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Extraction of enzymes from spent compost of *Pleurotus sajor-caju* and its potential use for decolourisation of synthetic dyes

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ABSTRACT Extraction of enzymes from 5-month old spent mushroom compost of *P. sajor-caju* by homogenization for eight min at 8000 rpm using tap water at pH 4 had an advantage over the use of an incubator shaker. Both lignin peroxidase (103.42 Ug^{-1}) and β-glucosidase (132.07 Ug^{-1}) were present at high levels and there was a further 5.5 fold increase of lignin peroxidase and 3.7 fold increase of β-glucosidase when concentrated. Other enzymes present in the spent mushroom compost at lower levels were laccase (0.15 Ug^{-1}), carboxymethyl cellulase (1.71 Ug^{-1}) and xylanase (6.62 Ug^{-1}). This mixture of concentrated enzymes which mainly consists of lignin peroxidase and β - glucosidase showed good potentials to decolourize crystal violet, trypan blue and amido black. The percentage decolourisation of remazol brilliant blue R was only significant at high concentration of lignin peroxidase (25 U) while no decolourisation was observed for methyl green. The study showed significant depolarization of all the dyes in the presence of 1 mM veratryl alcohol.

ABSTRAK Penghomogenan selama lapan minit pada 8000 rpm dengan menggunakan air paip pada pH 4, adalah lebih baik berbanding dengan penggunaan inkubator goyang untuk pengekstrakan enzim dari kompos cendawan *P. sajor-caju* yang berumur 5 bulan. Kedua-dua lignin peroksidase (103.42 Ug^{-1}) dan β-glukosidase (132.07 Ug^{-1}) hadir pada paras yang tinggi dan terdapat peningkatan sebanyak 5.5 kali ganda lignin peroksidase dan peningkatan 3.7 kali ganda β-glukosidase selepas proses pemekatan enzim yang diekstrak. Enzim lain yang hadir dalam kompos cendawan pada paras yang lebih rendah adalah lakase (0.15 Ug^{-1}), karboksimetil sellulase (1.71 Ug^{-1}) and xilanase (6.62 Ug^{-1}). Campuran enzim pekat ini yang terdiri kebanyakannya dari lignin peroksidase and β-glukosidase berpotensi besar untuk menyahwarna pewarna kristal ungu, tripan biru and amido hitam. Peratus penyahwarnaan remazol brilliant blue R hanyalah signifikan pada kepekatan lignin peroxidase (25 U) yang tinggi manakala tiada penyahwarnaan dilihat pada metil hijau. Kajian ini menunjukkan penyahwarnaan yang signifikan bagi semua pewarna dengan kehadiran 1 mM veratril alkohol.

(mushroom enzymes, spent mushroom compost, decolourisation)

INTRODUCTION

Spent mushroom compost consists of mycelium; extracellular enzymes produced by mushrooms during growth and unutilized lignocellulosic substrate used for mushroom production. For every 200g of mushroom produced, about 800g of spent compost is available. An average mushroom farm in Malaysia discards about 24 tonnes of spent compost per month. The disposal of spent mushroom compost is a major problem for mushroom farmers. Currently, the spent mushroom compost is disposed either by burning or dumping in nearby land. Both these disposal methods leads to environmental pollution. This residue, however, may be used for the production of value-added products including bulk enzymes [1]. The decolourisation potential of enzymes from fungi have been widely investigated, especially the use of laccase [2] and lignin peroxidases [3, 4]. The objectives of this study were to determine the levels of xylanase, cellulase, β-glucosidase, laccase and lignin peroxidase present in spent compost of *P. sajor-caju* at 5 months after inoculation, to maximize the extraction process for the enzymes present in spent mushroom compost and to use the enzymes for the decolourisation of crystal violet, methyl green, amido black, tryphan blue and remazol brilliant blue R (RBBR).

EXPERIMENTAL

Spent compost of P. sajor-caju

Five-month old discarded *P. sajor-caju* bags were collected from a farm in Semenyih, Selangor. Six bags were opened and the contents of the bags were placed in a plastic basin. The contents were manually broken up and mixed thoroughly. Enzymes were extracted from the resultant mixture. Duplicate samples were analysed.

