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ORIGINAL PAPER

Optimization of lignin peroxidase production and stability by *Phanerochaete chrysosporium* using sewage-treatment-plant sludge as substrate in a stirred-tank bioreactor

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Abstract A laboratory-scale study was carried out to produce lignin peroxidase (ligninase) by white rot fungus (*Phanerochaete chrysosporium*) using sewage-treatment-plant (STP) sludge as the major substrate. The optimization was done using full-factorial design (FFD) with agitation and aeration as the two parameters. Nine experiments indicated by the FFD were fermented in a stirred-tank bioreactor for 3 days. A second-order quadratic model was developed using the regression analysis of the experimental results with the linear, quadratic, and interaction effects of the parameters. Analysis of variance (ANOVA) showed a high coefficient of determination (R^2) value of 0.972, thus indicating a satisfactory fit of the quadratic model with the experimental data. Using statistical analysis, the optimum aeration and agitation rates were determined to be 2.0 vvm and 200 rpm, respectively, with a maximum activity of 225 U l^{-1} in the first 3 days of fermentation. The validation experiment showed the maximum activity of lignin peroxidase was 744 U l^{-1} after 5 days of fermentation. The results for the tests of the stability of lignin peroxidase showed that the activity was more than 80% of the maximum for the first 12 h of incubation at an optimum pH of 5 and temperature of 55°C.

Keywords Lignin peroxidase · Sewage treatment plant (STP) sludge · *Phanerochaete chrysosporium* · Optimization · Stirred tank bioreactor

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Introduction

All over the world, sewage is the largest source of organic pollution to water resources and their surrounding environments. In Malaysia, in terms of biological oxygen demand (BOD) load [1], sewage is the top polluter (64.4%), followed by animal-husbandry wastes (32.6%), agro-based residues (1.7%), and industrial effluent (1.3%). The management of the ever-increasing volume of domestic and industrial organic wastes is one of the prime environmental issues in Malaysia. Annually, approximately 4.2 million m^3 of sewage sludge is generated by the Indah Water Konsortium (IWK) in Malaysia, and this is expected to rise to 7 million m^3 by the year 2020 with a total management cost of 1 billion ringgit [2]. The country has yet to adopt a practical, economic, and acceptable approach for the management and disposal of sewage sludge. The current practice is either to co-dispose of it with solid waste at landfill sites or directly dispose of it in shallow trenches [3]. IWK's main concern is finding safe, scientific, and environmentally friendly disposal methods.

Sewage-treatment-plant (STP) sludge is a very good source of carbon, nitrogen, phosphorus, potassium, and other nutrients for many microbial processes. These add value to sludge by enabling the production of certain

valuable metabolic products [4, 5]. Currently several methods are practiced to utilize and dispose of STP sludge, such as land applications [6–8], methane production [9], biopesticides [10, 11], and biopolymers [12]. Besides these applications, lignin peroxidase (LiP) enzyme production using STP sludge as a major substrate seems to be a promising and encouraging alternative for better sludge management. This is a new environmental biotechnological approach for the biodegradation of sludge, which, in addition to producing LiP, would reduce treatment and production costs through the use of an environmentally friendly process [4, 13].

The production of ligninolytic enzymes using liquid media containing macro- and micronutrients (i.e., glucose, ammonium tartrate, $MgSO_4$, NaCl, $FeSO_4$, H_3PO_4, and trace elements such as nitriloacetic acid, $CoCl_2$, CaCl, $ZnSO_4$, etc.) has higher production costs because of the expensive media constituents [14–17]. Several studies have been done using low-cost raw materials with a high ligninolytic-enzyme activity. These include wheat straw [18, 19], olive pomace [20], sugarcane bagasse [21], grape stalks, grape seeds, and barley bran [22]. Thus, readily available and abundant STP sludge is being considered as a potential substrate for ligninolytic enzyme production, especially LiP, using white rot fungus (*P. chrysosporium)*.

