showed that the maximum lignin peroxidase was 744 UL<sup>-1</sup> in five day of fermentation. This enzyme activity was stable at pH 5.0 and temperature 55°C which maintained the activity more than 80% up to 12 hours of incubation. Optimization by one factor at-a time (OFAT) and statistical approach was carried out to evaluate the process conditions on decolorization of methylene blue dye using LiP enzyme in static mode. The OFAT technique indicated that the optimum conditions for decolorization of methylene blue (MB) dye was at temperature 55°C, pH 5.0 with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) concentration 4.0 mM. The addition of veratryl alcohol to the reaction mixtures did not show any positive effect on decolorization. The initial concentration of MB and the activity of LiP enzyme were further optimized using response surface methodology (RSM). The contour and surface plots suggested that the optimum initial concentration of MB and LiP activity predicted were 15-20 mg/L and 0.687 U/ml respectively for removal of 63-65%. The final validation in static and agitate mode showed that agitation gave higher removal in decolorizing MB. The mixtures solution was colorless as it reached the equilibrium time within 60 minutes with 90% removal compared to only 70% color removal in static mode at the same conditions: initial dye concentration 15 mg/L, LiP activity 0.687 U/ml, H<sub>2</sub>O<sub>2</sub> concentration 4.0 mM, at temperature 55°C in pH 5.0. In fact, this is a new biotechnological approach for the biodegradation and biosolids accumulation of sludge beside the production of industrial enzyme (LiP) which exhibits the benefit of low production cost as well as environmentally friendly.

## ملخص البحث

إن زيادة حجم فضلات المجاري وكلفة معالجتها سنوياً أصبحت من المشاكل البيئية الرئيسية في ماليزيا. تم إقتراح طريقة التحويل البيولوجي في معمل معالجة فضلات المجاري بواسطة الطريقة السائلة لحل هذه المشاكل بواسطة إنتاج أنزيم ليجنين بيروكسيديس. تم إجراء عدة تجارب مختبرية لإنتاج هذا الانزيم بواسطة فطريات وايت-روت المسماة فانيروجيت جرسوسبوريوم وإستخدام الفضلات كوسط رئيسي في هذه العملية. تم إستخدام خزان المفاعل بحجم لترين لإجراء التجارب كما تم إستخدام التصميم بكامل العوامل (أف أف دي) لغرض الحصول على أقصى قيم لعملية التزويد بالهواء والتحرك. تم التوصل الى أقصى قيم لعملية التزويد بالهواء 2 (في في أم) وسعة تحريك 200 دورة بالدقيقة مع أقصى إنتاج 225 وحدة باللتر في ثلاثة أيام تخمير باستخدام التحليل الاحصائي. تم التأكد من النتائج وتبين إن حجم الانزيم كان 744 وحدة باللتر في خمسة أيام تخمير. نشاط الانزيم كان ثابتاً عند درجة حامضية 5 وحرارة 55 درجة مئوية والتي ثبت فيها النشاط بنسبة 80% لغاية فترة حضانة 12 ساعة. تم إستخدام طريقة عامل واحد في الوقت الواحد (أف أف أي تي) وعملية الاحصاء لحساب أقصى ظروف للعملية وتأثيرها على قصر لون صبغة المثيلين الازرق باستخدام الانزيم في الوضع الستاتيكي. لم يظهر أي تأثير إيجابي على عملية القصر باضافة كحول فيراترايل الى خليط التفاعل. تم دراسة التركيز الاولي للمثيلين الازرق ونشاط الانزيم بواسطة (آر أس أم) والحصول على أقصى قيم والتي كانت 15-20 ملغم/لتر و0.687 وحدة باللتر بالتتابع لازالة 63-65%. تم التثبيت من الطريقتين الستاتيكية والمتحركة والتي وضحت إن التحريك أعطى أعلى إزالة للون. المحلول كان بدون لون عندما وصل الى وقت التوازن في 60 دقيقة مع نسبة إزالة 90% مقارنة مع 70% في الحالة الستاتيكية وفي نفس الظروف. التركيز الاولي للصبغة كان 15 ملغم/لتر، نشاط الانزيم 0.687 وحدة بالمللتر، تركيز البيروكسيد 4.0 ملي مول، الحرارة 55 درجة مئوية، ودرجة الحامضية 5. في الحقيقة، هذا يعتبر تقنية بيولوجية جديدة في التحليل وتجميع المواد البيولوجية الصلبة من النفايات بالاضافة الى إنتاج الانزيم والذي يعرض الفائدة من الانتاج قليل الكلفة وغير المضر بالبيئة.