Optimization of enzyme extraction and concentration

The method of extraction of enzymes in the spent mushroom compost using an incubator shaker was compared with extraction using a homogenizer. Extraction using an incubator shaker at 200 rpm was carried out for one hour using tap water at pH 6 as the extracting medium at two incubation temperature of 4 °C and 28 °C. Extraction using the homogenizer was carried out using tap water at pH 4 and homogenization was done at the speed of 8000 rpm for 8 min at 28°C. After extraction, the crude enzyme was concentrated using the Millipore stirred cells concentrator using 10,000 daltons cut off membrane. The activity of laccase, lignin peroxidase, xylanase, carboxymethyl cellulase and β-D-glucosidase were analyzed before and after concentration of the enzymes.

Enzyme assays

Laccase activity was determined by the increase in the absorbance due to the formation of tetramethoxy-azo-bis-methylenequinine resulting from the reaction of laccase with syringaldazine [5,6]. The substrate was 0.1 mmol syringaldazine in 50% ethanol (w/v). One unit of enzyme activity was defined as the amount of enzyme producing one unit change in absorbance/min/g of the substrate. Lignin peroxidase activity was measured by recording the increase in the absorbance at 310 nm due to the oxidation of veratryl alcohol to veratraldehyde by lignin peroxidase [7]. The reaction mixture consisted of lignin peroxidase, 100 mmol sodium tartrate buffer pH 3.0 and 2 mmol veratryl alcohol as substrate. The reaction was initiated by the addition of H_2O_2 at a final concentration of 0.5 mmol. The standard used was 3,4-dimethoxybenzaldehyde (veratraldehyde). Xylanase activity was measured using 1% (w/v) suspension of xylan from oat spelts in 50 mmol sodium citrate buffer pH 4.8 [8]. Xylose was used as the standard. Carboxymethyl cellulase (CMCase) activity was estimated by using 1% carboxymethyl cellulose (medium viscosity) as the substrate [9]. The liberated reducing sugars were determined using the dinitrosalicylic (DNS) acid reagent method as explained by Miller [10]. The standard used was glucose. β-D-glucosidase activity was determined using 0.5 mmol p-nitrophenyl-β-D-glucopyranoside in 50 mmol sodium citrate buffer pH 4.8 as the substrate [9]. The standard used was p-nitrophenol.

All the enzyme assays were carried out in duplicates. The enzyme activities were expressed as international units (U) and defined as the amount of enzyme required to produce 1 μmol product/min and were reported as Ug^{-1} of the substrate.

Decolourisation of dyes

The decolorisation of crystal violet, methyl green, amido black, tryphan blue and remazol brilliant blue R (RBBR) were tested using concentrated enzyme. Concentrated enzyme with lignin peroxidase activity was adjusted to enzyme units ranging from 5U to 25 U and were mixed with individual dyes in 50 mmol sodium tartrate buffer at the optimum pH of each dye in accordance with Avneesh *et al.*[11]. The optimum pH for crystal violet, trypan blue, amido black, RBBR and methyl green were 4, 3.5, 3.5, 3 and 2.5 respectively [11]. The concentration of the dye used in this experiment was 12.3 μmol according to Yesilada [12]. The reaction was initiated by adding 0.2 mmol H_2O_2 and incubated at different time intervals of 4, 8, 12 and 24 h. To observe the effect of mediators, 1 mmol of veratryl alcohol was added in one set whereas buffer was added in place of veratryl alcohol in another set of experiment. The total reaction volume used was 3 ml. The decrease in the absorbance was measured spectrophotometrically at the absorption maxima of each dye. All the experiments were run in triplicates.