Several white rot fungi such as *Oxysporus* sp., *Schizophyllum commune, Hypoderma* sp. [20], *Trametes versicolor* [22], *Ganoderma* sp. [23], and *Irpex lacteus* [24] have been reported to produce ligninolytic enzymes. Among the fungi tested for the ligninolytic system, the most common white rot fungus is *P. chrysosporium*, which can selectively degrade lignin and xenobiotic compounds [25–29]. *P. chrysosporium* produces ligninolytic enzymes that are widely used in the removal of dyes from industrial effluents [22], bio-bleaching [23], and treating hazardous waste [30].

The common bioreactors used in scaling-up the production of ligninolytic enzymes are submerged stirred-tank reactors [15], modified reactors with nylon-web carriers or a polyurethane system for the fungus [31, 32], hollow fiber reactors, silicone membrane reactors [30], packed-bed bioreactors [16], and trickle fixed-bed reactors [27]. The present study was carried out to identify the optimum operating conditions of the stirred-tank bioreactor for yielding maximum LiP activity using STP sludge as a substrate by statistical approach. Studying the interaction of the factors with the response (LiP activity) is superior to the one-factor-at-a-time (OFAT) method, which is time consuming, laborious, and expensive, for optimizing the process. This study also showed the optimum conditions of pH, temperature, and contact time for achieving stability of lignin peroxidase for industrial applications.

Materials and methods

Fermentation media

STP sludge as a major fermentation medium was collected from a secondary clarifier at the IWK sewage treatment plant in Kuala Lumpur, Malaysia. The sludge was stored in a container and kept in the cold room at 4°C. The total suspended solids (TSS) of sludge were 0.73% (w/w). A 1% (w/w) TSS of the sludge was used as the major substrate and supplemented by wheat flour (3% w/v), which acts as an easily biodegradable carbon source for the initial growth of microbes for LiP production. The fermentation medium was adjusted to pH 4.0 before sterilization at 121°C for 30 min.

Microorganism and its inoculum

The white rot fungus *P. chrysosporium* was used for the production of the LiP enzyme utilizing STP sludge as the major substrate. *P. chrysosporium* culture was grown on a potato dextrose agar (PDA) plate and incubated at 30°C for 5 days. Four plates (petri dishes) of fungal culture were successively washed with 100 ml sterile distilled water. The surface of the culture growth in the plates was gently rubbed with a sterilized glass rod and poured into a 250-ml sterilized Erlenmeyer flask. The determined concentration of inoculum was 400 mg/l, and the flask was kept in the chiller for further use [4].

Stirred-tank bioreactor for LiP production

A 3-l Biostat B 2 (Sartorius BBI Systems) fermenter with four baffles, two six-flat-blade impellers, and an inner vessel diameter of 130 mm was used for the production of LiP using STP sludge as medium. The fermenter was equipped with monitoring and control systems for agitation, aeration, temperature, pH, liquid level, and foaming.

Experimental procedures and statistical design for LiP production

A three-level full factorial design (FFD) was used to identify the experimental design using the two factors (independent variables) of aeration and agitation rate. The FFD determined that a set of nine experiments would be required to carry out the study. Table 1 shows the levels of maximum, minimum, and central levels with the coded and actual values for the parameters. A second-order polynomial model was developed using the experimental results. These include the linear, quadratic, and interaction effects. An analysis of variance (ANOVA) was used to test the model and to see if its coefficients were significant. The statistical software MINITAB, release 14, was used to analyze the regression

Table 1 Experimental range and levels of the independent variables in terms of actual and coded factors

Independent variables	Low (−1)	Medium (0)	High (+1)
Aeration rate (vvm), X_1	0	1	2
Agitation rate (rpm), X_2	0	100	200

model of experimental data, the coefficients of all effects, and the ANOVA. The *F* test, *P* value, *t* test, coefficient of determination (R^2), three-dimensional (3D) response surface, and two-dimensional (2D) contour plots were examined to evaluate the model as well as to determine the operating conditions of the bioreactor.

