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Full Length Research Paper

Laccase isozymes of *Pleurotus sajor-caju* culture on husk and bran of black sticky rice and their potential on indigo carmine decolourisation

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Extracellular laccases of *Pleurotus sajor-caju* grown on solid state medium consisted of husk and bran of black sticky rice, were partially purified by DEAE–cellulose chromatography. These laccases could be separated into three groups: unboundLac and bound fractions (pool1Lac and pool2Lac). The optimum pH of these laccases was studied using ABTS as substrate. It was found that the pH optimum for unboundLac fell in the range of 3–5 and 3–4 for pool1Lac and pool2Lac. The indigo carmine decolourisation capacity was compared between unboundLac and pool2Lac. It was found that the optimal pH for indigo carmine decolourisation were 5 and 3 for unboundLac and pool2Lac, respectively. In the range of various dye concentrations tested, it was found that indigo carmine at 10 µM with the enzyme activity of 0.01 U, gave the best dye decolourisation with 40.47% within 120 min for unboundLac and with 18.61% within 150 min for pool2Lac. High amount of enzyme used of these laccases might improve decolourisation ability.

Key words: Black sticky rice, husk, bran, *Pleurotus sajor-caju*, laccase, isozyme, purification.

INTRODUCTION

Laccase is a copper-containing polyphenol oxidase (EC 1. 10. 3. 2). It is structurally and evolutionarily related to the large blue copper protein group, which includes the plant ascorbate oxidases, and the mammalian plasma protein ceruloplasmin (Mayer, 2006). Laccase contains four copper ions, which are distributed among three different highly conserved binding sites, for its function, with each copper ion appearing to play an important role in the catalytic mechanism (Thurston, 1994). It catalyses the four-electron reduction of oxygen to water and this is typically accompanied by the oxidation of a substrate.

The biological role for laccase appears to vary depending on the type of organism (Thurston, 1994). In fungi, laccase has been well documented to act as a ligninolytic enzyme (Eggert et al., 1998); it is associated with pigment synthesis and sporulation in *Aspergillus fumigatus* (Sugareva, 2006), with fruiting body formation in *Volvariella volvacea* (Chen et al., 2004). The enzyme also has applications in the food industry (Selinheimo et al., 2007).

Several laccase genes, either as genomic or cDNA clones, have been isolated and characterized, including those from *Trametes versicolor*; (Jönnson et al., 1997), *Phlebia radiata* (Saloheimo et al., 1991), *Trametes villosa* (Yaver and Golightly, 1996), *Ganoderma lucidum* (Joo et al., 2008), Trametes sp. AH28-2 (Xiao et al., 2006), *Pycnoporus cinnabarinus* (Eggert et al., 1997), *Pleurotus sapidus* (Linke et al., 2005) and *Rhizoctonia solani* (Wahleithner et al., 1996). These genes typically display a high degree of identity with one another. In addition, the one cysteine and ten histidine residues, involved in binding the four copper atoms found in the majority of laccase molecules, are conserved, together with a small region around each of the four regions in which the copper ligands are clustered (Thurston, 1994). Several fungi code for more than one non-allelic variant, explaining in part the biochemical diversity of laccases.

Previous studies have shown there to be five laccase isoforms in *Pleurotus sajor-caju* at the transcript level (Fu

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et al., 1997). The expressions of these genes depend on environment of the fungus growth (Soden and Dobson, 2001). In this investigation we reported the extracellular laccase isoforms at the protein level. Then some laccase isozymes were tested for dye decolourisation potential.

MATERIAL AND METHODS

Microorganism

The white-rot fungus *P. sajor-caju* was obtained from the commercial mushroom farm in Ka La Sin province (northeast, Thailand). The stock cultures were maintained on potato dextrose agar medium and stored at 4°C with periodic subculture.

Enzyme extraction, activity assays and characterization

The fungus *P. sajor-caju* was grown on husk and bran of black sticky rice without any mineral supplementation for about 30 days at room temperature. The crude enzyme was extracted with distilled water, filtered through sheet cloth to remove solid stuffs and then subjected to centrifugation at 3,000 rpm for 15 min to discard the precipitate. The clear supernatant, crude enzyme was dialyzed against 20 mM Tris-HCl buffer, pH 7.5 prior to chromatographed on a DEAE-cellulose ion-exchange column equilibrated with the same buffer. The unbound proteins were washed out by the starting buffer. The bound proteins were eluted out later by the gradient of 0 - 1.0 M NaCl in the starting buffer. Fractions were collected, proteins were monitored by the absorbance at 280 and the ligninolytic enzymes were determined for their activities as following.

