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Integration of affinity precipitation with three phase partitioning methods for bioseparation of laccase from *Trametes versicolor*

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Abstract: Separation of *Trametes versicolor* laccase from a commercial preparation was carried out using Three-Phase-Partitioning (TPP) technique. The conditions were optimized and the most favorable ammonium sulphate concentration (70% w/v), crude to t-butanol ratio (1.0:2.0) and pH (4.5) resulted in 56.22% yield with 1.65-fold purity of laccase. For further improvement in purification, an improved version of TPP called Macroaffinity-Ligand-Facilitated Three-Phase Partitioning (MLFTPP) of Trametes versicolor laccase was performed in the presence of the polymer chitosan. The chitosan shows a selective binding to the laccase and is precipitated in the interfacial layer as chitosan-laccase complex. The MFLTPP conditions were then optimized for chitosan and the best conditions were found to be 70% w/v ammonium sulphate concentration, 1.0:1.0 ratio of polymer solution to t-butanol and pH 6.0 that gave 92.3% recovery and 4.97-fold purity. The study was further widened by comparing the efficiency of TPP and MLFTPP in purifying laccases from 3 different sources *Trametes veriscolor*, *Pleurotusostreatus*, *Pseudomonas desmolyticum.* This study, thus, demonstrates the potential of MLFTPP as an industrially exploitable, bioseparation technique for the separation of laccase from a less-established fungal source *Trametes veriscolor*.
Keywords: Three-Phase-Partioning (TPP); Laccase; Macroaffinity-Ligand-Facilitated Three-Phase Partitioning (MLFTPP); Chitosan; Affinity precipitation.

Introduction

Laccase (p-diphenol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the group of copper-containing oxidases with significant applications in industrial and environmental biotechnology that works by catalysing one electron oxidation of wide range of phenolic substrates with simultaneous reduction of dioxygen molecule to water[1].This implies that laccase has a major role in a large number of processes involving aromatic compounds, ranging from natural processes like lignification, delignification, detoxification etc., to biotechnological processes like dye decolorization, bleaching of paper, synthesis of biomaterials, coal solubilisation, ascorbic acid determination, clarification of wine and fruit juices and as a biosensor[1]. Laccase yields obtained by recombinant expression of laccase genes[2] are relatively low for the demands in industrial applications. Osma et al.,[3]clearly explained the cost analysis of upstream process of white rot fungus *Trametes* sp laccase. Downstream process (DSP) costs are an extremely important factor as these constitute upto 80% of the overall production costs of proteins/enzymes[4]. The demand for the simple and high benefit/cost ratio approaches for downstream processing of laccase with high yield and purity is a top priority.

Three phase partitioning (TPP) is described as an efficient integrated bioseparation technique which acts as a bridge between upstream and downstream processing and limits the number of individual unit operations resulting in a high recovery of product from minimally processed culture broths[5]. Application of TPP technique for the purification of laccase has been studied recently[6-9] but their use on a commercial industrial scale is yet to be looked into. Many variations of TPP with better efficiency than TPP, in separation of proteins have also been studied. MLFTPP is one such variant, which employs a more selective procedure for protein separation using awater soluble polymer. The use of water soluble polymers like alginate, esterified alginate, chitosan and eudragit S-100 for the purification of commercially available industrial enzymes like pectinase, amylase, glucoamylase, pullulanase, cellulase and xylanase, respectively resulted in enhanced process selectivity[10-13].This study was aimed at using Three-Phase-Partioning (TPP) technique for the separation of *Trametes versicolor*-laccase from a commercial preparation and investigating the potential use of soft-ligand like chitosan for a more selective purification of laccase using the technique of Macroaffinity-Ligand-Facilitated Three-Phase Partitioning (MLFTPP).

Materials and Methods

Materials

Commercial preparation of laccase from *Trametes versicolor*, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and chitosan were purchased from Sisco Research Laboratories. All other chemicals used were of analytical grade and obtained from various commercial suppliers.*Trametes versicolor* MTCC 138 and *Pleurotus ostreatus* MTCC 1804 were obtained from Microbial type culture collection (MTCC), Punjab, India and maintained on malt extract agar. *Pesudomonasdes molyticum* NCIM 729 was obtained from National Collection of Industrial Microorganism (NCIM), Pune, India and maintained on nutrient agar.