RESULTS AND DISCUSSIONS

Optimisation of enzyme extraction and concentration

Table 1 shows the levels of enzymes present in five-month old spent mushroom compost when extracted using an incubator shaker and a homogenizer. Among the enzymes assayed, the levels of lignin peroxidase was highest when extracted with the incubator shaker or a

homogenizer at levels ranging from 55.36 – 96.21 Ug^{-1}. β-glucosidase showed high activity of 92.63 Ug^{-1} when extracted with the homogenizer. Five months old bags of *P. sajor-caju* were a good source of extracellular enzymes namely lignin peroxidase and β-glucosidase at which the yield in terms of the fruit bodies was low and the bags were discarded by the mushroom farmers. The method of extraction of enzymes in tap water using a homogenizer at room temperature for 8 min at 8000 rpm was faster and more economical compared to the extraction using an incubator shaker.

Table 2 shows the levels of enzymes in spent mushroom compost when extracted using the homogenizer before and after concentration using the Millipore stirred cells. After concentration there was an increase in the activities of all the enzymes. Laccase showed 16 fold increase in concentration followed by lignin peroxidase (5.5 fold) and β - glucosidase (3.7 fold). This mixture of concentrated enzymes which mainly consist of lignin peroxidase and β - glucosidase, were tested for its ability to decolourize various individual dyes.

Table 1. Enzyme activities in spent mushroom compost when extracted using an incubator shaker and a homogenizer

Method of extraction	Laccase (Ug^{-1})	Lignin Peroxidase (Ug^{-1})	Xylanase (Ug^{-1})	Carboxymethyl Cellulase (Ug^{-1})	β-glucosidase (Ug^{-1})
Incubator shaker at 200 rpm for one hour using tap water pH 6 at 4° C.	0.25	91.41	6.48	1.94	23.31
Incubator shaker at 200 rpm for one hour using tap water pH 6 at 28° C.	0.11	55.36	5.11	1.14	No activity observed
Homogenizer at 8000 rpm for 8 min using tap water pH 4 at 28° C.	0.05	96.21	6.96	2.99	92.63

Table 2. Enzyme activities before and after concentration of enzymes using Millipore stirred cells

Enzyme	Activity before concentration (Ug^{-1})	Activity after concentration (Ug^{-1})	Concentration fold
Laccase	0.15	2.41	16
Lignin peroxidase	103.42	572.86	5.5
β - glucosidase	132.07	497.65	3.7
Carboxymethyl Cellulase	1.71	3.41	2
Xylanase	6.62	7.85	1.1

Decolourisation of dyes using concentrated crude enzyme

Decolourisation of triphenyl methane, azo and polymeric dyes were observed in our study using concentrated enzyme in which the concentration of lignin peroxidase was adjusted to 5U, 15U and 25U.

Lignin peroxidase decolourizes crystal violet both in the presence and absence of veratryl alcohol. Fig. 1 shows the percentage decolourisation of crystal violet was higher in the presence of veratryl alcohol in the range of 28.0 – 48.8 % whereas in the absence of veratryl alcohol the range of percentage decolourisation was 16.1 – 25.8%. Decolourisation of crystal violet increases, as the concentrations of lignin

peroxidase were increased from 5 U to 25 U, however the increase in percentage decolourisation was less than 10% as the time of incubation of enzyme with the dye was increased from 4 to 24 h.

A 92%, 82%, 86% and 62% decolourisation of crystal violet over 3 days by *Coriolus versicolor, Funalia trogii, Laetoporus sulphureus* and *Phanerochaete chrysosporium* ME446 respectively, have been reported by Yesilada [12] when 12.3 μmol of the dye was added to the cultures. The researcher further reported that only 3 μg residual crystal violet dye was observed after 24 h using 6 U and 30 U of commercial horseradish peroxidase [12]. A complete decolourisation of crystal violet after 72 h has been reported by Bumpus *et al.* [13] when 12.3 μmol of the dye was added to the nitrogen limited ligninolytic cultures of *Pha chrysosporium* and also using purified lignin peroxidase. However, Vasdev *et al.* [14] have reported a 96% decolourisation of crystal violet after 96 h when the fungus *Cyathus bulleri* was grown in liquid medium to which 72 μmol of the dye was added. The researchers also observed 83 - 90% decolourisation after 240 h using active supernatant, ultrafiltered and dialyzed extracellular culture filtrates. The researchers observed a high laccase activity during the decolourisation of dye also suggesting the involvement of this enzyme in decolourisation. The researchers reported a 90 μmol of dye decolourized by *Cyathus bulleri*, which is quite high as compared to 12.3 μmol of dye decolourized by *Pha chrysosporium*.