Laccase (Lcc) activity was spectrophotometric assayed at 32°C of oxidized ABTS as previously described (Khammuang and Sarnthima, 2007).

Manganese peroxidase (MnP) activity was measured with the oxidation of MBTH (3-methyl-2-benzothiazolinone hydrazone) and DMAB (3, 3-dimethylaminobenzoic acid) in the presence of H_2O_2, a modified method of Ngo and Lenhoff (1980). The results were corrected by activities in the reactions without manganese (Mn-independent peroxidase, MnIP) where manganese sulfate was substituted by ethylenediaminetetraacetate (EDTA) to chelate Mn present in the samples. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1.0 □mol of product per minute. All assays were performed in duplicate, with an average sample mean deviation of less than 10%.

Estimation of the optimum pH of each enzyme pool (crude, unbound, pool 1 and pool 2) was measured with ABTS substrate in 50 mM sodium citrate-phosphate buffer (pH 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0).

Protein assays

Protein concentration was determined by the Bio-Rad Protein Assay Reagent (Bio-Rad) with bovine serum albumin as a standard according to Bradford (1976).

Decolourisation experiments

Indigo carmine dye was tested for decolourisation at wavelength with maximum absorbance of the dye (610 nm) by unboundLac and pool2Lac enzymes. The pH optimum for each enzyme was investigated first at 32°C. The effect of initial concentrations of dye on decolourisation ability was evaluated ranged from 10 – 40 µM with the enzyme activity of 0.01 U. Experiments were monitored immediately after enzyme addition and periodically time interval using a JENWAY 6400 Spectrophotometer (LABQUIB, England). Dye decolourisation was expressed in terms of percentage calculated according to the following equation:

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is an absorbance at λ_{max} of each dye immediately measured after enzyme addition and A_t is an absorbance at λ_{max} of each dye after each time intervals.

RESULTS

Laccase production

The laccase productivity of *P. sajor-caju* grown on a medium of husk and bran of black sticky rice was slightly increased at the first 20 days, then rapidly increased and reached the highest level at day 30 (Figure 1).

Purification and characterization of the laccases

The crude enzyme was passed through a DEAE-cellulose column. After washing off unbound proteins, the bound proteins were eluted with 0 - 1.0 M NaCl gradient. Two peaks showing laccase activity were eluted out from a DEAE-cellulose column. These two peaks were designed as pool1Lac (fraction no. 9 - 20) and pool2Lac (fraction no. 21 - 31). In the unbound protein part, laccase activity was also detected. Laccase activity of unbound was designed as unboundLac (Figure 2).

Native polyacrylamide gel electrophoresis

After purifications, each fraction was subjected to a native-PAGE and staining for both laccase activity and protein patterns (Figure 3). It was found that crude enzyme contained at least three laccase isozymes. Unbound fraction contained only upper two laccase isozymes while both pool1Lac and pool2Lac contained three laccase isozymes like in crude enzyme.

Optimal pH of laccase isozymes

Each laccase isozyme was tested for optimal pH using ABTS as substrate. It was found that pH optimal for unboundLac, pool1Lac and pool2Lac were 3 - 5, 3 - 4 and 5, respectively (Figure 4).

Optimal pH for indigo carmine decolourisation

Indigo carmine decolourization at various pH by unboundLac and pool2Lac were observed. It was found that the optimal pH of indigo carmine decolourisation for unboundLac was pH 5 while pool2Lac gave pH 3 as the highest indigo carmine decolorisation (Figure 5).

