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Optimisation of Lignin Peroxidase Production Using Locally Isolated *Pycnoporus* sp. Through Factorial Design

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Abstract: Lignin peroxidase has been extensively studied and has been reported to produce by white rot fungus. The highest lignin peroxidase producer from local isolates, identified as *Pycnoporus* sp. was selected for the optimisation study. Factorial design approach was significant to determine the optimum conditions that significantly influenced the production of lignin peroxidase by *Pycnoporus* sp. Several factors were selected in a range indicated by -1 and +1 for lower and upper level, respectively. The results of ANOVA were analysed to check for the significant factors. Optimum condition for the highest lignin peroxidase activity of 51.1 U L^{-1} was obtained at 24 mM of nitrogen concentration, agitation speed at 110 rpm, pH 3.5, inoculum concentration of 6×10^6 spores mL^{-1} and with the addition of inducer (veratryl alcohol). Considering the results obtained, this statistical design was effective in improving the lignin peroxidase production from *Pycnoporus* sp.

Key words: Ligninolytic, lignin peroxidase, optimise, *Pycnoporous* sp.

INTRODUCTION

Lignocellulosic biomass offers an inexpensive and abundant source of renewable resources. It includes agricultural residues such as Palm Kernel Cake (PKC) and Empty Fruit Bunch (EFB) from palm oil industry, rice straw and rice husk from paddy industry and forestry residue. Lignocellulosic biomass deriving from mentioned agricultural sub-sectors have and will continue to provide large quantities of valuable nutrients to sustain livestock, particularly ruminant production in Malaysia and supports the evolving of recent technology on biomass based ethanol production (Angenent *et al*., 2004). Effective conversion or biodegradation of lignocellulosic materials requires 3 sequential steps:

- Size reduction
- Pre-treatment/fractionation
- Enzymatic hydrolysis (Zhang and Lynd, 2004)

One of the most important and difficult challenges is to overcome the recalcitrance of natural lignocellulosic materials, which must be enzymatically hydrolysed (Moreira, 2005; Mosier *et al*., 2005; Wyman *et al*., 2005) Lignocellulosic materials contained a complex structure of lignin. Removal of lignin was important in order for enzymatic hydrolysis to occur as it acts as a barrier to most of agricultural wastes.

Lignin is a three-dimensional heterogeneous polymer stored in the plant cell wall of all vascular plants. It is the most abundant renewable aromatic biopolymer in the biosphere. Owing to its recalcitrant nature, lignin is remarkably resistant to degradation by most microorganisms, an important factor limiting the rate of degradation of lignocellulosic materials. Filamentous fungi, primarily the white-rot fungi and related basidiomycete fungi are most efficient terrestrial microorganism capable of catalyzing lignin biodegradation. Studies of lignin biodegradation are important for possible biotechnology application, since lignin polymers are major obstacles to the efficient utilization of lignocellulosic materials in a wide range of industrial processes (Eriksson *et al*., 1990). Although, removal of lignin can be done using chemical and physical pre-treatment, it is not naturally occur as lignin compound and chemical reagent itself will cause environmental pollution. Therefore, biotechnology methods that are environmental friendly are preferred as a tool in any processes.

Various white-rotting fungi isolated have the ability to degrade lignin by producing extracellular oxidative enzymes known as lignin peroxidase (ligninase) EC

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1.11.1.14, a generic name for a group of isoenzymic peroxidases that catalyze the oxidative depolymerization of lignin. Another two extracellular ligninolytic peroxidases (manganese peroxidase, MnP, EC 1.11.1.13) and one phenol oxidase of lactase type (benzenediol: oxidoreductase, EC 1.10.3.2) also have been intensively studied in various white-rot fungi for lignin biodegradation and dye decolourisation (Maganhotto de Souza Silva *et al*., 2005). In this study, attempt has been made to focus on lignin peroxidase enzyme production due to its capability to catalyze the oxidation of variety of compound with high reduction potential (Viral *et al*., 2005). This enzyme was produced by the fungi during secondary metabolism in response to environmental stress and the level of nutrient condition. Thus, several factors are believed could optimize the viability and potential of white rot fungus.

Optimisation of enzyme production in fermentation technology through statistical analysis of factorial design and Response Surface Methodology (RSM) is a common practice nowadays. This technique has been applied for the enhancement and optimisation of culture conditions (Cacchio *et al*., 2001; Rosli *et al*., 2003) and media composition by Roshanida *et al*. (2004) and for various fermentation processes. For a broad application, the cost of enzyme is one of the main factors determining the economics process. Reducing the costs of the enzyme production by optimising the fermentation medium is the basic research for industrial application. The objective of this study is to optimise the parameter involved in lignin peroxidase production using locally isolated *Pycnoporus* sp.

MATERIALS AND METHODS

Inoculum preparation: The pure culture was grown on malt extract agar until spore's production. Spore inoculum was prepared aseptically by the addition of approximately 8 mL of sterile distilled water onto agar plates and gently shakes for a short period. The spores were then collected using sterile glass rod by scrapping the agar surface to release the bounded sporangium. The desired concentrations (based on the level analysed in factorial design) of spores were measured using hemocytometer and the preparation was done freshly prior to fermentation.