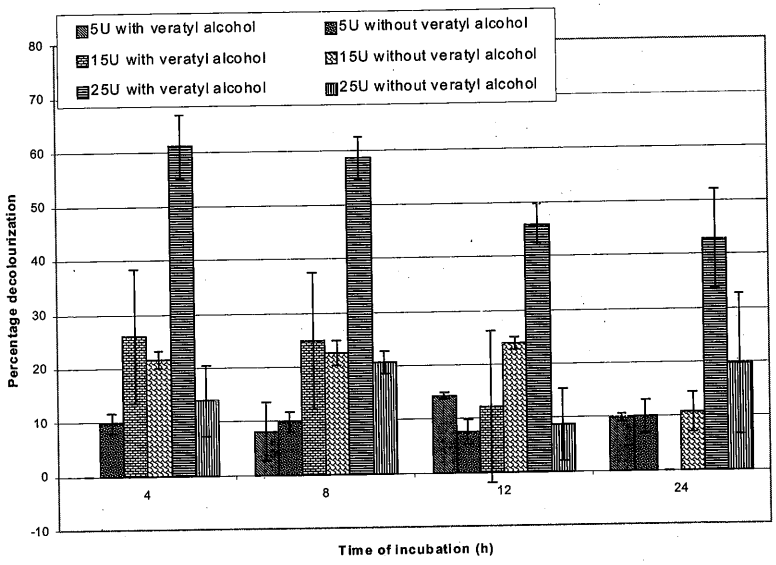

Fig 1. Percentage decolourisation of crystal violet by different concentrations of concentrated enzyme in the absence and presence of veratryl alcohol

The percentage decolourisation of trypan blue and amido black by lignin peroxidase was also higher in the presence of veratryl alcohol ranging from 40.1 – 60.8% and 13.0 – 48.8% respectively, whereas in the absence of veratryl alcohol the percentage decolourisation was 36.5 – 44.2% and 8.8 – 30.9 respectively (Fig. 2 & 3). In the presence of veratryl alcohol, the percentage decolourisation of both the dyes significantly increased as the concentration of lignin peroxidase was increased. However, in the absence of veratryl alcohol the percentage decolourisation of trypan blue showed no significant increase as lignin peroxidase was increased as compared to amido black. The time of incubation did not significantly affect the percentage of decolourisation of trypan blue and amido black.

A complete decolorisation of amido black and tryphan blue within 14 days have been reported by Pointing *et al.* [15] when *Pycnoporus sanguineus*, *Trametes versicolor* and an unidentified member of the corticeaceae (HKUCC 4062) were grown in the liquid medium supplemented with 0.01% (w/v) dye. A 23-48% mineralization of azo dyes after 12 days by the lignin degrading fungus *Pha chrysosporium* under nitrogen limiting conditions have also been reported by Spadaro *et al.* [16].

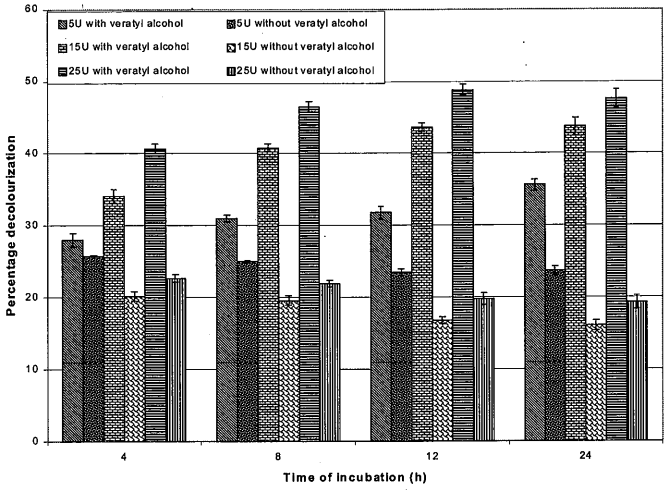

Figure 2. Percentage decolourisation of trypan blue by different concentrations of concentrated enzyme in the absence and presence of veratryl alcohol

