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Purification and recovery of laccase produced by submerged cultures of *Trametes versicolor* by three-phase partitioning as a simple and highly efficient technique

Agata Wasak[1*], Radosław Drozd[1], Bartłomiej Grygorcewicz[1,2], Dorota Jankowiak[1], Rafał Rakoczy[3]

[1]*West Pomeranian University of Technology Szczecin, Faculty of Biotechnology and Animal Husbandry, Department of Immunology, Microbiology and Physiological Chemistry, Piastów Avenue 42, 70-311 Szczecin, Poland*
[2]*Pomeranian Medical University in Szczecin, Department of Microbiology, Immunology, and Laboratory Medicine, Powstańców Wielkopolskich 72, 70-111 Szczecin, Poland*
[3]*West Pomeranian University of Technology Szczecin, Faculty of Chemical Technology and Engineering, Institute Intitute of Chemical Engineering and Environmental Protection Processes, Piastów Avenue 42, 71-065 Szczecin, Poland*
[*]*Corresponding author: e-mail: agata.wasak@zut.edu.pl*

In this work, three-phase partitioning (TPP) was used for the purification of laccase from liquid cultures of *Trametes versicolor*. For determining the optimal conditions of TPP process, parameters such as initial pH (6.5, 7.0, 7.5, 8.0), ammonium sulphate saturation (20%–80%) and the water phase to *tert*-butanol ratio (1:0.5, 1:1, 1:2), were analyzed. The best conditions with 73% recovery and 24-fold purification was obtained with the use of 50% saturation with ammonium sulphate, water phase to *tert*-butanol ratio of 1:1 and initial pH 7.0. The molecular mass of the purified laccase secreted by analyzed strain *T. versicolor* was found for ≈ 66 kDa. The results showed that TPP is an efficient method for the fractionation and purification of laccase obtained from liquid cultures of *T. versicolor* and it allows for obtaining the relatively pure enzyme without the use of time-consuming and costly chromatographic methods.

Keywords: purification, recovery of laccase, *tert*-butanol, three-phase partitioning, *Trametes versicolor*.

INTRODUCTION

Laccases (EC 1.10.3.2) are important industrial enzymes with great biotechnological potential. Due to their ability to the catalyzing the oxidation reaction, laccases are used in numerous applications e.g.: delignification, degradation, paper pulp bleaching, and recently are used in the biosensors design and obtain biofuel cells[1]. Development of laccase applicability results in an increase in its extraction and effective purification on a large scale. In purification process of these enzymes, standard techniques of protein purification are used: membrane filtration, ion exchange, dialysis, gel filtration, or other electrophoretic and chromatographic techniques[2, 3]. Methods such as filtration or salting out are usually used at the first step in protein purification process. These methods are intended to reduce the volume of liquid e.g. after cultivation with minimal loss of enzyme activity and allow for the separation of desired protein from the mixture of lipids, carbohydrates, and small peptides. The filtration may be affected by fouling of the membrane, and in consequence, decrease the efficiency of purification[3]. Therefore, concentration by salting out requires a long time for protein aggregation and low values of proteins purification level are obtained. Finally, highly purified protein solutions are obtained by means of various chromatographic techniques, which are time consuming and require a cost insensitive equipment[4, 5].

Down-stream processing takes up to 80% of the overall production costs of industrial enzymes. An effective process of purification is an important step in post-production. Despite the development of numbers of enzymes purification techniques, in many cases obtaining of the effective process is still challenging[6]. In order to solve purification process problems, scientists focused on the development of inexpensive, rapid, and efficient methods with a limited number of steps for an economical laccase purification[5].