Culture media: The carbon limited medium formulation used was adapted from Janshekar and Fiechter (1988). Culture medium with 10% (v/v) inoculum was added aseptically into the medium for the production of lignin peroxidase. The medium were autoclaved at 121°C, 15 psi for 15 min. Trace element, vitamin and thiamine HCl, which are heat labile compound were autoclaved separately at 121°C, 15 psi for only 10 min. The solution was added aseptically after sterilization prior to fermentation.

Table 1: Factors and levels analysed in factorial experiment

Factor coded	Name	Units	Type	Low (-1)	High (+1)
A	Nitrogen concentration	mM	Numeric	8.00	24.00
B	Agitation	rpm	Numeric	50.00	110.00
C	pH		Numeric	3.50	5.50
D	Inoculum concentration	Spores mL^{-1}	Numeric	1×10^{4}	6×10^{6}
E	Inducer	mM	Categorical	No veratryl	Veratryl

Growth condition of spore inoculum: Agitated liquid culture were prepared in 250 mL Erlenmeyer flasks containing 150 mL working volume with 10% (v/v) inoculum of desired spores concentration. All flask were sealed with cotton gouge and tubing for aeration purpose. The fungus was grown at 37°C in shaking incubator. Culture condition was modified according to the condition required in optimisation step (Table 1). Each culture flask was then flushed with sterile air for 10 min every day after inoculation time (Tien and Kirk, 1988).

Sample assays

Lignin peroxidase activity: Lignin peroxidase (LiP) activity was determined as the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde (Tien and Kirk, 1988). One unit of activity corresponds to 1 micromole veratraldehyde formed from the oxidation of veratral per minute under the assay conditions with molar extinction coefficient of $\varepsilon_{310} = 9300/M/cm$.

Nitrogen concentration: The concentration of nitrogen in the sample was determined by the Nessler method (HACH Company, 1991). The range of nitrogen concentration that could be detected by this assay method was between 0 to 2.5 mg L^{-1}. Sample with high nitrogen concentration was diluted prior the assay. The reagent for Nessler was available in the kits form and it was consist of mineral stabilizer, polyvinyl alcohol dispersing agent and Nessler reagent. For each assay, 25 mL of sample was required (usually the sample volume was top up with water to reach the require volume). The analysis was started by adding 3 drops of mineral stabilizer into the sample and mixed well. Followed by adding 3 drops of polyvinyl alcohol dispersing agent and mixed well. Then the mixture was left for 1 min before the reading was taken by using DR 2000 spectrophotometer (HACH Company) at wavelength of 425 nm. Nitrogen concentration could be determined directly using DR 2000 Spectrophotometer and the concentration unit was shown in mg L^{-1}. The reaction for blank sample was carried out by replacing the 25 mL of the sample with 25 mL of deionised water.

Preliminary optimisation prior to factorial design: Prior to experimental design using factorial analysis, the optimum temperature for the best growth and lignin peroxidase production was investigated. The effect of temperature on lignin peroxidase production was conducted at 30, 37 and 45°C, respectively, based on the previous study by Tekere *et al*. (2001). The experiments were all done in the incubator shaker up to 20 days of fermentation and the samplings were done for 24 h interval.

Optimisation of lignin peroxidase production by factorial design using Design-Expert 6.06: In order to enhance the production of lignin peroxidase, Fractional Factorial Design for five independent variables was adopted. The experimental design was analysed using statistical software of Design-Expert 6.06. Two level Fractional Factorial Design were used to obtain the combination of values that can optimize the response between the region of three dimensional observation spaces, which allow one to design a minimal number of experimental runs (Montgomery, 1991). It is also a statistically based method that involves simultaneous adjustment of two level experimental factors, high or low. It offers a parallel testing scheme that is much more efficient than one factor at a time. The variables applied to the design were inoculum concentration, initial pH, nitrogen concentration, agitation speed and the addition of 1 mM veratryl alcohol as inducer. The addition of veratryl alcohol was done after a complete consumption of glucose and in the early stages of secondary metabolism. These factors were selected according to previous studies which were shown to be importance and criteria affecting lignin peroxidase production (Faison and Kirk, 1985; Ventakadri and Irvine, 1990;Viral *et al*., 2005).

The variables considered for the design are shown in Table 1. Each independent variable was investigated at a high and a low level leading to a total 26 sets of experiments carried out in this study (Table 2). The center point was included in the matrix and statistical analysis was used to identify the effect of each variable on lignin peroxidase production. The statistical analysis was performed by Analysis of Variance (ANOVA). All response surfaces graph were drawn with the third variable at the center point level. The experiments were randomized for statistical reason. Samples were collected every 24 h interval and the highest lignin peroxidase activity considered as the response. Three milliliter aliquots from each culture broth were taken after inoculation and at 24 h intervals for analysis by using sterile plastic tips. The extracellular fluids were centrifuged at 13,000 rpm for 10 min. All the samples were handled in 4°C to avoid enzyme denaturation. The samples were kept in -20°C until further used for determination of lignin peroxidase activity, protein, glucose oxidase and nitrogen content.