Optimum culture conditions for the production of laccase from various species

The media components employed for laccase production from *Pseudomonas desmolyticum* were (g/l) tryptone 10; yeast extract 5; NaCl 10 and $CuSO_4 \cdot 7H_2O$ 0.005 (pH 4.5). A 72 h age and 15% size was used for inoculation. Flasks were incubated (30 °C, 150 rpm) in a rotary shaker for 14 days. Samples were withdrawn aseptically at regular time intervals and analyzed for laccase activity. The media components employed for laccase production from *Trametes versicolor* MTCC 138 and *Pleurotus ostreatus* MTCC 1804 were (g/l) malt extract 10; yeast extract 40; Glucose 4; Tyrosine 0.005; and $CuSO_4 \cdot 7H_2O$ 0.001 (pH 6.3). A 108 h age and 10% size was used for inoculation[9]. Flasks were incubated (30 °C, 150 rpm) in a rotary shaker for 14 days. Samples were withdrawn aseptically at regular time intervals and analyzed for laccase activity. After approximately three weeks, the produced laccase from the fungal sources, was purified from the crude extracts by employing and TPP and MLFTPP in the presence of chitosan.

Optimization of TPP of Trametes versicolor crude extract

In the initial set of optimisation experiments, TPP was carried out by saturating 1ml of crude extract of *Trametes versicolor* (35.6U at pH 4.0) to 30 - 90% (w/v) using ammonium sulphate, followed by addition of t-butanol to bring 1:1 ratio of crude extract to t-butanol, at room temperature and pH 4.0. In the second set of optimisation, t-butanol was added to obtain 1.0:0.25 to 1.0:2.0 (v/v) ratios at room temperature, 70% saturation of ammonium sulphate and pH 4.0. pH optimisation was done by carrying out the experiment with 70% ammonium sulphate concentration and 1:2 ratio of crude extract to t-butanol, at different pH 3.0–6.5. After shaking vigorously, the mixture was incubated for 1 h at room temperature after which, the separation of the mixture into three phases was apparent. The interfacial precipitate was collected and was suspended in 2ml of 0.1M sodium acetate buffer, pH 4.0. Protein concentration and enzyme activity were determined by taking those of crude extract as 100%.

Optimisation of MLFTPP conditions for chitosan

Purification of laccase enzyme from crude extract using MLFTPP technique performed by saturating 0.2% w/v chitosan-enzyme solution (38.15U at pH 4) with various conditions of ammonium sulphate concentration, crude extract to t-butanol ratio, pH, etc as described earlier for TPP.

Laccase assay

Laccase activity was estimated using 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) as substrate and the reaction was carried out at room temperature. The reaction mixture of 1 ml contained 1mM ABTS, pH 4.0 buffer (0.1 M), and 0.1 ml aliquots of diluted enzyme extract (interfacial layer dissolved in 2 ml of pH 4 acetate buffer). Oxidation of ABTS was indicated through an absorbance increase at 420nm (ε = 36,000M−1 cm−1).One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1µM of ABTS per min at 25°C. Laccase activities were expressed in U/ml. Protein content was estimated by the Folin–Lowry method[14] using bovine serum albumin as standard protein. %Yield and fold purification were calculated as follows:

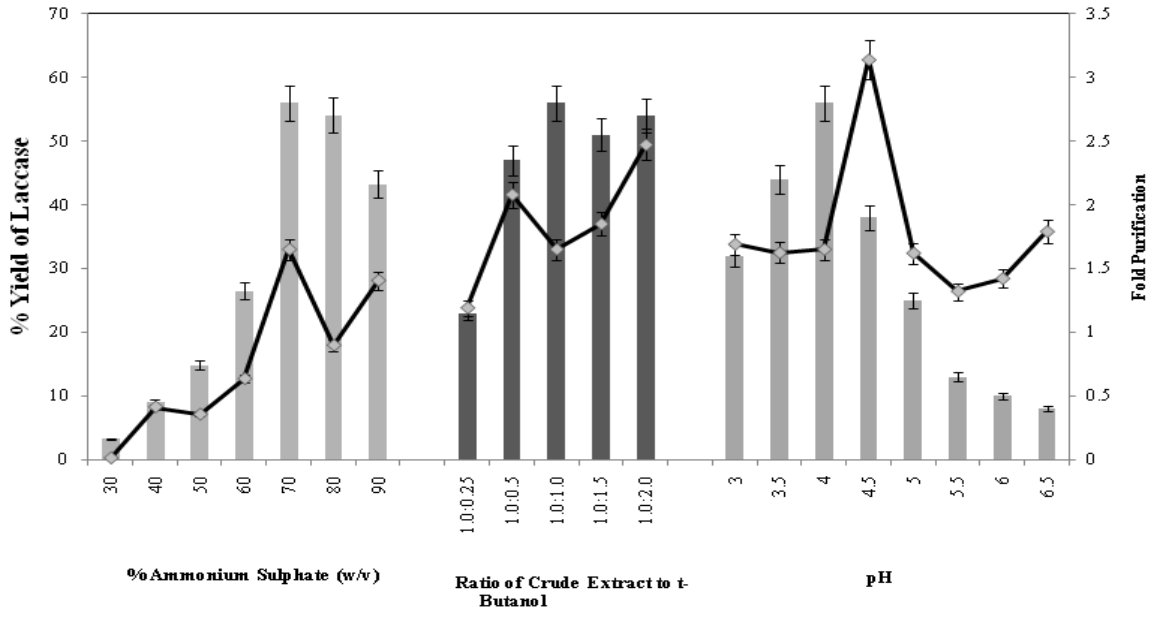
$$\%\ Yield = \frac{Total\ activity\ in\ purified\ sample\ x\ 100}{Total\ initial\ activity}$$

$$Fold\ purification = \frac{Specific\ activity\ of\ puriifed\ sample\ \left(\frac{U}{\mathbf{mg\ protein}}\right)}{Specific\ activity\ of\ initial\ sample\ \left(\frac{U}{\mathbf{mg\ protein}}\right)}$$

Results and Discussion

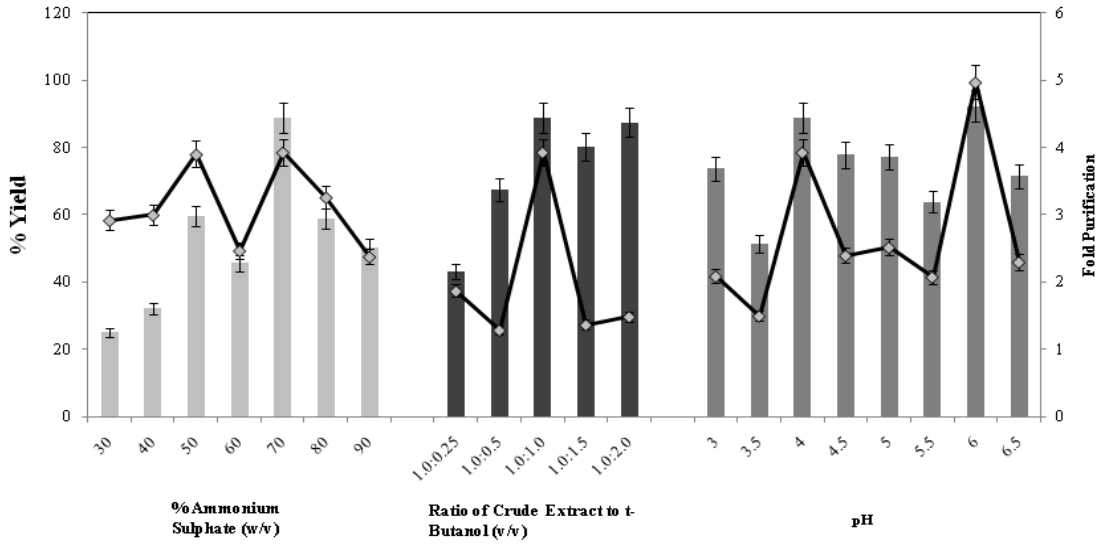
It is a well-known fact that bioseparation accounts for a major percentage of overall production costs of enzymes/proteins[4]. Most reported separation methods for laccase purification were developed for characterization purposes and hence are applicable only on a lab-scale and have many inherent disadvantages. The broadening application necessitates discovery of new strains producing laccase and development of efficient purification techniques for large-scale production and to achieve desired yield and purity. TPP proves an efficient tool for both. MLFTPP technique aims at providing high efficient separation at low costs. The general applicability of this technique to a wider range of enzymes and proteins has to be tested. This experiment proves to be an important step in this regard where the efficiency of chitosan to purify laccase from the crude extract of *Trametes versicolor* was tested. It is clear that MLFTPP in the presence of chitosan performed better,in terms of fold purification and yield, compared to the conventional TPP.Chitosan, being an inexpensive polymer makes this MLFTPP approach, a potentially easily scalable process. Naturally occurring polymers (e.g., alginate, chitosan, j-carrageenan) and synthetic polymers (e.g., methylmethacrylates) have been used for direct precipitation of proteins in MLFTPP[10-13]. TPP purification was optimized as shown by Figure 1, by the traditional technique of varying one variable at a time to achieve optimal yield and purity. Optimisation of MLFTPP with chitosan is summarised in Figure 2, where it can be seen that the parameters like ammonium sulphate concentration, pH, and t-butanol volume have a major influence on the purification of the laccase.