The media and process conditions were all optimized: temperature 30°C, initial pH 4.0, substrate (STP sludge) concentration 1% (w/w TSS), co-substrate (wheat flour) concentration 3% (w/w,) and inoculum concentration 3% (v/w) [33, 34]. Sterilized STP sludge (1.5 l; 121°C for 30 min) was incorporated in the bioreactor under the optimum conditions. The experiment was carried out according to the FFD. The fermentation broth was collected every day and filtered through filter paper (Whatman no. 1). The filtrate was centrifuged at 10,000 rpm at 4°C for 10 min and kept at −20°C for further analysis.

Experiment for validation

The validation of the statistical approach for the production of LiP by *P. chrysosporium* was carried out in a 2-l Biostat B 2 (Sartorius BBI Systems) fermenter with a working volume of 1.5 l. The optimized medium (pH 4) was sterilized at 121°C for 30 min. The medium was inoculated with 3% of inoculum, and fermentation was carried out at 30°C for 6 days with an uncontrolled pH. The impeller speed was set to 200 rpm and air was sparged into the medium at 2.0 vvm. Samples of fermentation broth were withdrawn every 24 h, and the filtrate was analyzed for LiP activity, pH, content of reducing sugar, and chemical oxygen demand (COD).

Stability test for LiP obtained by optimum fermentation process

The crude LiP obtained by the fermentation of sludge under the optimum operating conditions of the stirred-tank bioreactor was used for the stability test. This study was to determine the optimum pH, temperature, and total contact time for the stability of LiP activity.

Effect of pH

The effect of pH on the LiP stability was measured in the range 3.0–11.0. The buffer systems used were 50 mM glycin/HCl (pH 3), 50 mM sodium acetate/HCl buffer (pH 5), 50 mM Tris/HCl–NaOH buffer (pH 7), 50 mM Tris/NaOH buffer (pH 9), and 50 mM glycin/NaOH buffer (pH 11). The LiP was incubated in 1 ml of various buffers at 35°C for 30 min. After incubation, the enzyme was immediately cooled in an ice bath and the residual activity assayed. The activity was expressed as percentage, with the higher activity as 100%.

Effect of the temperature

The effect of temperature on LiP stability was measured at various temperatures ranging from 25 to 75°C. The enzyme was incubated in 1 ml 50 mM sodium acetate buffer (pH 5.0) at 25, 35, 45, 55, 65, and 75°C for 30 min. After each period of incubation, the enzyme was immediately cooled in an ice bath and the residual activity determined. The highest activity of the enzyme was noted as 100%.

Effect of the contact (incubation) time

The effect of contact (incubation) time on LiP stability was used together with the optimum pH and temperature obtained from this study. The enzyme was incubated in 1 ml 50 mM sodium acetate buffer (pH 5.0) at 55°C for 1, 3, 6, 12, 24, 48, 72, 96, and 120 h of operation. After each incubation period, the enzyme was immediately cooled in the ice bath and the residual activity determined. The enzyme activity was expressed as a percentage with the highest activity noted as 100%.

Analytical analysis

LiP activity assay

LiP activity was determined spectrophotometrically according to Tien and Kirk [25]. The reaction mixture contained 10 mM veratryl alcohol diluted with 1.5 ml distilled water, 50 µl enzyme sample, and 0.25 M sodium tartrate buffer (pH 2.5). The reaction was started by adding 5 mM H_2O_2. One unit (U) was defined as 1 µmol of veratryl alcohol oxidized in 1 min, and activity was reported as U/l.