Table 2: Fractional factorial design of 5 variables in coded units

		Factors				
Std.	Run	1 (A)	2 (B)	3 (C)	4 (D)	5 (E)
13	1	-1	-1	1	1	1
16	2	1	1	1	1	1
9	3	-1	-1	-1	1	-1
6	4	1	-1	1	-1	1
10	5	1	-1	-1	1	1
14	6	1	-1	1	1	-1
2	7	1	-1	-1	-1	-1
1	8	-1	-1	-1	-1	1
5	9	-1	-1	1	-1	-1
18	10	0	0	0	0	1
22	11	0	0	0	0	1
24	12	0	0	0	0	1
21	13	0	0	0	0	-1
23	14	0	0	0	0	-1
25	15	0	0	0	0	-1
26	16	0	0	0	0	1
17	17	0	0	0	0	-1
20	18	0	0	0	0	1
19	19	0	0	0	0	-1
3	20	-1	1	-1	-1	-1
11	21	-1	1	-1	1	1
12	22	1	1	-1	1	-1
8	23	1	1	1	-1	-1
15	24	-1	1	1	1	-1
4	25	1	1	-1	-1	1
7	26	-1	1	1	-1	1

Fixed variables: Temperature, 37°C and glucose concentration, 3 g L^{-1}. (A) Nitrogen concentration (mM), (B) Agitation speed (rpm), (C) pH, (D) Inoculum concentration (spores mL^{-1}) and (E) Inducer. The statistical analysis were performed by Analysis of Variance (ANOVA). All response surfaces graph were drawn with the third variable at the center point level

RESULTS

Preliminary determination of optimum temperature: Prior to experimental design using factorial analysis, the best growth and lignin peroxidase production under particular temperature were investigated. The effects of temperature on lignin peroxidase production were done using 30, 37 and 45°C, which these values were selected based on the previous study done by Tekere *et al*. (2001). Figure 1 show that the highest lignin peroxidase production was obtained at 37°C with 0.301 U L^{-1}, followed by 30°C with 0.172 U L^{-1} and no activity detected at 45°C. Mycelial pellet of *Pycnoporus* sp. grew well in 30 and 37°C except at 45°C with no growth.

Determination on the effect of lignin as a growth substrate: Figure 2 shows the profile of lignin peroxidase production as compared with and without the addition of lignin compound. Lignin compound was prepared by chemical treatment of Empty Fruit Bunch (EFB) from palm

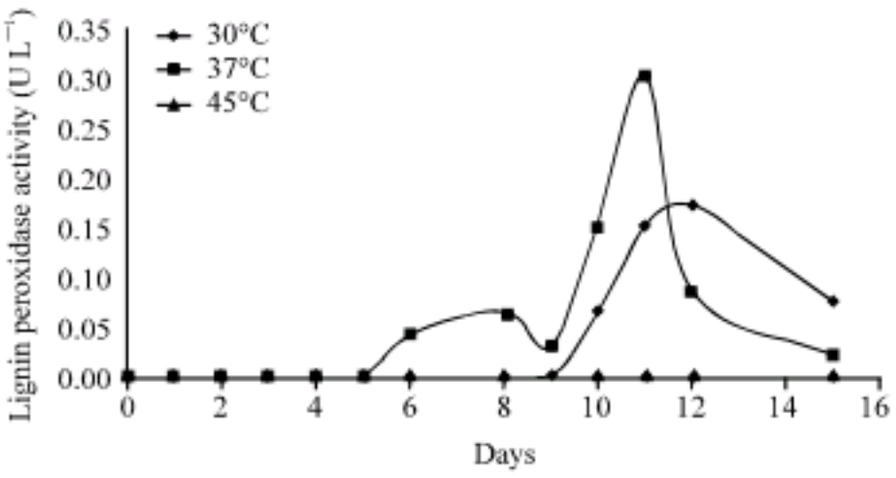

Fig. 1: Lignin peroxidase production in different temperature

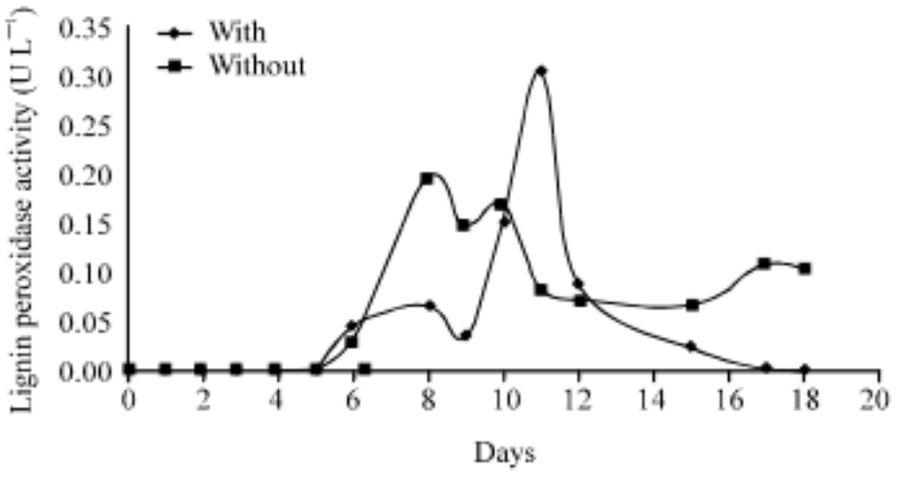

Fig. 2: Profiles of lignin peroxidase activity with and without the present of lignin compound

oil mill wastes treated with 4% natrium hydroxide. The highest lignin peroxidase activity can be obtained in the absent of lignin at 0.3 U L^{-1} as compared with the addition of lignin with only 0.19 U L^{-1}.