Fig.1: Optimisation of parameters for conventional TPP of *T.versicolor* Laccase



Ammonium sulphate saturation was optimised by taking different systems with constant pH as 4.0 and ratio of crude extract to t-butanol as 1:1and varying amounts of ammonium sulphate (30, 40, 50, 60, 70, 80 and 90%(w/v)). Maximum yield and purification was attained at 70% (w/v) ammonium sulphate saturation. Ratio of crude extract to t-butanol was optimised bytaking five different conditions with 1.0:0.25, 1.0:0.5, 1.0:1.0, 1.0:1.5, 1.0:2.0 (v/v)at pH 4.0 and optimised ammonium sulphate concentration of 70% (w/v) saturation. 1.0:2.0 (v/v)ratiowas considered the best since it gave the maximum fold purification. Final optimisation of pH was done by varying the parameter as 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 at constant optimised ammonium sulphate saturation and ratio of crude extract to t-butanol. Maximum fold purification was attained at pH 4.0. (The bars depict % yield and the solid lines represent fold purification).

Fig.2: Optimisation of parameters for MLFTPP of *T.versicolor* Laccase with chitosan as the affinity ligand.



Optimisation of ammonium sulphate saturation was done by taking 7 different systems with (30, 40, 50, 60, 70, 80 and 90% (w/v)) at pH 4.5 and 1.0:1.0 ratio of enzyme-polymer solution to t-butanol. 70% (w/v) was seen as the best since it gave the maximum fold purification and yield. Ratio of enzyme-polymer solution to t-butanol was optimised by varying it from 1.0:0.25 to 1.0:2.0 at pH 4.5 and optimised ammonium sulphate concentration of 70% (w/v). The optimisation ended with taking 8 different systems with varying pH from 3.0 to 6.5 at other optimised parameters of ammonium sulphate saturation and ratio of enzyme-polymer solution to t-butanol. Maximum yield and fold purification was attained at pH 6.0.(The bars depict % yield and the solid lines represent fold purification).

The optimised conditions for TPP of laccase, which gave maximum fold purification and yield, as shown in Figure 1, were 70% (w/v) ammonium sulphate saturation (with 1.65 fold purification and 56% yield),1:2 ratio of crude extract: t-butanol (2.472 fold purification and 54% yield) and pH 4.5 (3.14 fold purification and 38% yield).Figure 2 shows the optimisation of the same parameters for purification of laccase by MLFTPP in the presence of chitosan. 70% (w/v) ammonium sulphate saturation, 1:1 ratio of enzyme-polymer solution to t-butanol (with 3.93 fold purification and 88.78% yield, in both the cases) and pH 6.0 (with 4.97 fold purification and 92.3% yield) were taken as the best since they gave the highest fold purification. The theoretical aspects regarding the significance of each of the parameter optimised have already been discussed[15,16].It is clear from the results that there is enhanced enzyme activity in both MLFTPP and TPP. Earlier this was attributed to the removal of inhibitors during the purification step. However, when the pure Proteinase K (a serine protease) was subjected to TPP and its structure analyzed by X-ray diffraction at 1.5 A^0 resolution[17], a higher overall temperature factor (B factor) was observed, as a result of which side chains of several amino acid residues in the binding site were found to adopt more than one conformation. This resulted in the protein existing in an excited state which explained its increased enzyme activity.Comparison of the results from conventional TPP and MLFTPP shows that purification of *Trametes versicolor* laccase, by MLFTPP with chitosan, resulted in the better combination of laccase yield (92.3%) and purity (4.97) than conventional TPP (with 1.65-fold purification and 56.22% yield).