Chemical oxygen demand (COD) determination by HACH

A 2.5-ml sample was placed in a culture tube or ampule and mixed with 1.5 ml digested solution to measure COD [35]. Sulfuric acid reagent (3.5 ml) was carefully added to the ampule. It was then placed in a block digester preheated at 150°C for 2 h and cooled to room temperature. The absorbance of the developed color was measured at

Table 2 Experimental and predicted values of LiP production obtained by the second-order polynomial model

Run	Actual (coded) values		LiP activity (U/l)		
	Aeration	Agitation	Observed	Predicted	Error (%)
1	2 (+1)	100 (0)	199.3	189.0	5.2
2	1 (0)	100 (0)	152.2	149.7	1.6
3	2 (+1)	200 (+1)	225.1	231.9	−3.0
4	0 (−1)	100 (0)	97.7	110.7	−13.3
5	2 (+1)	0 (−1)	162.9	166.5	−2.2
6	1 (0)	0 (−1)	123.5	127.9	−3.6
7	0 (−1)	200 (+1)	156.9	152.1	3.1
8	0 (−1)	0 (−1)	97.7	89.7	8.2
9	1 (0)	200 (+1)	193.8	191.8	1.0

600 nm. The absorbance values were translated into a COD solution equivalent using a standard graph.

Reducing sugar estimation

A 1.5-ml sample was added to an ampule along with 3 ml of DNS reagent and placed in a boiling water bath for 5 min to estimate reducing-sugar content [36]. The tube was immediately cooled in an ice bath after boiling. The absorbance of the developed color was measured at 540 nm. The absorbance values (after subtraction of the reagent blank) were translated into glucose equivalents using the standard graph (glucose range: 0.1–1.0 g/l).

Results and discussion

Optimization of operating conditions of the stirred-tank bioreactor by FFD for LiP production

The statistical design approach of three-level full factorial design was used to study the interactive effects of aeration and agitation on LiP production by *P. chrysosporium*. Table 2 summarizes the experimental and predicted LiP activities (response) for the optimization of the operating conditions. The predicted LiP was determined by developing a second-order regression model that considered the linear, quadratic, and interaction effects of the parameters. The results obtained from the FFD were analyzed to develop the regression equation, which shows the dependent variable of LiP activity (Y) as a function of aeration (X_1) and agitation (X_2) as follows:

$$Y(\text{LiP activity, U/l}) = 89.7 + 38.1X_1 + 0.108X_2 + 0.14X_1^2 + 0.00102X_2^2 + 0.0075X_1X_2 \quad (1)$$

The FFD results showed that the best combination of aeration and agitation rates were 2.0 vvm and 200 rpm, respectively, which gave a maximum LiP activity of 225.05 U/l. The predicted yield (determined from Eq. 1) was 231.9 U/l, which is slightly higher than the experimental results.

Using the statistical software, the regression equation and determination coefficient (R^2) were evaluated to test the fit of the experimental design [37]. The model showed a high R^2 of 0.972. This indicated a satisfactory adjustment of the quadratic model to the experimental data, and indicated that approximately 97% of the variability in the dependent variable (LiP) could be explained by the model (Table 3). The adjusted R^2, which is more suited for comparing models with different numbers of independent variables, was 0.925. The analysis of variance (ANOVA) shown in Table 3 indicated that the F- and P values were 20.6 and 0.016, respectively. This indicated that the model fit was significant ($P < 0.05$). The regression sum of squares, which is a measure of how a particular model fits each point in the design, was 1.55×10^4.

The t-distribution and the corresponding P values of the coefficients for the linear, quadratic and interaction effects were evaluated to determine their significance. The pattern of interactions between the variables is indicated by these coefficients. The variables with low P values contribute to the model, whereas those with high P values can be neglected and eliminated from the model. Larger t values and smaller P values indicated the high significance of the corresponding coefficient or factor [38]. The t and P values for the linear, quadratic and interactive terms are presented in Table 4. It can be seen that the linear aeration term (X_1) had the largest effect ($P < 0.140$), followed by the squared agitation term (X_2^2) ($P < 0.324$), the agitation rate (X_2) ($P < 0.613$), and others.