Statistical design approach: The statistical combination of the factors and the maximum lignin peroxidase production are shown in Table 3 with the total 26 set of experiments. The maximum lignin peroxidase production was obtained after 10 to 20 days fermentation time.

The second-order polynomial regression model containing 5 linear and 10 interaction terms was employed by using Factorial Design, Design Expert 6.0.4 (Stat Ease, Inc). The model was tested for adequacy and the quality of fit by the Analysis of Variance (ANOVA) as shown in Table 4. Production were identified on the basis of confidence level above 95% ($p<0.05$). The behaviour of the present system described by Eq. 1, which includes all the interaction terms regardless of their significance:

$$Y = \beta_0+\beta_1 A+\beta_2 B+\beta_3 C+\beta_4 D+\beta_5 E+\beta_{12} AB+\beta_{13} AC+\beta_{14} AD+ \beta_{15}AE+\beta_{23}BC+\beta_{24}BD+\beta_{25}BE+\beta_{34}CD+\beta_{35}CE+\beta_{45}DE \quad (1)$$

where, Y is predicted response, yield of lignin peroxidase (U L^{-1}), A, B, C, D and E are independent variables of nitrogen concentration, agitation speed, initial pH, inoculum concentration and inducer, β_0 is coefficient constant for offset term, β_1, β_2, β_3, β_4 and β_5 are coefficient constant for linear effects, β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35} and β_{45} are coefficient constant for interaction effects.

The model will evaluate the effect of each independent variable to the response.

The comparison of lignin peroxidase activity in different combination variables along with the experimental design was shown in Table 3. The best lignin peroxidase activity at 51.1 U L^{-1} was achieved in medium containing 24 mM nitrogen concentration, 110 rpm of agitation speed, initial pH at 3.5, 6×10^6 spores mL^{-1} inoculum concentrations and with the addition of veratryl alcohol as an inducer.

The coefficient of determination (R^2) value provides a measure of how much variability in the observed response values can be explained by the experimental factor and their interaction (Table 4). As a practical rule,

Table 3: Fractional factorial design of 5 variables in coded units along with the observed response

		Factors					Lignin peroxidase activity
Std.	Run	1 (A)	2 (B)	3 (C)	4 (D)	5 (E)	(U L^{-1})
13	1	-1	-1	1	1	1	0.15
16	2	1	1	1	1	1	0.23
9	3	-1	-1	-1	1	-1	0.30
6	4	1	-1	1	-1	1	0.00
10	5	1	-1	-1	1	1	0.15
14	6	1	-1	1	1	-1	0.12
2	7	1	-1	-1	-1	-1	0.00
1	8	-1	-1	-1	-1	1	0.00
5	9	-1	-1	1	-1	-1	0.00
18	10	0	0	0	0	1	2.12
22	11	0	0	0	0	1	1.16
24	12	0	0	0	0	1	0.00
21	13	0	0	0	0	-1	0.00
23	14	0	0	0	0	-1	0.00
25	15	0	0	0	0	-1	9.46
26	16	0	0	0	0	1	10.60
17	17	0	0	0	0	-1	0.00
20	18	0	0	0	0	1	0.00
19	19	0	0	0	0	-1	2.90
3	20	-1	1	-1	-1	-1	0.13
11	21	-1	1	-1	1	1	0.30
12	22	1	1	-1	1	1	51.10
8	23	1	1	1	-1	-1	0.19
15	24	-1	1	1	1	-1	0.00
4	25	1	1	-1	-1	1	0.09
7	26	-1	1	1	-1	1	0.82

Fixed variables: Temperature 37°C and glucose concentration, 3 g L^{-1}, (A) Nitrogen concentration (mM), (B) Agitation speed (rpm), (C) pH, (D) Inoculum concentration (spores mL^{-1}) and (E) Inducer

Table 4: Analysis of Variance (ANOVA) for the production of lignin peroxidase enzyme

Source	Sum of square	df	Mean square	F-value	p-value	R^2
Model	2366.26	15	157.75	6.65	0.0035	0.9173
Residual	213.45	9	23.72			
Lack of fit	66.38	1	66.38	3.61	0.0939	
Pure error	147.08	8	18.38			
Correlation total	2582.94	25				