## ABSTRAK

Kenaikan kuantiti isipadu enapcemar kumbahan yang terhasil dan jumlah kos pembiayaan untuk menguruskannya setiap tahun menjadi salah satu isu alam sekitar yang serius di Malaysia. Biopenukaran keadaan cecair (LSB) terhadap enapcemar daripada loji rawatan kumbahan (STP) merupakan antara cadangan untuk mengatasi permasalahan ini melalui penghasilan produk seperti enzim lignin peroksida (LiP). Kajian berskala makmal telah dijalankan bagi menghasilkan enzim LiP oleh fungi *Phanerochaete chrysosporium* menggunakan enapcemar kumbahan sebagai bahan utama. Kajian ini dilakukan dengan menggunakan tangki reaktor berpusar (STR) dua liter. Rekabentuk faktor penuh (FFD) digunakan untuk mengoptimumkan kadar pengudaraan dan agitasi proses ini. Melalui analisis statistik, kadar pengudaraan dan agitasi yang paling sesuai dicatatkan adalah 2.0 vvm dan 200 rpm dengan jumlah aktiviti 225 UL<sup>-1</sup> dalam tempoh tiga hari. Eksperimen pengesahan menunjukkan penghasilan maksimum enzim LiP adalah pada hari kelima sebanyak 744 UL<sup>-1</sup>. Aktiviti enzim LiP didapati stabil pada pH 5.0 dengan suhu 55°C, yang mana 80% aktiviti dapat dikekalkan selepas 12 jam inkubasi. Pengoptimuman secara kaedah satu faktor pada satu masa (OFAT) dan statistik dijalankan bagi menentukan keadaan proses penyahwarnaan methylene biru (MB) menggunakan enzim LiP dalam keadaan statik. Keadaan optimum untuk penyahwarnaan MB menggunakan kaedah OFAT adalah pada suhu 55°C, pH 5.0 dan kepekatan hidrogen peroksida (H<sub>2</sub>O<sub>2</sub>) 4.0 mM. Penambahan veratryl alkohol pada larutan campuran tidak menunjukkan sebarang kesan positif pada penyahwarnaan MB. Metodologi tindak-balas permukaan (RSM) digunakan untuk mengoptimumkan kepekatan asal MB dan aktiviti enzim LiP. Plot kontur dan plot permukaan menunjukkan kepekatan asal MB dan aktiviti enzim LiP yang optimum adalah 15-20 mg/L dan 0.687 U/ml untuk menyahwarnakan antara 63-65% pewarna MB. Ekperimen pengesahan dalam keadaan statik dan agitasi menunjukkan penyahwarnaan MB adalah lebih tinggi dalam keadaan agitasi. Larutan campuran bertukar jernih selepas mencapai masa seimbang 60 minit dengan penyahwarnaan sebanyak 90% berbanding dalam keadaan statik yang hanya menyahwarnakan pewarna MB 70% dalam keadaan yang sama: kepekatan asal MB 15 mg/L, aktiviti LiP 0.687 U/ml dan kepekatan H<sub>2</sub>O<sub>2</sub> 4.0 mM pada suhu 55°C dalam pH 5.0. Hakikatnya, ini adalah kaedah bioteknologi yang baru untuk biodegradasi dan pengumpulan biopepejal enapcemar disamping penghasilan enzim industri (LiP) yang memberi faedah dengan kos penghasilannya yang rendah disamping mesra alam sekitar.

## APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality as a thesis for degree of Master of Science (Biotechnology Science)

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## DECLARATION PAGE

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Mariatul Fadzillah binti Mansor

Signature .....

Date .....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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# **CHAPTER ONE**

## **INTRODUCTION**

### **1.1 PROBLEM STATEMENT**

Sewage is the largest contributor of organic pollution to water resources as well as to surrounding environments all over the world. Therefore, the sludge treatment and disposal in proper ways are probably the most difficult tasks. Increasing civilization and urban development generate large amount of sludge and its disposal is requires since it affects environment. The U.S. Environmental Protection Agency (U.S. EPA, 1999) reported, quantity of domestic sludge (biosolids) produced annually in the United State has increased dramatically, from roughly 4.6 million dry tons in 1972 to 6.9 million dry tons in 1998 and expected to increase to 8.3 million dry tons by the year 2010. According to Department of Environmental Malaysia (DOE, 1996), sludge contribution is top listed (64.4%); followed by animal husbandry wastes (32.6%), agro based (1.7%) and industrial effluent (1.3%) in term of biological oxygen demand (BOD) load.