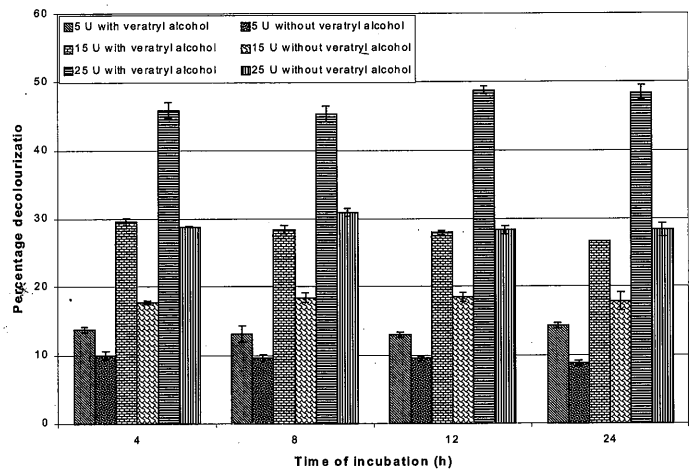

Figure 3. Percentage decolourisation of amido black by different concentrations of concentrated enzyme in the absence and presence of veratryl alcohol

The percentage decolourisation of RBBR was less than 30% at low lignin peroxidase concentrations in the presence or absence of veratryl alcohol (Fig. 4). Lignin peroxidase at 25U showed significant decolourisation of RBBR in the presence of veratryl alcohol at 4, 8, 12 and 24 h with values of 61.1, 58.6, 45.9 and 42.8% respectively.

In this study, no decolourisation was observed for methyl green using concentrated enzyme in the presence or absence of veratryl alcohol at all the

concentration of enzyme used. However, Ollikka *et al.* [4] reported more than 80% decolourisation of methyl green using crude enzyme in the presence of 2 mmol veratryl alcohol after 15 min of incubation by the fungus *Pha chrysosporium.* A similar decolourisation rate was observed by these researchers for methyl green using the isoenzymes of lignin peroxidase in the presence of veratryl alcohol.

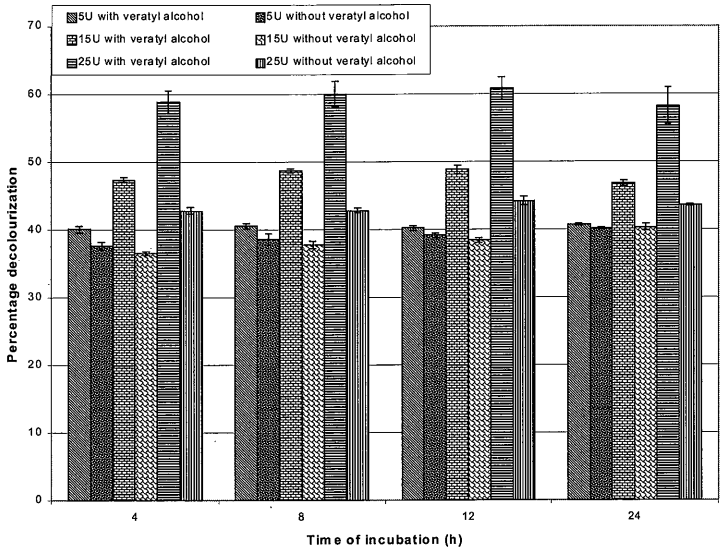

Figure 4. Percentage decolourisation of remazol brilliant blue R by different concentrations of concentrated enzyme in the absence and presence of veratryl alcohol