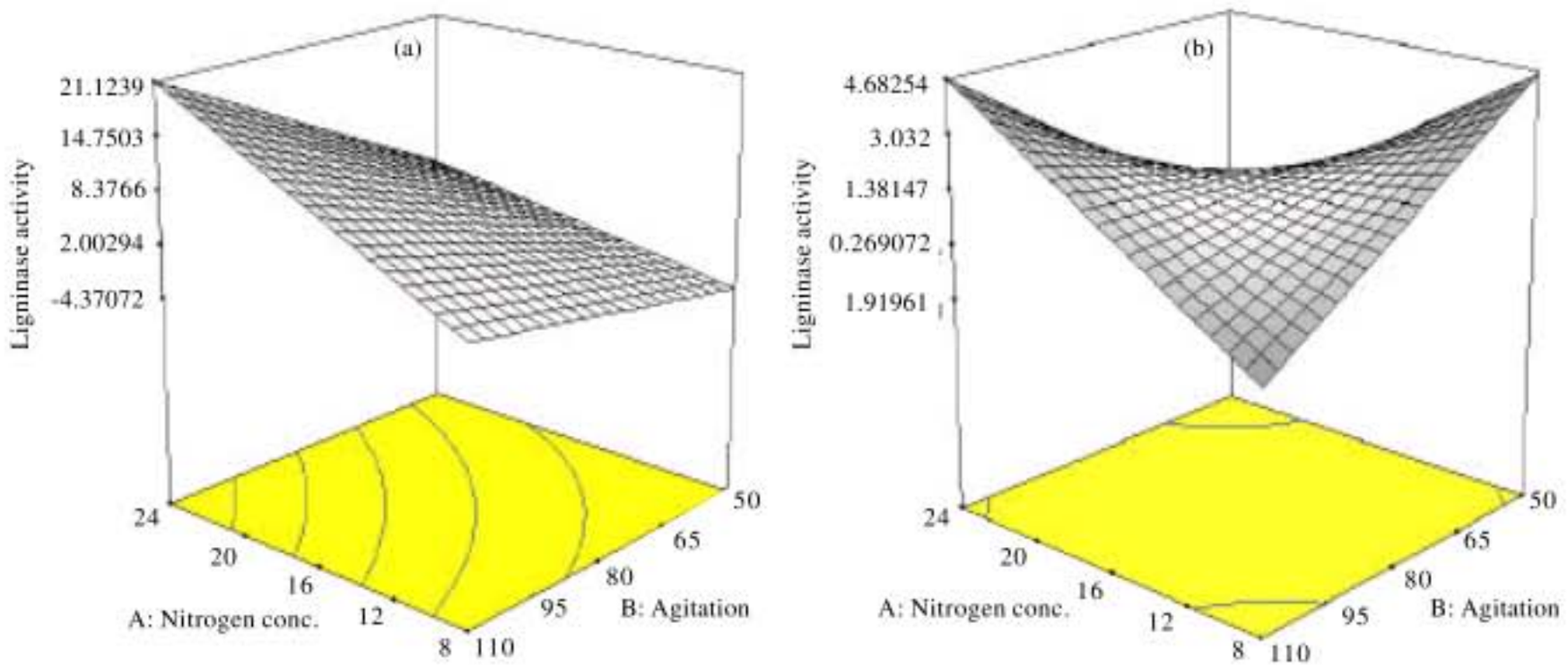

Fig. 3: Response surface of lignin peroxidase activity as a function of nitrogen concentration and agitation speed while keeping initial pH and inoculum concentration at center point, (a) with veratryl alcohol and (b) without veratryl alcohol

Table 5: Estimated coefficient and p-value calculated from the model

Factors	Coefficient estimate	p-value
A	3.14	0.0299
B	3.26	0.0254
C	-3.16	0.0290
D	3.20	0.0276
E	-1.87	0.0821
AB	3.16	0.0290
AC	-3.19	0.0279
AD	3.22	0.0267
AE	-3.24	0.0261
BC	-3.14	0.0299
BD	3.10	0.0312
BE	-3.12	0.0307
CD	-3.26	0.0254
CE	3.24	0.0259
DE	-3.20	0.0273

the model has statistical significance when the $p<0.05$. p-value is the probability that the magnitude of a contrast coefficient is due to random process variability. A low p-value indicates a real or significant effect. The model R^2 of 0.9173 suggested that the fitted linear plus interaction effect could be explain 91.7% of the total variation. The p-value below than 0.05 indicates that the present model is in good prediction of the experimental results. Thus, the model regression is adequate in explaining the functional relationship between the response and the independent variables. For the lack of fit, the value of $p>0.05$, lack of fit is good when the $p>0.05$.

The empirical relationship between the yield of lignin peroxidase and the test variables is explained by regression equation (Eq. 2) in term of coded factors:

$$Y = 3.35+3.14A+3.26B-3.16C+3.20D-1.87E+3.16AB-3.19AC+3.22AD-3.24AE-3.14BC+3.1BD-3.12BE-3.26CD+3.24CE-3.20DE \quad (2)$$

Table 5 shows estimated coefficient and p-value of the experimental variables. The p-values serves as tool for checking the significance of each of the variables. The result showed that among variable tested only inducer (E) had a non-significant effect in term of linear effects. However, its influence could not be totally overruled because of its interactive effect with other variables.

The effects of inducer on lignin peroxidase production: Figure 3 and 4 show the comparison of lignin peroxidase production with and without the addition of veratryl alcohol as an inducer in a 3D response surfaces for all variables. Response surface shown the effect of pair wise interaction of the parameters, when the third parameter is kept at center point. The main goal of response surface is to find the optimum values of the variable so the response is maximised. It was observed that lignin peroxidase production was greatly influenced by the addition of veratryl alcohol with all the figures showed almost 5 fold increment compared to those without veratryl alcohol.

The effect of agitation speed: The effects of agitation speed on lignin peroxidase production were studied ranging from 50 to 110 rpm. From Table 5, agitation speed is significant in term of linear and interaction effect with the $p<0.05$. In correlation with the response surface graph of Fig. 3 with the addition of veratryl alcohol, it was observed that lignin peroxidase production increased as the agitation speed increase along with the increase in nitrogen concentrations.