Response surface and contour plots are presented to study the interaction among the various factors used and to find out the optimum value of each factor for maximum production [37, 39]. The 3D response surface and 2D contour plots with the effects of aeration and agitation rates on the response

Table 3 Analysis of variance (ANOVA) for the quadratic model

$R^2 = 0.972$; R^2 (adj) $= 0.925$

Source	Degrees of freedom	Sum of squares	Mean squares	F values	P values
Regression	5	15,527.9	3,105.6	20.6	0.016
Residual error	3	452.2	150.7		
Total	8	15,980.2			

Table 4 Regression analysis showing the coefficients and *t* and *P* values

Predictor	Coefficient	Standard error coefficient	*t* value	*P* value
Constant	89.68	11.02	8.14	0.004
Aeration, X_1	38.12	19.09	2.00	0.140
Agitation, X_2	0.1075	0.191	0.56	0.613
X_1^2	0.142	8.68	0.02	0.988
X_2^2	0.0010	0.0009	1.18	0.324
X_1X_2	0.0075	0.0614	0.12	0.910

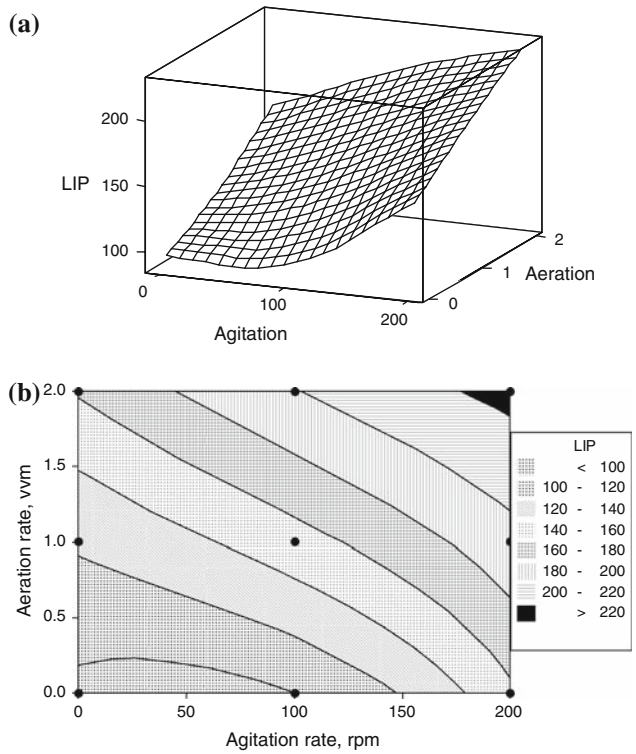

Fig. 1 Response surface and contour plots showing the effect of aeration (vvm) and agitation (rpm) on the production of lignin peroxidase (U/l). **a** 3D response surface. **b** 2D contour plot

(LiP) are shown in Fig. 1. The maximum yield is the area defined by the smallest ellipse in the contour plot. A perfect interaction between the independent variables can be shown when elliptical contours are obtained [40]. Maximum enzyme activity was predicted at 231.86 U/l at corresponding maximum levels of aeration (2 vvm) and agitation (200 rpm). Higher rates of aeration and agitation may provide better mixing as well as enhanced mass and heat transfer thereby accelerating enhanced growth and enzyme production. The influence of higher agitation rate on ligninolytic enzyme (laccase) production from *Streptomyces* species has been reported [41, 42].