The management of the ever-increasing volume of domestic and industrial organic wastes has been one of the prime environmental issues in Malaysia. Approximately 4.2 million cubic meters of sewage sludge is produced by Indah Water Konsortium (IWK) annually in Malaysia and the total cost of managing is estimated at RM 1 billion (Kadir and Velayutham, 1999). This sludge volume is expected to rise to 7 million cubic meters by the year 2020. Malaysia has yet to adopt a practical, economic and acceptable approach in managing and disposing sewage sludge. The present practice is either to co-dispose it with solid waste at landfill sites or direct

disposal in shallow trenches (Zain, Basri, Suja and Jaafar, 2001). The safe and environmental-friendly disposals of this huge quantity of sludge are the main concern of IWK.

## **1.2 JUSTIFICATION OF RESEARCH**

In recent years, interest has switch on to the methods or processes based on the resource recovery approach known as recycling and reusing of organic waste produced from different sources of domestics as well as industry. The concept of product recovery from organic residues by applying biological-based treatment is becoming more popular to be used for various purposes. Previously composting as a resource recovery was used as an acceptable alternative for the sludge treatment due to its potential use for land application as biofertilizer and soil conditioner (Fang, Wong, J.W.C., Li and Wong, M.H., 1998; Molla, 2002). Bioconversion of sewage treatment plant sludge by liquid state bioconversion (LSB) is being proposed as solution to these problems through the recovery of products especially ligninolytic enzymes such as lignin peroxidase (LiP) and manganese peroxidase (MnP). This is a new biotechnological approach for the biodegradation and biosolids accumulation of sludge beside the production of industrial enzyme which exhibits the benefit of being very low treatment and production cost and environmentally friendly (Alam, Fakhru'l-Razi and Molla, 2003a; Alam, Muyibi, Jamal, Jalal, 2004).

Submerged cultivation is generally recognized as the most economic way for production of extracellular enzymes (Kapich, Prior, Botha, Galkin, Lundell and Hatakka, 2004). However, agitation in many cases leads to inhibition of ligninolytic activity in *P. chrysosporium* (Kirk and Farrell, 1987; Venkatadri and Irvine, 1990). Previously, the difficulty in producing ligninolytic enzymes has led to the addition of

some detergents into medium composition to stimulate enzyme production. The research by Venkatadri and Irvine (1990) shown the detergents Tween 20, Tween 40, Tween 60, Tween 80 and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) were able to protect both purified ligninase and extant ligninase in culture fluids (free of biomass) against mechanical activation. Enhanced activity may also be obtained by addition of veratryl alcohol, manganese salts, organic acid chelators, polypropylene glycol, phospholipids, and saturated fatty acids (Kirk, Croan, Tien, Murtagh and Farrell, 1986; Janshekar and Fiechter, 1988; Venkatadri and Irvine, 1990, 1993). The high cost in isolation, purification and addition of detergents and chemicals in producing ligninolytic enzymes prior to the expensive price of commercial enzymes (Durán and Esposito, 2000).

Several studies have been conducted using lignocellulosic materials as substrates for the produce of high activity of ligninolytic enzymes. The examples of the addition of the lignocellulosic substrates into culture medium are wheat straw, hemp woody (Kapich et al., 2004), olive pomace (Haddadin, Al-Natour, Al-Qsous and Robinson, 2002), wheat straw (Arora, Chander and Gill, 2002), grape stalks, grape seeds and barley bran (Lorenzo, Moldes, Couto and Sanroman, 2002). The lignocellulosic materials used are usually from the agricultural wastes. As such, the productions of ligninolytic enzymes are low in costs and also can help reducing the agricultural wastes.

Potentially less expensive alternative lignocellulolytic enzyme could be produced by a number of filamentous fungi utilizing the abundant domestic and industrial organic residue such as STP sludge and POME available at Malaysia. The most common fungus studied for lignin degradation is *Phanerochaete chrysosporium* (Haddadin et al., 2002; Arora et al., 2002; Kapich et al., 2004) which this fungus can

degrade lignin selectively (Ward, Hadar, Dosoretz, 2004). *Phanerochaete* (usually pronounced as fan-er-oh-KEE-tee) *chrysosporium* is a white rot causing basidiomycete. It has a relatively high optimum growth temperature, degrades lignin, forms copious asexual spores and sexual fruiting structures, and lacks laccase activity. These properties have made *P. chrysosporium* the most known lignin degrader (Janshekar and Fiechter, 1988). Degradation of lignin and lignin model compounds by *P. chrysosporium* has been shown to be associated with an extracellular peroxidase sometimes called lignin peroxidase or ligninase. The enzyme requires H<sub>2</sub>O<sub>2</sub> for activity (Tien and Kirk, 1984). *P. chrysosporium* produces variable amounts of LiP isoenzymes depending on the culture conditions, but separation methods may also cause variations.