The effect of nitrogen concentration: Table 5 showed that nitrogen concentrations were significant in term of linear

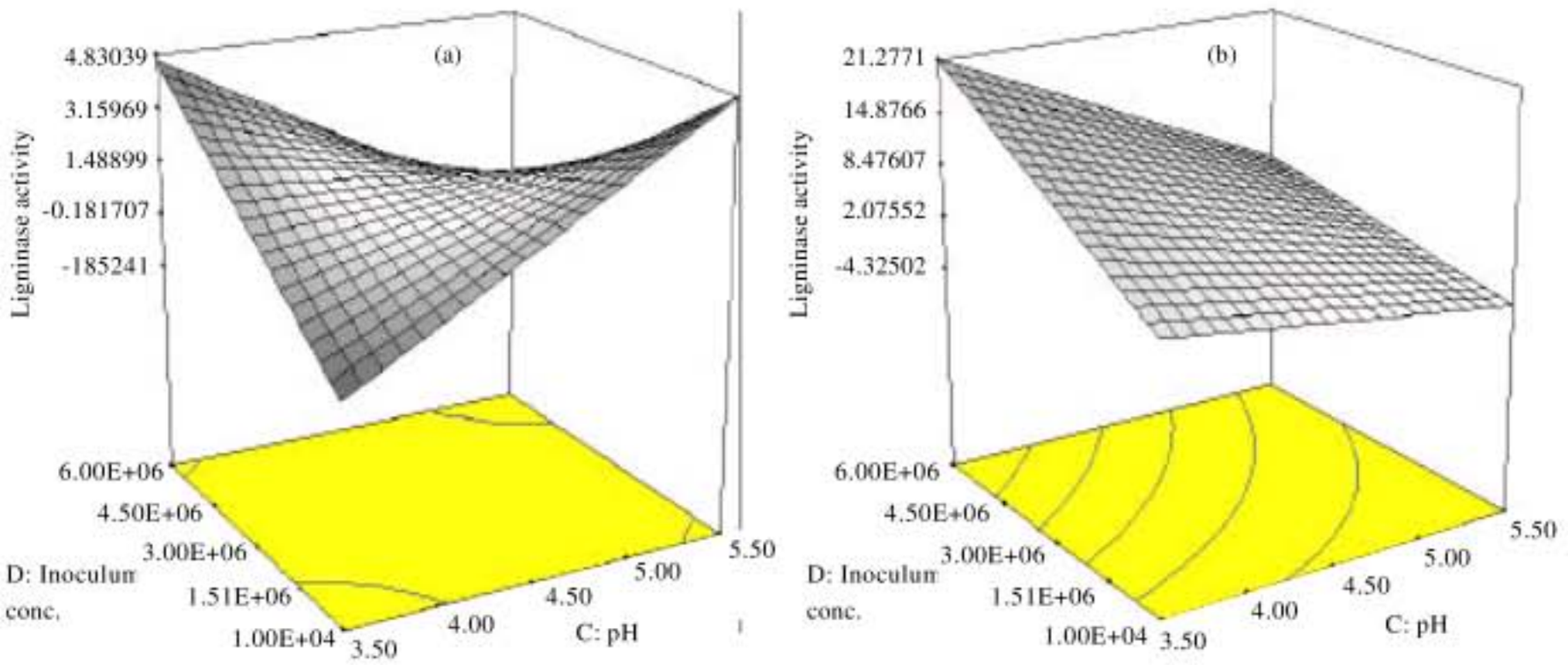

Fig. 4: Response surface of lignin peroxidase activity as a function of inoculums concentration and initial pH while keeping nitrogen concentration and agitation speed at center point, (a) with veratryl alcohol and (b) without veratryl alcohol

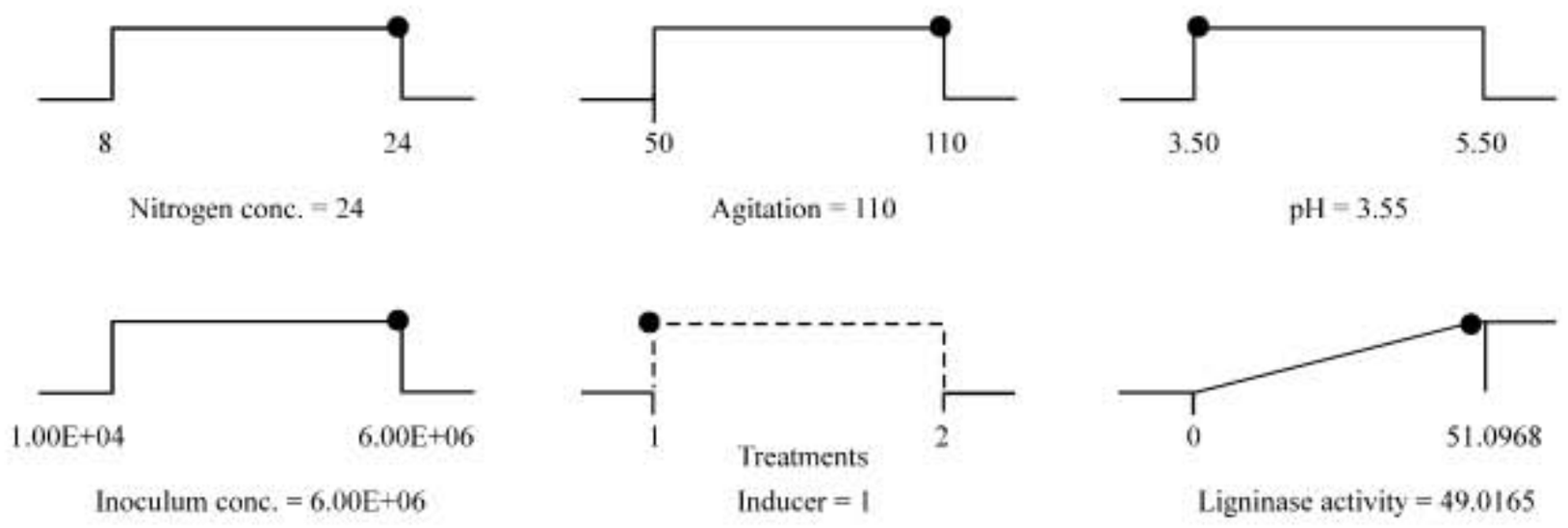

Fig. 5: Predicted optimum condition of lignin peroxidase production by *Pycnoporus* sp. with (●) represent the optimum value