Three-phase partitioning (TPP) is a simple method, useful for the fractionation, concentration and initial purification of proteins[7]. TPP can be applied between upstream and downstream processing. This method allows for reduction the individual steps in purification procedure and results in high recovery of desired enzymes directly from raw culture media[8]. TPP technique uses a combination of water-miscible aliphatic alcohol e.g.: methanol, ethanol, 1-propanol, 2-propanol, and most generally *tert*-butanol (*t*-butanol) with high concentrations of salt to precipitate proteins from filtrates of liquid cultures. TPP can be performed at room temperature, it needs much lower concentration of salts and gives much higher level of purification fold, than that obtained in different procedures of salting out[9]. The most widely used salt for fractioning and purifying of proteins is ammonium sulphate, because of its high ionic strength and solubility. *T*-butanol binds to the precipitated proteins in aqueous solution and forms protein-*t*-butanol co-precipitates. This process increases the buoyancy of proteins, which finally float in the inter-layer, that is formed from a dense salts aqueous solution[9, 10]. Precipitation of enzymes by TPP can also result in their enhanced activity, that probably results from structural changes, and higher conformational flexibility, due to binding them with *t*-butanol[11]. Furthermore, compounds such as phenolic, tannins, pigments, lipids and other different inhibitors of enzymatic activity, are extracted to *t*-butanol phase, which has a direct impact on protein purity[11, 12]. The efficiency of protein purification depends on their natural properties e.g.: hydrophobicity, isoelectric point, solubility, concentration, and purification conditions: concentration of salt, temperature, pH and aqueous solution to *t*-butanol ratio. Understanding of the relation between all of these parameters is essential to design the effective purification process and obtaining a high yield and recovery of enzyme activity[6].

The study presents purification process of laccase from a liquid culture of white rot fungus strain *Trametes versicolor* using an economical and rapid method called three-phase partitioning. We optimized the parameters such as concentration of ammonium sulphate, crude extract phase to *t*-butanol ratio, and pH.

MATERIAL AND METHODS

Reagents

All used chemicals were analytical grade reagents. *Tert*-butanol (*t*-butanol), ammonium sulphate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were purchased from Sigma Chem. Co. (St. Louis, MO, USA), hydrogen peroxide was procured from Chempur, and potato extract was purchased from Difco.

Fungal strain and laccase production

The strain of *Trametes versicolor* (strain 5_22) was isolated from hardwood of beech collected from the area of Wkrzańska Forest (Poland). The species identyfication was carried out accroding to morphological characteristic of fruit body. The fungus was maintained on slants with potato dextrose agar medium (PDA) at 4°C. Five agar plugs (1 cm^3) with mycelium were used as the inoculum. The strain was grown in stationary condition, in 1000 mL Erlenmeyer flasks filled with 500 mL of optimum culture medium consisting of 1.5 g/L potato extract, 10 g/L glucose, 0.1 g/L $MgSO_4$, 0.6 g/L KH_2PO_4; pH 6.0. The flasks were then incubated at 25°C for 8 days. After five days of cultivation, $CuSO_4$ (final concentration 1 mM) was added to enhance the laccase production. After fermentation, the broth was filtered (Whatman No. 1 paper), and obtained supernatant was lyophilized, and stored at –20°C in freeze drying systems prior to the analysis.

Laccase activity assays

The activity of laccase was analysed using 0.5 mM ABTS as a substrate. The reaction was carried out in a 50 mM acetate buffer pH 4.0, at 2 min, at constant temperature 30°C. Oxidation of ABTS was measured spectrophotometrically at 420 nm (ε = 36.000 M^{-1} cm^{-1}) using the Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). One unit of the enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol ABTS per min. All measurements were performed in triplicate.

Protein determination

Protein determinations were made according to the method of Bradford[13], using bovine albumin as the standard.

Three-phase partitioning

TPP experiment was carried out employing various concentrations of ammonium sulphate (20%, 30%, 40%, 50%, 60%, 70%, 80%) (w v^{-1}), aqueous phase crude extract to *t*-butanol ratio (aq/tb ratio) (1:0.5, 1:1, 1:2) (v v^{-1}), and pH (6.5, 7.0, 7.5, 8.0). The scheme of experiment is shown on Figure 1.

The lyophilizates (crude extract solution, 15 U/mg) were dissolved in appropriated buffer (50 mM phosphate buffers, pH 6.5; 7.0; 7.5 and 8.0). After that, the crude extract was saturated with ammonium sulphate (20% for the beginning) (w v^{-1}), and vortexed until the salt dissolve following by addition of different aq/tb ratios (1:0.5; 1:1; 1:2) (v v^{-1}), at 25°C. The mixture was stirred gently and then allowed to stand for 10 min at 25°C. After 10 min, the mixture was centrifuged at 3500 rpm for 10 min at 4°C to form three phases: upper phase – *t*-butanol; middle phase – interfacial precipitate; bottom phase – aqueous phase with $(NH_4)_2SO_4$. The bottom aqueous phase and the interfacial phase were analysed for laccase activity and total protein content. The interfacial phase was gently separated and centrifuged at 13200 rpm for 10 min at 4°C. Pellets were dissolved in 20 mM phosphate buffer (pH 7.0), and laccase activity was determined in such prepared solutions. Next, samples with the enzyme activity were dialysed overnight in 20 mM phosphate buffer (pH 7.0) at 4°C. All experiments were performed in triplicate.