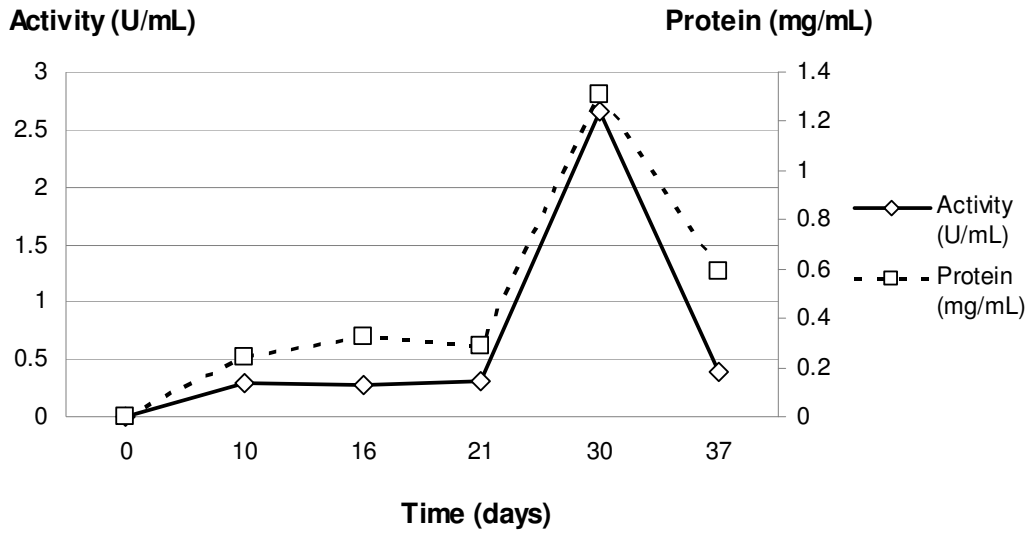

Figure 1. Laccase activity at various culture times when *P. sajor-caju* is grown on rice husk and rice bran of sticky rice medium at room temperature with the highest activity at day 30.

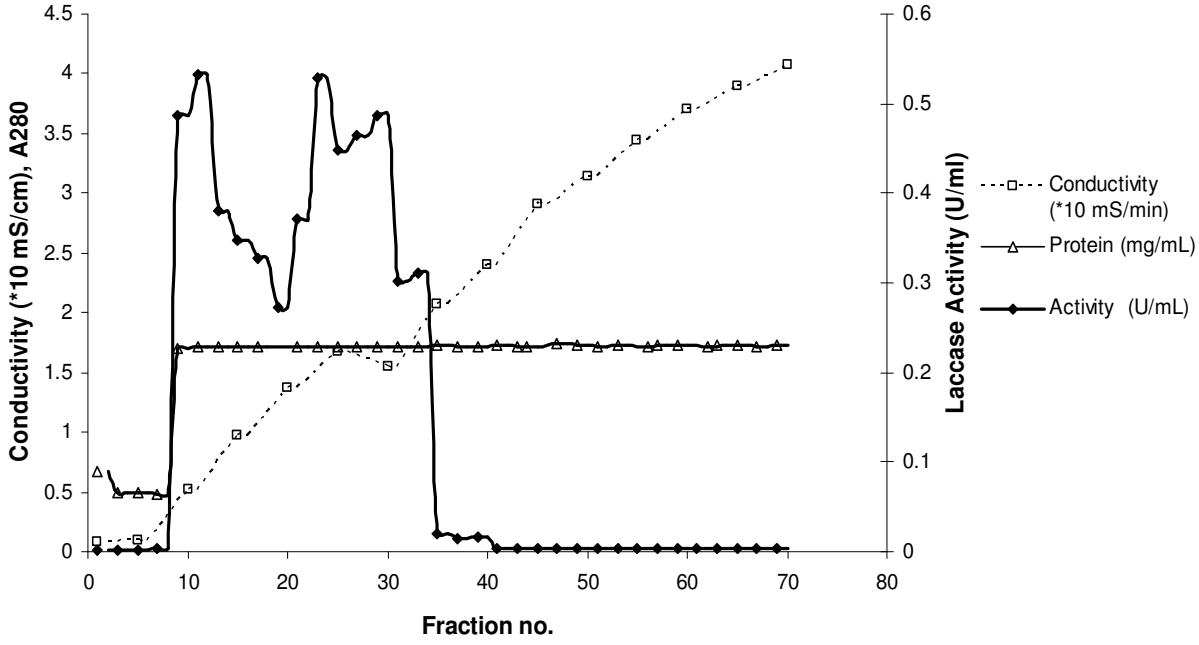

Figure 2. DEAE-cellulose elution profiles with two peaks of laccase activity observed.

Indigo carmine decolourisation percentage

Indigo carmine decolourisation percentages at various initial dye concentrations at 10, 20, 40, and 80 µM by laccase isozymes of unboundLac at 0.01 U (Figure 6a) and pool2Lac at the same amount (Figure 6b) were performed. The highest percentage of dye decolourisation for unboundLac was obtained with about 30% decolourisation of 10 µM of dye within 30 min. On the other hand, the highest indigo carmine decolourisation percentage was observed with about only 18% of 10 µM dye concentration within 150 min.