Validation of the model through the production of LiP and biodegradation of sludge in a stirred-tank bioreactor

LiP production was carried out in a laboratory-scale 3-l fermenter with a 1.5-l working volume. The optimum fermentation conditions were used to evaluate the model in terms of the maximum activity of LiP for 6 days. The LiP activity, removal of COD, and content of reducing sugar are shown in Table 5. LiP was detected as early as the first day (Table 5) and increased with fermentation time. The highest LiP activity obtained in the bioreactor was 742.7 U/l after 5 days of fermentation, and it decreased (595 U/l) on the final day (after 6 days). In some cases, production of LiP in conventional stirred-tank reactors and shallow stationary cultures in large flasks was unsuccessful or gave a low yield [14, 43]. Janshekar and Fiechter [15] reported the production of LiP by *P. chrysosporium* in 30-l submerged stirred-tank reactors with low yield of 40–60 U/l. Scaled-up production of LiP with high yield using other bioreactors such as hollow fiber reactors, silicon membrane reactors, and packed-bed columns has also been reported [16, 30–32].

The COD was an important factor in evaluating the organic content of the STP sludge. Table 5 shows the removal of COD in treated sludge by fungal treatment using *P. chrysosporium*. The COD removal had increased to 93% at day five. The maximum utilization of organic material by the fungus with the highest production of LiP was obtained in the same fermentation period. This might have happened due to a good relationship of product and substrate uptake by the *P. chrysosporium*. Alam et al. [4] studied biosolids accumulation and biodegradation of domestic wastewater-treatment-plant sludge by developed liquid state bioconversion process where the result showed highest removal of COD of 93% at day five.

The content of reducing sugar was observed to determine the substrate utilization after microbial treatment of the sludge. The concentration of reducing sugar increased until day three of fermentation (7.3 g/l) and then decreased at the end of the fermentation time (Table 5). The concentration of reducing sugar decreased with the increase in fungal growth during the fermentation.

Stability test for LiP activity produced by *P. chrysosporium* using sludge as substrate in a stirred-tank bioreactor

Figure 2 shows the pH stability of crude extracellular LiP of *P. chrysosporium*. A 0.5-ml (743 U/l) enzyme sample was incubated in 1 ml of various pH buffers for 30 min. This was based on the previous stability study of the lipase enzyme created using thermophilic fungi isolated from

Table 5 The maximum production of LiP and biodegradation of sludge using optimum operating conditions in the bioreactor

Fermentation period (days)	LiP activity (U/l)	Removal of COD (%)	Reducing sugar (g/l)
0	0 ± 0.0	0 ± 0.0	2.3 ± 0.24
1	60.0 ± 3.4	64 ± 2.1	2.3 ± 0.11
2	117.9 ± 5.3	71 ± 1.1	5.1 ± 0.21
3	286.6 ± 11.1	77 ± 1.9	7.3 ± 0.17
4	516.6 ± 9.2	82 ± 1.0	5.6 ± 0.12
5	743.7 ± 8.9	93 ± 2.2	4.6 ± 0.18
6	595.0 ± 7.3	89 ± 1.5	3.6 ± 0.20

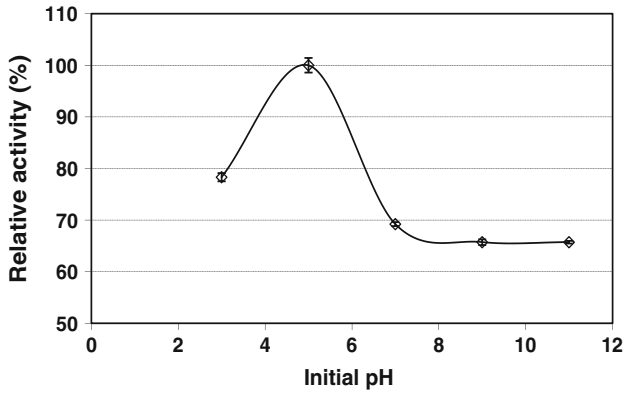

Fig. 2 Effect of pH on lignin peroxidase activity obtained by liquid state bioconversion of domestic wastewater sludge