and interaction effect with the $p<0.05$. The studied range of nitrogen concentrations between 8 to 24 mM resulted in the increase of lignin peroxidase production as the nitrogen concentrations were increase along with the increase in agitation speed (Fig. 3a). In relation, lignin peroxidase activity was at optimum as the carbon concentration was fixed at 3 g L^{-1} and nitrogen concentration at 24 mM.

The effect of initial pH: pH was found to be significant in term of linear and interaction effects with the $p<0.05$ (Table 5). Lignin peroxidase production was found to increase, while pH decreased from 5.5 to 3.5 along with the increase of inoculum concentration (Fig. 4a) with the addition of veratryl alcohol.

The effect of inoculum concentration: Inoculum concentration apparently increase lignin peroxidase production at higher concentration as it showed a significant result in linear and interaction effects (Table 5). Figure 4a with the present of veratryl alcohol shows that the lignin peroxidase activity increased along with the decreased in initial pH.

Lignin peroxidase production using optimised condition: Figure 5 shows the optimum condition predicted by the model for all variables with the highest lignin peroxidase production was theoretically at 49.02 U L^{-1} which is in close match with 51.1 U L^{-1} in practice.

DISCUSSION

Thermal instability of lignin peroxidase at higher temperature is apparent from the rapid loss in activity as temperature increases (Fig. 1). It was suggested that at 37°C the best growth was performed that reflects the best

temperature for lignin peroxidase production. As proposed by Tien and Kirk (1984), lignin peroxidase was proven stable to be conducted at 37°C, while Tekere *et al*. (2001) determined that the optimum growth of *Pycnoporus sanguineus* was the best at temperature ranging from 37 to 40°C. With no growth found in 45°C culture, it was assumed that this strain is not a thermophilic fungi.

Lignin is rich in carbon source but it is not a growth substrate for microorganisms which are reported to degrade lignin. Lignin degrading fungi are not able to use lignin as a sole energy source, but are dependent on a readily available co-substrate and the presence of an alternate energy or carbon source (Boominathan and Reddy, 1992). Ligninolytic system in *P. chrysosporium* as a model reference has been considered non-inducible by lignin (Faison and Kirk, 1985). Figure 2 shows that the addition of lignin compound substituting the simple sugar of glucose will significantly reduce lignin peroxidase production as it not acting as a growth substrate. However, there is a slight increase of activity with the presence of lignin compound while lignin peroxidase activity in the medium without the present of lignin compound started to decrease at the end of the fermentation days suggested that lignin peroxidase activity were produced to use up the lignin. As the aim of the present study is to achieve the best lignin peroxidase production, the highest lignin peroxidase activity can be obtained in the absent of lignin at 0.3 U L^{-1} as compared with the presence of lignin with only 0.19 U L^{-1}.

The inducer (veratryl alcohol) used in the present study show that it does significantly enhance lignin peroxidase production. Veratryl alcohol is a natural secondary metabolite of white-rot fungus, besides acting as a mediator (Viral *et al*., 2005) and protecting enzyme against inactivation (Wariishi and Gold, 1989). It is also enhances the lignin peroxidase enzyme production (Faison and Kirk, 1985; Linko and Zhong, 1991). Lignin peroxidase catalyses oxidation of veratryl alcohol to veratraldehyde (VA^+) cation radical which is a powerful charge transfer reagent that can oxidized large hydrophobic molecules like lignin and other recalcitrant molecules by indirect oxidation (Pointing, 2001). It is in agreement with the previous studies done by Faison and Kirk (1985) and Linko and Zhong (1991), which using veratryl alcohol to enhance the lignin peroxidase production.

In contrary with Shimada *et al*. (1981), agitation appear not to effect lignin peroxidase production and in agreement with Jager *et al*. (1985) to which Tween 80 was added to give a protective effect to the lignin peroxidase enzyme against mechanical inactivation. The formations of mycelial pellet were influenced by the agitation speed applied. The higher the agitation speed the smaller the mycelial pellet obtained. Study shows that lignin peroxidase production by *Pycnoporus* sp. was morphology dependant. It could clearly observe that culture with higher lignin peroxidase production was generally associated with the present of mycelium pellet 1-1.5 cm diameter size. Darah and Ibrahim (1998) also revealed the present of small mycelial pellet in lignin peroxidase and manganese peroxidase production. Studies carried out by Michel *et al*. (1990) and Jimenez-Tobon *et al*. (1998) showed that *Phanerochaete chrysosporium* grew mostly in the form of small pellet was known to favour manganese peroxidase and lignin peroxidase production. Generally, pellet size was given more attention because it's determined the surface area for oxygen transfer. The changes in morphology during growth affect nutrient consumption and oxygen uptake in submerged culture. Pelleted growth exhibits low viscosities. Higher power inputs are required for filamentous as compared to pellet growth in achieving adequate agitation and oxygen transfer. Frequently, the central region of larger pellet undergoes autolysis as a result of nutrient limitation. This autolysis could have a significant effect on both cellular metabolism and product synthesis. An important aspect to consider during pellet growth was fragmentation or cells breakup. It has been observed that the initial increased in pellet concentration in fungal culture was followed by the rapid decreased which coincides with the decreased in specific growth rate. The breakup was caused by cell lysis within pellets whereby the stability is lost and it become more susceptible to damage by mechanical forces. Thus, small pellet as opposed to large ones would generally be considered desirable in developing filamentous fungal fermentations (Papagianni, 2004).