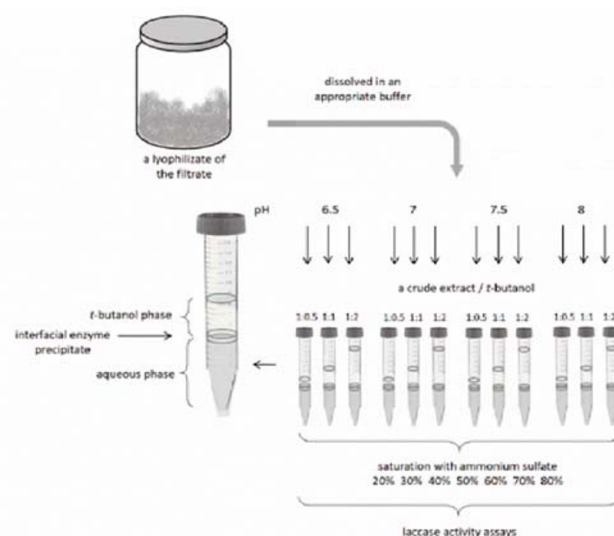

Figure 1. Flowchart for the optimization of the TPP process

The % recovery and purification fold were calculated as follows:

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

$$Fold\ purification = \frac{Specific\ activity\ of\ purified\ sample\ *}{Specific\ activity\ of\ initial\ sample}$$

* - U/mg protein

SDS–PAGE gel electrophoresis

The molecular weight of laccase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel according to Laemmli[14]. Laccase samples were diluted in denaturing loading buffer (1% SDS, 10% glycerol, 10 mM Tris-Cl, pH 6.8, 1 mM EDTA, 14.4 mM 2-mercaptoethanol, 0.1 mg/mL bromophenol blue) and incubated for 10 min at 100°C. The molecular weight was estimated with proteins marker: carbonic anhydrase ≈ 29 kDa, ovalbumin ≈ 45 kDa, albumin beef ≈ 66 kDa, phosphorylase B of rabbit ≈ 97 kDa, β – galactosidase from *E. coli* ≈ 116 kDa, myosin from rabbit muscle ≈ 200 kDa. After electrophoresis, protein bands were visualized by silver staining.

Zymogram gels

Native polyacrylamide gel electrophoresis (Native-PAGE) was performed under non-denaturing conditions at 4°C according to protocol by Patel et al.[15] with some modification. After electrophoresis, the gel was equilibrated in acetate buffer (50 mM, pH 4.0), transferred to 0.5 mM solution of ABTS, and incubated until the green bands appeared. Finally the gel was scanned and densitometric analysis was performed using ImageJ software.

RNA isolation

The RNA was isolated from wild type *Trametes versicolor* grown in PD (potato dextrose) medium at 25°C for 8 days. After 5 days, $CuSO_4$ (1 mM) was added, the mycelium was centrifuged and washed in RNAlater® (Sigma-aldrich). Total RNA was extracted from liquid nitrogen powdered mycelia using the Total RNA mini (A&A). The cDNA was synthesized by reverse transcription from 1 μg of total RNA using 5 mM oligo(dT) primers with SuperScript IV Reverse Transcriptase with ezDNase (Invitrogen), following the instructions of the manufacturer. The laccase gene was amplified by a PCR experiment using the obtained cDNA as a template with primers 5'-ATGTCGAGGTTTCACTCTCTTTTCGC-3' and 5'-TTACTGGTCGCTCGGGTCGCGCGCGTC-3' designed according to the sequence of *lcc*2 gene (GenBank accession No. Y18012.1), Cassland[16] by Jolivalt et al.[17] and Bertrand et al.[15] modified by deletion of restriction sites. Jolivalt et al.[17] suggested the temperature profile: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and then by 20 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min, with a final step at 72°C for 7 min. The amplified cDNA was sequenced in both directions by primer-walking method. The obtained cDNA sequence of laccase was deposited in GenBank genetic sequence database with accession No MH496506.