DISCUSSION

Several lignocellulosic agricultural wastes were used as solid substrates and mixed in different compositions for

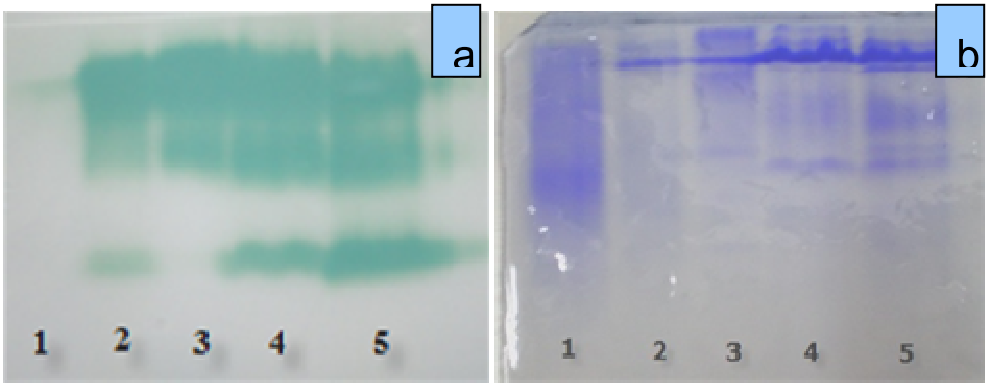

Figure 3. Laccase isozyme patterns of various fractions after native-PAGE and stained with ABTS (a) and corresponding protein patterns after stained with Coomassie Brilliant Blue R-250 (b) lane 1) marker proteins, lane 2) crude enzyme, lane 3) unboundLac, lane 4) pool1Lac, lane 5) pool2Lac.

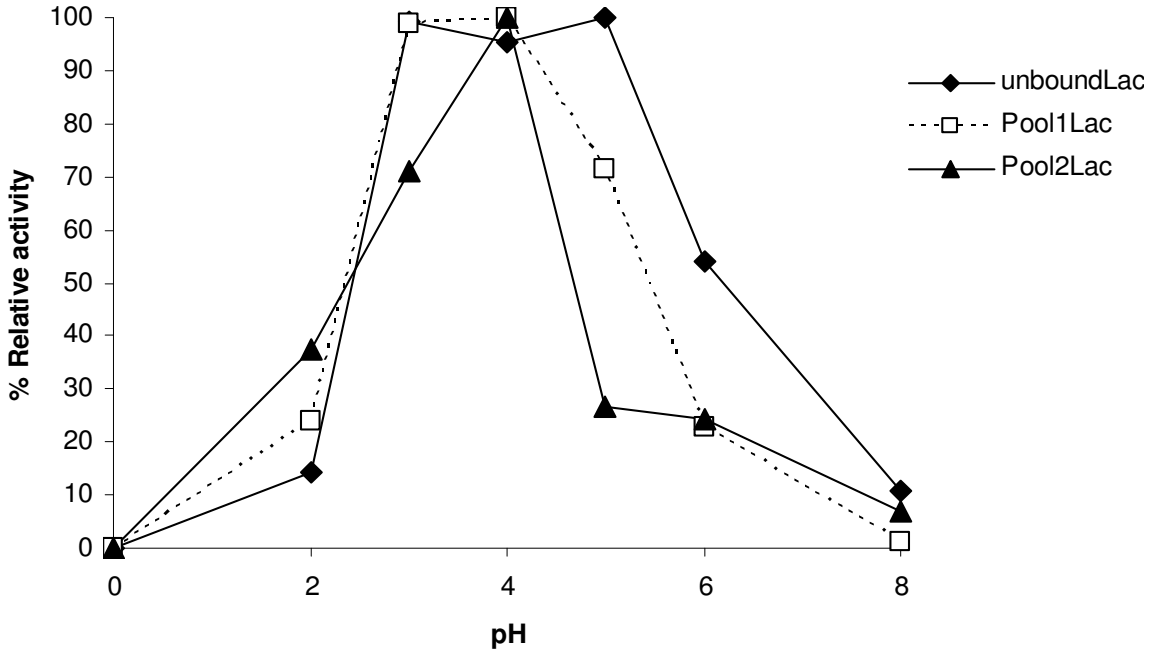

Figure 4. pH optimum of various fractions of laccases using ABTS as substrate.