Veratryl alcohol (3,4-dimethoxybenzylalcohol) is synthesized *de novo* from L – phenylalanine by *Pha chrysosporium* and its formation coincides with the onset of ligninolytic activity [17,18]. Furthermore, veratryl alcohol has been shown to stimulate ligninolytic activity as well as ligninase production [19]. The role of veratryl alcohol as a mediator in lignin degradation has been proposed by Harvey *et al.* [20]. The researchers suggested that veratryl alcohol is oxidized to a radical cation which in acidic media, is not rapidly degraded. Therefore, in the presence of a second oxidizable substrate it can act as a one-electron oxidant and in the process of electron transfer, is regenerated. [20]. In our study, significant decolourisation of all the dyes were observed in the presence of 1 mmol veratryl alcohol. A decrease in the decolourization was observed when veratryl alcohol was omitted from the reaction mixtures (Figs. 1- 4). Our results are in accordance with Ollikka *et al.* [4] who reported a decrease in the ability of the purified isoenzymes of lignin peroxidase to decolourize methylene blue, methyl green, methyl orange, toluidine Blue O and Poly S-119 when veratryl alcohol was omitted from the reaction mixture. No decrease in the decolourisation ability of the crude lignin peroxidase was observed by the researchers when veratryl alcohol was omitted from the reaction mixture. However, a decrease in the decolourisation ability of the crude lignin peroxidase was observed by the researchers when diafiltered crude lignin peroxidase was used, which was restored when pure veratryl alcohol was added to the reaction mixture. The researchers further reported that in the presence of 1 mmol veratryl alcohol, the crude lignin peroxidase was able to decolourize methyl green almost completely. In another study, Paszczynski and Crawford [21] also reported that ligninase of *Pha chrysosporium* oxidized azo dyes i.e. Biebrich Scarlet and Tetrazine to a very limited extent. After veratryl alcohol was added to the reaction mixtures, oxidation of both substrates began, leading to total decolourisation of the dyes. The researchers also reported that a spectrum of native enzyme was observed in the reaction mixture that contained veratryl alcohol

whereas, only the spectrum of LiPII (lignin peroxidase compound two) was observed in the reaction mixture that did not contained veratryl alcohol and only the azo dye was available as a reductant. Based on the results, the researchers suggested the probable role of veratryl alcohol could be that LiPI (lignin peroxidase compound one), formed during oxidation of H_2O_2 by lignin peroxidase, oxidized polyaromatic azo dye forming LiPII which was then reduced back to the native enzyme by oxidation of veratryl alcohol. A similar role may also be played by veratryl alcohol in our study thus giving a higher decolorisation rate of dyes in the samples supplemented with veratryl alcohol.

High amounts of lignin peroxidase can be extracted from spent compost of *P. sajor-caju* which was discarded by mushroom farms. This enzyme have been shown to have potential use for the application of decolourisation of dye wastewaters. The use of lignin peroxidase for the decolorisation of textile waste-water has been reported by Darah *et al.* [22]. The researchers reported a 50 % decolorisation of the textile waste-water after 15 days using 55 Uml^{-1} of lignin peroxidase. A 50 % reduction in chemical oxygen demand after 14 days was also observed by the researchers using cell free extracts [22]. In an another report, Kirby *et al.* [23] observed the decolorisation of an artifical textile effluents after 7 days by growing *Pha chrysosporium* using glucose as the primary source of carbon. The role of lignin peroxidase however, was not clear although high lignin peroxidase of 100 UL^{-1} was produced by the strains [23].

Although lignin peroxidase have been reported to have potential for the decolorisation of dyes as well as textile effluents, a further study is needed to find the effect of heavy metals (present in textile waste water) on lignin peroxidase activity. Pointing *et al.* [15] through their experiments have reported that *Pha chrysosporium* when grown in the presence of Cd^{2+}, Cu^{2+}, and Zn^{2+}, completely lost its ability to decolourize polymeric dye Poly-R suggesting a possible inhibitory effect of metals on the lignin modifying enzyme system of the fungi.

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