A study conducted by Rajeeva and Lele,[6], focussed on developing a downstream protocol for processing laccase from the fermentation broth of *Ganoderma* sp. WR-1 by comparing different techniques like

TPP, ultra filtration, diafiltration and chromatography. The TPP protocol followed in this study was a two-step procedure where the first step removes all the other contaminating proteins and the second step concentrates the laccase at the interface [6]. In the following study that was aimed exclusively at developing TPP protocol for the purification of laccase from the same strain, a purification fold of 13.2 and yield of 60% were obtained[7]. Both these studies justified the compromise in yield using TPP, since this technique eliminated all the drawbacks posed by the other traditional techniques[7]. Optimisation of parameters of TPP is another important aspect required for standardising yield and purification. Purification of laccase from *Pleurotus ostreatus* by TPP was optimised using a statistical approach called Response Surface Methodology (RSM) that gave the information of interaction between variables, information for design and process optimisation simultaneously[8]. The study purified laccase 7.22 fold with 184% yield. Another study performed for biochemical characterisation of the TPP-purified laccase, demonstrated a 27.8-fold purification and 161% yield[9].

Table: 1 Comparison of purification of laccase from various microbial sourcesby TPP and MLFTPP

	Enzyme Activity (U/mL)	**Protein Concentration (mg/mL)**	**Specific Activity (U/mg)**	**Fold Purification**	**Yield (%)**
Commercial laccase					
Crude	7.12	0.0405	175.8	1	100
TPP	4.002	0.0174	290.07	1.65	56.22
MLFTPP	6.57	0.0075	873.72	4.97	92.3
P. desmolyticum					
Crude	33	16.8	1.96	1	100
TPP	11.4	2.4	4.75	2.42	34.54
MLFTPP	30.9	3.48	8.88	4.52	93.63
T. versicolor					
Crude	63	18	3.5	1	100
TPP	48.6	3.48	13.96	3.99	19.33
MLFTPP	48.9	3.96	12.35	3.53	22
P. ostreatus					
Crude	59	39	1.51	1	100
TPP	36.9	7.08	5.21	3.45	18.15
MLFTPP	57.9	8.28	6.99	4.62	21.23

Production of laccase from three different sources was achieved by growing them in appropriate media for a period of about three weeks. The resulting crude extract was subjected to TPP and MLFTPP and the results exhibited an unpredictable trend (Table. 1). MLFTPP proved better in all the three cases apart from the commercial source in terms of yield. Maximum yield of 93.63% and an increased purification fold of 4.52 were seen in the case of MLFTPP of bacterial laccase in the presence of chitosan, while TPP resulted in 2.42-fold purification and 34.54% yield. These results indicate a high affinity between the bacterial laccase and chitosan which contributed to an enhanced enzymatic recovery compared to that obtained with TPP. Both the fungal strains exhibit similar yield of around 22% at the end of MLFTPP in the presence of chitosan. These were more than the individual yieldsof 19.33% and 18.15% obtained from the two sources *T.versicolor* and *P.ostreatus*, respectively, by TPP. These were par below the 56.22% and 92.3% yield obtained from the TPP and MLFTPP of commercial source of *T.versicolor* respectively, which indicates absence of inhibitors in the commercial source. These results thus demonstrate *T.versicolor* as a potential source of laccase among the other established sources like *P.desmolyticum* and *P.ostreatus*.

Conclusion

Laccase, an important commercial enzyme has wide applications in biotechnology ranging from bioremediation to green chemistry. The present study shows application of TPP for purification of Laccase from different species. *Trametes versicolor,* being a potential source wasfirst time subjected purification by three phase partitioning in our work.For further improvement of yield, MLFTPP was also employed and optimised for the same. MLFTPP in this case was based on the affinity between the enzyme and chitosan. It was evident from the purification fold and yield that MLFTPP method outperformed the conventional method of TPP. The optimised conditions for MLFTPP for laccase from the commercial source was further validated by testing with three different species. The results indicated a high affinity of chitosan for bacterial laccase that contributed to

the maximum recovery. This method thus worked out to be a simple, robust and cost-effective method for downstream process of laccase from any microbial source.

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