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Recovery of lignin peroxidase from submerged liquid fermentation of *Amauroderma rugosum* (Blume & T. Nees) Torrend using polyethylene glycol/salt aqueous two-phase system

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***Amauroderma rugosum* is a wild mushroom species widely distributed in tropics and is classified under the class of Basidiomycetes. Basidiomycetes are well-known for their abilities of producing lignocellulolytic enzymes such as lignin peroxidase (LiP), laccase (Lac) and manganese peroxidase (MnP). Different factors such as nutrient sources, incubation period and agitation affect the production of lignocellulolytic enzymes. The *A. rugosum* produced LiP in the medium supplemented with potato dextrose broth (PDB), 0.5% yeast and 1.0% saw dust at 26.70 ± 3.31 U/mL. However, the LiP activity was increased to 106.32 ± 5.32 U/mL when supplemented with 150 μm of copper ($CuSO_4$). The aqueous two-phase system (ATPS) is a simple, rapid and low cost method for primary extraction and recovery of LiP. A total of 25 systems made from five different molecular weights of polyethylene glycol (PEG)/dipotassium hydrogen phosphate (K_2HPO_4) were tested. PEG 600 produced the highest top phase purification factor (P_{FT}) of 1.33 ± 0.62 with yield of 72.18 ± 8.50%. The optimization of the ATPS parameters, such as volume ratio V_R, pH and crude enzyme loading are the factors controlling the phase partition. Our results showed that significant improvement (P_{FT} of 6.26 ± 2.87 with yield of 87.31 ± 3.14%) of LiP recovery can be achieved by optimized the parameters.**

[**Key words:** Aqueous two-phase system; Lignin peroxidase; Polyethylene glycol; Purification factor; Mushroom; Submerged fermentation]

Lignin peroxidase (LiP) is an enzyme that was first extracted from white-rot fungus *Phanerochaete chrysosporium* in 1983 (1,2). Its ability to degrade the rigid lignocellulose, lignin-related aromatic compounds, and even non lignin-related compounds (3) has attracted huge interest, especially for biotechnological application. Currently there are several instances of LiP being used in the biotechnology industry such as biopulping, biobleaching, bioremediation, cosmetics, and nanotechnology applications (4,5). According to Maciel et al. (5), this enzyme can be found naturally in Ascomycetes and Basidiomycetes as the only known enzyme being able to degrade lignin. *A. rugosum* is a wild white rot fungi belongs to the order of Polyporales—the largest pore fungi under the Basidiomycetes phylum. Submerged fermentation is a cost effective process to produce mycelia biomass and allows for mass production of bioactive compounds (6). Moreover, the agricultural by-products from the submerge fermentation process can be used as substrate, thus further reducing operation cost. There are several methods for the purification of extracellular enzyme from fungus, for example exclusion chromatography, ion exchange chromatography, affinity chromatography, and the ultrafiltration method (7). However, most of these processes involve multiple step purification, are time-consuming, and very often laborious. Moreover, some of these techniques could potentially affect the conformation and biological activities of the biomolecules, which would then result in low yield and high operational cost (8–14) (Table 1).

Industry demands low cost, efficient, and simple purification processes. The aqueous two-phase system (ATPS) process can therefore be used to extract and purify mushroom LiP. The ATPS method is a common purification methodology often employed in biotechnological purification processes to purify enzyme, protein, DNA, and even carbon nanotubes (15,16). For the purification of enzymes, this method has been used to extract enzymes such as cyclodextrin glycosyltransferase (17) and lipase (18) from bacteria. Besides, the ATPS method has also successfully recovered fibrinolytic enzyme and laccase from different fungus (8,19–22). The simplicity and efficiency of ATPS compared to other purification methods makes it one of the more popular purification methods for biomolecules (23). The chemical used in ATPS is mostly water-miscible and low in toxicity, for example dextran, polyethylene glycol (PEG), and salts such as dipotassium hydrogen phosphate (K_2HPO_4). Recent developments in ATPS technology has come up with an alcohol-based ATPS (24), temperature sensitive ATPS like the usage of thermoseparating polymers (e.g., ethylene oxide propylene oxide (EOPO)) (25), as well as a recycle-able ATPS system

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TABLE 1. Purification of lignin peroxidase from white-rot fungi.