growing *P. sajor-caju*. Among them the interested medium composition for laccase production was husk and bran of black sticky rice (data not shown). Thailand is a leading country in rice production; there are numerous types of rice in Thailand including black sticky rice. Thus we would like to use such rice wastes for more benefit. This type of medium also has not been reported for laccase production. When grown on this medium, *P. sajor-caju* produced at least three laccase isozymes. The highest level of laccase productivity by *P. sajor-caju* grown on this medium occurred at day 30 and was slightly slower than others previous report from Fernanda et al. (2007). These authors found that *P. sajor-caju* grown on sago 'hampas' gaved maximum amount of laccase after 6 days using 4-week-old inoculums at a density of 10%. However, laccase production in medium consisted of husk and bran of black sticky rice in our report has not been optimized yet. Laccase activity in *P. sajor-caju* is affected by nutrient nitrogen and carbon, and by the addition of copper and manganese to the culture medium. In addition, 2,5-xylidine, ferulic acid, veratric acid and 1-hydroxybenzotriazole induced laccase activity by the fungus. Four laccase isozyme genes have been cloned from *P. sajor-caju*. While some laccase isoforms were inducible, others appeared to be constitutively expressed (Soden and Dobson, 2001). In the media we

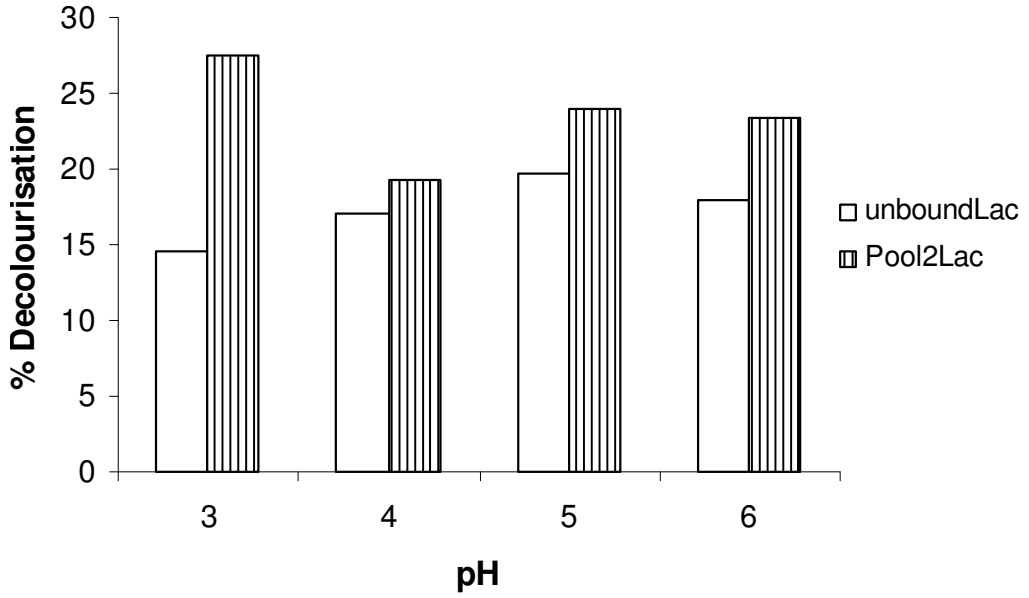

Figure 5. Indigo carmine decolourisation percentages at various pH values by unboundLac and pool2Lac.