Cultures can be more closely monitored in bioreactors than in shake flasks for a better control of the whole process. Several parameters information can be collected such as oxygen requirements of the cells, their shear sensitivity and foaming characteristics. The main objective in scaling-up the process is to identify the limitations imposed by the reactor on organism activity and the prime concern is whether or not the reactor can provide conditions for optimal activity. Janshekar and Fiechter (1988) have demonstrated ligninase production in a 42 liter stirred tank reactor only when polypropylene glycol was present in the culture medium. Attempts by Janshekar and Fiechter (1988) to scale-up the process to a 300 liter reactor resulted in no observable ligninase production.

Linko (1988a) was able to produce ligninase in 10 liter bioreactor in which mixing was provided with an air and/or oxygen supply without an impeller. While Linko study (1988b) shows the continuously production of lignin peroxidase by nylon-web or polyurethane immobilized *Phanerochaete chrysosporium* in a modified

bioreactor, agitated either with the air and oxygen flow alone or in combination with a mechanical stirrer. Venkatadri and Irvine (1993) demonstrated the use of hollow fiber reactor and a stirred tank reactor modified into a unique silicone membrane reactor for the cultivation of *P. chrysosporium* and production of high levels of LiP. The production of LiP in packed bed bioreactor operated in semi continuous mode was studied by Feijoo, Dosoretz and Lema (1995). Bosco, Ruggeri and Sassi (1996) demonstrated the LiP production in trickle fixed bed reactor with working volume 250 ml of liquid medium at batch mode.

The ligninolytic enzymes have wide application that currently used in removal of dyes from industrial effluents (Young and Yu, 1997; Trupkin, Levin, Forchiassin and Viale, 2003; Mohorčič, Teodorovič, Golob and Friedrich, 2006; Ferreira-Leitão, Carvalho and Bon, 2007), bio-bleaching (Silva, Melo and Oliveira, 2005) and for treating hazardous waste (Huang, Wang, Liu, Hu, Qu and Gao, 2003; Torres, Bustos-Jaimes and Borgne, 2003; Akhtar and Husain, 2006). Textile dyeing effluents containing recalcitrant dyes are polluting waters due to their color and by the formation of toxic or carcinogenic intermediated such as aromatic amines from azo dyes that have to be eliminated before release into natural water streams. A special problem is encountered in the application synthetic dyes which have a complex aromatic molecular structure and are designed to be resistant to physical, chemical and microbial fading (Wesenberg, Kyriakides and Agathos, 2003). Therefore, elimination of dyes from textile dyeing effluents currently represents major ecological concerns. Enzymatic methods used to solve these problems generally have low energy requirements, are easy to control, can operate over a wide range of conditions and have a minimal environmental impact (Torres et al., 2003).

Application of LiP enzymes to several bioprocesses requires stability of such enzymes. It was the important factor in determining both the economic and technical feasibility of application for industrial uses and is also critical factor in optimizing commercial production of enzymes. Couto, Moldes and Sanromán (2006) detected the optimum value of LiP was pH 4.2 and temperature 34°C using orthogonal design. Bosco, Capolongo and Ruggeri (2002) also found that a mixture of LiP isoenzymes from immobilized cultures of *P. chrysosporium* catalyzed oxidation reactions at acidic pH and at temperature between 25°C to 60°C. In contrast to findings by Ikehata, Buchanan, Pickard and Smith (2005), the peroxidase from *Coprinus sp.* was found most stable at 50°C and under basic conditions (up to pH 10). The study on lipases enzyme stability from *Rhizopus oryzae* and *rhizopus rhizopodiformis* isolated from palm oil mill effluent (POME) found the enzyme have optimum temperature 45°C and pH 6.0 (Razak, Salleh, Musani, Samad and Basri, 1997). The data from the stability studies will be a great importance for the application of such enzymes to several biotechnological processes.

### **1.3 SCOPE OF RESEARCH AND BENEFITS**

One of the major goals in this study is to reuse the organic residue STP sludge for the production of value added products that will provide solutions to waste management problems through effective and efficient bioconversion of sludge. Currently, high costs of enzyme production have hindered the industrial application of ligninolytic enzymes through various expensive media to producing such enzymes. Efficient bioconversion process using low costs substrate STP sludge may provide low treatment and production cost for various industrial applications. So far, no investigations has been done on liquid state bioconversion of renewable substrate STP