Fungus	Purification methods	Yield (%)	Purification fold	Source
Pycnoporus sanguineus MTCC-137	Ultrafiltration and anion exchange chromatography	10.85	2.26	9
Bjerkandera sp. strain BOS55	Ammonium sulphate precipitation, cation and anion exchange chromatography	2.0	167	10
Phanerochaete sordida YK-624	Ultrafiltration, Anion-exchange and gel permeation chromatography	177	7.4	11
Schizophyllum commune	Ammonium sulphate precipitation and gel filtration chromatography	5.2	2.34	12
Phanerochaete chrysosporium	Ammonium sulphate precipitation and ion-exchange chromatography	48	2	13
Loweporus lividus MTCC-1178	Ultrafiltration and anion exchange chromatography	12.6	4.9	14

(e.g., alcohol, EOPO, ionic liquid) (26). This shows the versatility of ATPS in catering to various forms of purification need.

A simple and efficient extraction process is vital to produce the LiP enzyme in large quantity from the *Amauroderma* mushroom. Therefore, the aim of this study is to investigate the optimum condition for recovery of LiP using ATPS.

MATERIALS AND METHODS

Polyethylene glycol (PEG) with different molecular weights (M_W) (ranging from 600, 1000, 1500, 3350 and 8000 g/mol), veratryl alcohol, 3,4-dimethoxybenzaldehyde (veratraldehyde), bovine serum albumin, and Coomassie Brilliant Blue G-250 reagent were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), and copper sulphate ($CuSO_4$) were purchased from Merck (Darmstadt, Germany), while potato dextrose agar (PDA) and the potato dextrose broth (PDB) were purchased from Difco.

Fungus strain The mycelium culture of *A. rugosum* (KUM 61131) was obtained from Mushroom Research Centre, University of Malaya. The mycelium culture was maintained in the PDA plate by periodical subculture on every 14th day.

Preparation of mushroom mycelia for submerged fermentation The submerged fermentation of *A. rugosum* was produced by inoculating *A. rugosum* mycelium subculture into PDB. Various types of substrates such as potato dextrose, glucose, malt, yeast, peptone, saw dust, bamboo leaves, or an inducer (copper sulphate at concentrations of 0, 50, 100, 150, 200 and 300 μM) were added in different ratios for optimum LiP production. The submerged culture was incubated at 25°C with shaking (120 rpm) for 14 days. After 14 days of incubation, the mycelia were filtered with Whatman filter paper No. 1 and stored in a refrigerator at 4°C for not more than 2 days to reduce the degradation.

Determination of lignin peroxidase activities The LiP activity was determined according the method described by Have et al. (10). The enzyme mixture contained 0.2 mL of enzyme (crude or partial purified) in 2.4 mL of 100 mM sodium tartrate (pH 3.0) and 0.2 mL of 30 mM veratryl alcohol. The reaction was initiated by adding 0.2 mL freshly prepared 0.5 mM hydrogen peroxide. The enzyme activity was measured at $\lambda = 310$ nm after 5 min of incubation. The veratraldehyde was used as standard. One unit (U) was defined as the amount of 1 μmol of veratryl alcohol that converts to veratraldehyde per min per ml of the substrate.

Determination of total protein concentration The total protein concentration was determined using the method described by Bradford (27). The bovine serum albumin (BSA) was used as the standard. For protein determination, 0.2 mL of the enzyme sample was added to 5 mL of Coomassie Brilliant Blue G-250 reagent, and the mixture was incubated for 5 min at room temperature (26 ± 2°C). The absorbance of the enzyme sample was measured at 595 nm.