Lignin peroxidase production also seems affected by the nitrogen concentration as it shown to have a strong regulation effects (Gold and Alic, 1993). This result (Fig. 3a) contrary with Jager *et al*. (1985) stated that higher nitrogen concentration delayed or completely suppressed development of lignin peroxidase activity. Under carbon limitation media in access of nitrogen concentration the lignin peroxidase production were found to increase. This is in agreement with Hamman *et al*. (1997) stated that higher titre of lignin peroxidase were found in *Phanerochaete flavido-alba* in the carbon limited media in excess nitrogen. On the other hand, the increased in lignin peroxidase activity could occur as a consequence of the availability of nitrogen in the carbon limited cultures, that has been demonstrated by Dosoretz *et al*. (1993). The negative effects of a decrease in ammonium

availability on *P. flavido-alba* lignin peroxidase and mangan peroxidase activities were observed in carbon limited medium. Under these nutritional stressful conditions, a phenomenon of disappearance and reappearance of soluble ammonia has been described in *P. chrysosporium* nitrogen limited cultures (Dosoretz *et al*., 1993; Rothschild *et al*., 1995). It suggested that the reappearance of soluble ammonia results from an autolytic process involving proteolysis of cell proteins, after complete glucose depletion as an alternative energy source (Hamman *et al*., 1997).

Initial pH is one of the factors affected lignin peroxidase enzyme production (Fig. 4a). Results show a close agreement with several studies done by Tamara *et al*. (1993), Rimko *et al*. (1998), Fakoussa and Hofrichter (1999) and Mutsumi *et al*. (2003). Tamara *et al*. (1993) conducted isoelectric focusing (IEF) analysis of purified lignin peroxidase isoenzymes of *Phlebia ochraceofulva* using a broad pH range (3.5-10) and showed that the lignin peroxidase have isoelectric (pI) values from 4.2 to 3.5 and even lower. Rimko *et al*. (1998) also stated that one of the isoenzymes produced by *Bjerkandera* sp. strain BOS55 would oxidise veratryl alcohol optimally in the presence of H_2O_2 at near pH 3.0. Fakoussa and Hofrichter (1999) reported that the pH range of lignin peroxidase was 2-5, with an optimum between pH 2.5 to 3.0. The pH dependence of the oxidation of veratryl alcohol by *Phanerochaete sordida* YK-624 was also studied by Mutsumi *et al*. (2003) and the highest rate of veratryl alcohol oxidation or lignin peroxidase activity was also observed at pH 3.0. However, when the pH increased, the lignin peroxidase activities decreased and no activity was detected at pH 7. The pI values of lignin peroxidase isozymes from *Bjerkandera adusta* were estimated to be 3.0 and 3.1.

Inoculum is known to be one of the factors that affect fungal culture morphology, fungal growth and enzyme production (Darah and Ibrahim, 1998; Papagianni, 2004). Influence of pellet size was mainly depending on the spore inoculum size (Papagianni, 2004). It was observed that higher inoculum size resulted in a rapid formation of numerous small pellets while low inoculum size resulted in large pellet size.

Lignin peroxidase production was optimum using 24 mM nitrogen concentration, 110 rpm of agitation speed, initial pH at 3.5, 6×10^6 spores mL^{-1} inoculum concentration and enhanced by the addition of veratryl alcohol with activity 51.1 U L^{-1}. It was shown that the model is adequate to predict the optimisation of lignin peroxidase production from *Pycnoporus* sp. The use of veratryl alcohol in general was proven to enhance lignin peroxidase activity. In general, all variables give significant effect to the production of lignin peroxidase in order to determine optimal conditions to obtain extract with high lignin peroxidase. Therefore, factorial design approach could be an alternative option to enhance lignin peroxidase production.

CONCLUSION

Factorial design approach was useful to determine the optimum conditions that significantly influenced the production of lignin peroxidase by *Pycnoporus* sp. The variables having most significant effect of lignin peroxidase production were identified using 2 level fractional factorial designs. From this study, the combinations of all factors used in this analysis showed significant effect on lignin peroxidase production. The final conditions to produce lignin peroxidase after optimisation step were 24 mM of nitrogen concentration, agitation speed at 110 rpm, pH 3.5, inoculum density of 6×10^6 spores mL^{-1} and addition of inducer (veratryl alcohol). These parameters produced theoretically 49 U L^{-1} lignin peroxidase compared to 51.1 U L^{-1} in practice. However, repetition production of lignin peroxidase using optimised condition was able to achieve up to 81.1 U L^{-1}.

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