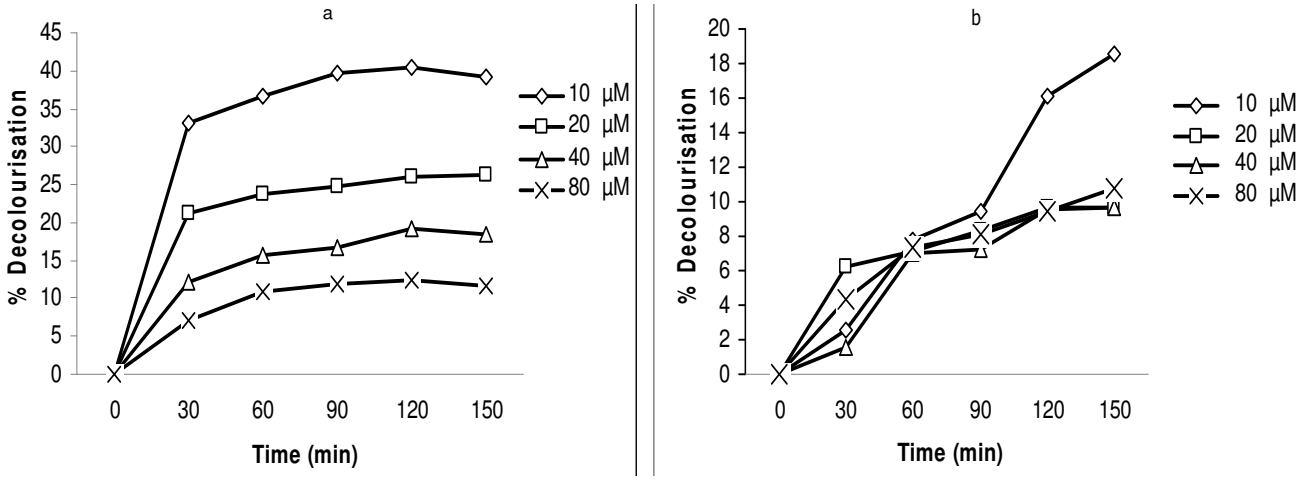

Figure 6. Indigo carmine decolourisation percentages at various initial dye concentration in 100 mM phosphate–citrate buffer, pH 5.0 by unboundLac, 0.01 U (a) and pH 3.0 by pool2Lac, 0.01 U.

tested, laccase isoforms were checked at the protein level. Some laccase isoforms were observed in all media types which showed constitutive expression of these isozymes. Some isozymes were inducible since found in a certain media types. More studied have to be done for clarifying which laccase isozyme is inducible or constitutively expressed at the protein level. The other ligninolytic enzymes also had been assayed from the crude extract. Lignin peroxidase was not found, but Mn peroxidase and Mn independent peroxidase was found in much lower activity when compared to laccase activity.

In this research, the each fraction showed quite clear difference in characteristics and pH optimum for catalysis. These suggest the presence of different isozymes. The decolourisation ability of unboundLac is better than that of pool2Lac. From our result, we can conclude that Indigo carmine dye can be effective decolourisation in different pH conditions according to the isozymes. UnboundLac works best at pH 5.0 which is in agreement with Indigo carmine decolourisation by *Trametes hirsute* (Dedeyan et al., 2000) and by *T. villosa* (Basto et al., 2007). Whereas pool2Lac enzyme of *P. sajor-caju* in our work, has pH optimum at a more acidic condition (pH 3.0). Because the yield of partial purified enzyme of each fraction is in small amount, the decolourisation experiments were studied only by unboundLac and pool2Lac.

However, the amount of enzyme activity use in both fractions was very low in the dye decolourisation experiment compared to previous use in other reports. Therefore, decolourisation percentages of Indigo carmine dye were quite low. Basto et al. (2007) observed that the best Indigo carmine decolourisation was obtained when the dye solution was treated with ultrasound and *T. villosa* laccase stabilized with polyvinyl alcohol yielding about 65% of decolourization after 1 h. However, only 20% decolourization was obtained with the enzyme alone (Basto et al., 2007). In our work, at the same pH value, decolourization could be higher than 35% after 1 h when unbound enzyme used. Our results are more effective and economic as well as environmental friendly. That is because of the enzyme's ability to work at lower temperature, with low amount of biocatalyst and no need ultrasound and other chemical for stabilization. Higher efficiency might be achieved if higher amount of enzymes is used.

The complete decolourisation of indigo carmine has been reported by Couto and Sanromán (2005) which was obtained by an alginate-immobilized laccase from *T. hirsuta* after more than 30 h of treatment or about 3 days with immobilization on stainless steel sponge (Couto et al., 2004). Camarero and co-workers (2005) also reported a 100% indigo carmine decolourisation in less than 1 h when mediators were used with laccase from *T. villosa*. It is possible that decolourisation by these *P. sajor-caju* enzymes might be more successful if redox mediators were applied. Many reports show the high efficiency in dye decolourisations by the purified enzymes. Purification of each isozyme leads to more understanding about them. This knowledge will be very useful for setting the conditions where they work best either for each or in a combination. Purification of each isozyme from *P. sajor-caju* is on the way in our laboratory in order to understand more about its catalytic properties and comparison study of decolourisation ability of each isozyme.

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