Preparation of ATPS Several ATPS systems were prepared using the PEG and salt. The PEG and salt were added in a predetermined % w/w into water to form the stock solution. One gram of crude enzyme was then added to top up to a total weight of 10 g. The two stock solutions were then mixed together based on a different quantity, and centrifuged at 4000 $\times g$ for 10 min to form a two-phase system. A phase diagram for different molecular weights of PEG (600 g/mol, 1000 g/mol, 1500 g/mol, 3350 g/mol, and 8000 g/mol) and potassium phosphate salt at pH 7.0 was obtained using the method described by Albertsson (28). The tie-line-length (TLL) was computed based on the binodal curve shown on the phase diagram. Both the upper and bottom phase of the ATPS system were then tested for enzyme concentration. The experiment was repeated to investigate the effect of different parameters such as volume ratio (V_R), pH (pH 6–9), crude enzyme concentration (10–20%), and addition of salt (NaCl at 1–4%).

Determination of volume ratio, purification factor, and yield The V_R was defined as the ratio of volume in the top phase (V_T) to the volume of the bottom phase (V_B) (Eq. 1):

$$V_R = \frac{V_T}{V_B} \tag{1}$$

Purification factor (P_{FT}) was calculated by the ratio of specific activity in the top phase to the specific activity (Eq. 2).

$$P_{FT} = \frac{\text{specific activity in top phase}}{\text{specific activity in crude extract}} \tag{2}$$

The partition coefficient (K) of the LiP was the ratio of the LiP concentration in top phase and bottom phase (Eq. 3).

$$K = \frac{C_T}{C_B} \tag{3}$$

where C_T and C_B represented the LiP activity (U/mL) in the top and bottom phase, respectively.

The yield of LiP in top phase was determined using (Eq. 4).

$$Y_T = \frac{100}{1 + \left[\frac{1}{V_R * K}\right]} \tag{4}$$

Where K represented the partition coefficient and V_R was the volume ratio.

Characterization of protein Molecular mass of the crude enzyme and top phase enzyme of ATPs was analysed using the sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). The SDS-PAGE was prepared using 12% resolving gel and 5% stacking gel, based on the method described by Laemmli and Favre (29). Electrophoresis was then carried out at 100 V for 120 min. Then gel was stained using the Coomassie Brilliant Blue R-250. The Native-PAGE was carried out to determine the LiP activity by excising out the bands.

Statistical analysis All the data were recorded as mean ± standard deviation in triplicates. Data were analysed using SPSS package (PASW Statistics 18 for windows) as one way analysis of variance (ANOVA). The differences between each sample were performed by Duncan test where $p < 0.05$ was considered as significantly different.

RESULTS AND DISCUSSION

Optimization of culture medium for submerged fermentation Process optimization on fermentation medium and process conditions play important roles to increase the product yields and to ensure the product quality in industrial fermentation (30). Rogalski et al. (31) reported that the ligninolytic enzymes production by white rot fungi is dependent on the growth condition. Almost all the formulated media tested produced LiP and the activity ranged from 3.13 ± 0.84 U/mL to 26.70 ± 3.70 U/mL. The highest LiP activity detected was PDB, 1% saw dust and 0.5% yeast. In general, by increasing both the carbon and nitrogen concentrations, the enzyme activities could be increased as well (Table 2). As for the media containing PDB, 1% saw dust and 0.5% yeast, it contained a natural nitrogen source in the form of yeast. Yeast extract is naturally enriched with peptides, all kinds of amino acids, nucleotides, and also the soluble components of yeast cells (32). Besides, the inclusion of lignocellulosic compounds such as wheat straw and hemp wood were reported to enhance LiP production in the PDB media (33). The media with added saw dust (C:N = 95:1) or bamboo leaves shown in Table 1 induced the level of LiP. The high LiP activity could also be attributed to the nature of the *A. rugosum* itself. Hariharan and Nambisan (34) reported that optimization of different process parameters such as nutrient sources, incubation period and agitation enhanced the production of LiP in *Ganoderma lucidum*.

Effects of copper in LiP production The addition of copper into the media was able to increase the LiP production in *A. rugosum*. As shown in Fig. 1, the medium without copper showed the lowest LiP activity with 3.13 ± 0.75 U/mL. On the other end, the highest

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