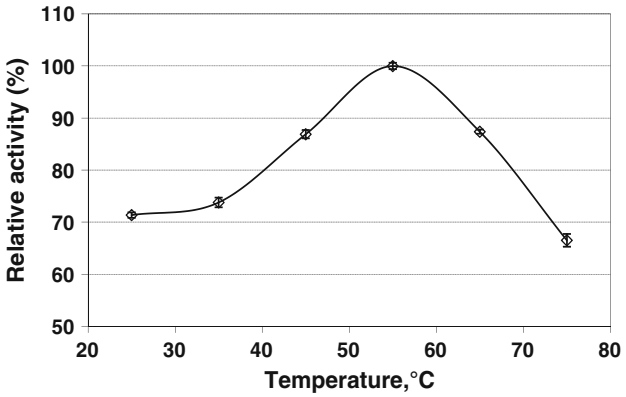

Fig. 3 Influence of temperature on the stability of lignin peroxidase activity

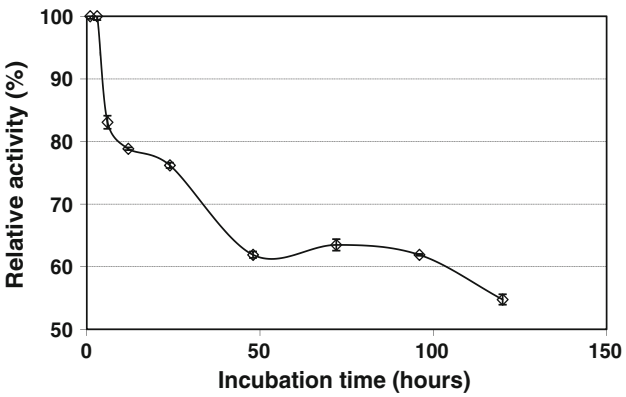

Fig. 4 Effect of incubation time on stability of lignin peroxidase activity

palm oil mill effluent [44]. After 30 min of incubation, the activity of LiP was stable at pH 5.0. The LiP activity was lower at a low pH of 3 and was high below pH 7. More than 70% of activity was lost at pH 7–11. Couto et al. [45] studied the stability of LiP enzyme produced by *P. chrysosporium* and found an optimum pH of 4.2. Other authors have made similar observations [46].

The effect of temperature on the enzyme stability was studied from 25 to 75°C at pH 5 (Fig. 3). The results showed that 100% LiP activity was retained at 55°C after incubation. The LiP activity dropped more than 75% at temperatures below 35°C and lost about 66% of its activity at a temperature of 75°C. Other authors found the LiP enzyme to have an optimum stable temperature of 34°C [45]. Bosco et al. [46] found that a mixture of LiP isoenzymes from immobilized cultures of *P. chrysosporium* catalyzed oxidation reactions at acidic pH and temperatures between 25 and 60°C.

The stability of LiP was studied over 5 days at a pH of 5.0 and temperature of 55°C. Figure 4 shows the effect of incubation time (contact) on LiP activity under the optimum stability conditions. The LiP activity was maintained at 100% for the first 3 h of the incubation period. The activity decreased to less than 80% after 12 h of incubation. After 48 h, the LiP activity remained steady at around 60% and remained constant through 120 h of incubation time.

Conclusions

Results obtained from this study show that an FFD was able to ascertain the optimum operating conditions of the bioreactor to achieve maximum activity of LiP. An aeration rate of 2.0 vvm and an agitation rate of 200 rpm gave

the highest activity of 743 U/l after 5 days of fermentation, which was 3.5-fold higher compared to the FFD (225 U/l) at day three. With an optimum yield of LiP, the biodegradation was observed to achieve the maximum removal of COD of 93% after 5 days of treatment. The enzyme stability test indicated that the LiP enzyme was most stable at a pH of 5.0 and a temperature of 55°C. The LiP activity was reduced by up to 40% after 48 h of incubation. This study shows an alternative solution for sludge management through the production of LiP, which could offer a major source of revenue in the future.

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