Prediction of electrostatic properties of laccase from *Trametes versicolor* 5_22 strain

Prediction of the physicochemical properties of laccase structure was performed using 3D structure laccase from *Trametes versicolor* PDB code; 1KYA[18] which shares 99.9% identity with an amino acid sequence of laccase from *Trametes sp*. strain (supplementary materials) used in research. Prediction of tritable amino acid pKa values and modeling of the structure with the protonation state in the pH range from 6.5 to 8.0 was performed using PROPKA on the PDB2PQR server[19]. The electrostatic potential on the protein molecular surface was calculated using the Adaptive Poisson-Boltzmann Solver (APBS) incorporated into the molecular modeling software, UCSF Chimera 1.9v[20].

Statistical analysis

Statistical analysis of the results was performed with the use of analysis of variance (ANOVA). The differences between means were considered as significant with p-values < 0.05. All statistical analyses was conducted with Statistica 12 software.

RESULTS

The effects of aq/tb ratio (v v^{-1}), pH, and saturation with ammonium sulphate (w v^{-1}) on the recovery and purification of laccase

Figure 2 shows effects of pH of the aqueous phase (6.5; 7.0; 7.5; 8.0) on laccase partitioning.

At pH 6.5 and aq/tb ratio of 1:1 (v v^{-1}) (Fig. 2A), we observed two fractions with relative laccase activity of more than 88%. Similar results were obtained at pH 7.5 and aq/tb ratios of 1:1 and 1:2 (v v^{-1}), with enzyme activity at 79% (Fig. 2C) and above 80% at pH 8.0 for the same aq/tb ratios (v v^{-1}) (Fig. 2D). The best fractionation was observed at pH 7.0 (Fig. 2B), at which homogeneous profiles were obtained for each tested aq/tb ratio (v v^{-1}) with the highest enzyme activity in a single fraction.

The recovery of laccase during TPP was significantly influenced by initial buffer pH (Fig. 3). Recovery increased from pH 6.5 to 7.0, then gradually decreased as pH arose. Purification showed the same trend for pH above 6.5 (Fig. 3A). It can be seen that difference between the highest and the second highest recovery value was 10% for aq/tb ratio 1:0.5, 17% for 1:1, and 33% for 1:2 (v v^{-1}). The highest recovery and purification were obtained at aq/tb ratios of 1:1 (Fig. 3B) and 1:2 (v v^{-1}) (Fig. 3C). The results show that the TPP 1:1 or 1:2 aq/tb ratio (v v^{-1}) resulted in a comparable efficiency in the fractionation process.

We examined the influence of aq/tb ratios (v v^{-1}) and saturation with ammonium sulphate (w v^{-1}) on laccase recovery and purification at pH 7.0 (Fig. 4).

SDS–PAGE gel electrophoresis

SDS-PAGE analysis was applied to determine the molecular weight of purified laccase: 66 kDa. At pH 7.0 (columns 4, 5 and 6), purified laccase was shown to be homogeneous with single protein band with addition of low amount of second protein. At pH 6.5 (columns 1, 2 and 3), in addition to the expected laccase, another two low-weight proteins were observed (Fig. 5).

Zymogram gels

During optimization of TPP parameters, two isozymes of laccase were visible in all analyzed combinations of tested parameters. Densitometric analysis of zymograms from TPP with initial pH 7.0 showed that the activity of both isoenzymes at aq/tb (v v^{-1}) ratios of 1:1 (Fig. 6B) and 1:2 (Fig. 6C) was greater than at 1:0.5 (Fig. 6A).

Prediction of electrostatic properties of laccase

Changes of pH strongly affect the protein charges, that could have an impact on salting out the proteins by ammonium sulphate. Negatively charged proteins usually show better solubility than positively charged ones[21]. The predicted laccase net charge at analysed pH, vary in short in the range from – 21 to – 25 (e) and the molecular surface are mostly negatively charged with the occurrence of neutral patches. This can partially explain better recovery of laccase at initial pH 6.5 and 7.0 than 7.5 and 8.0 where the recovery was the lowest (Fig. 7).

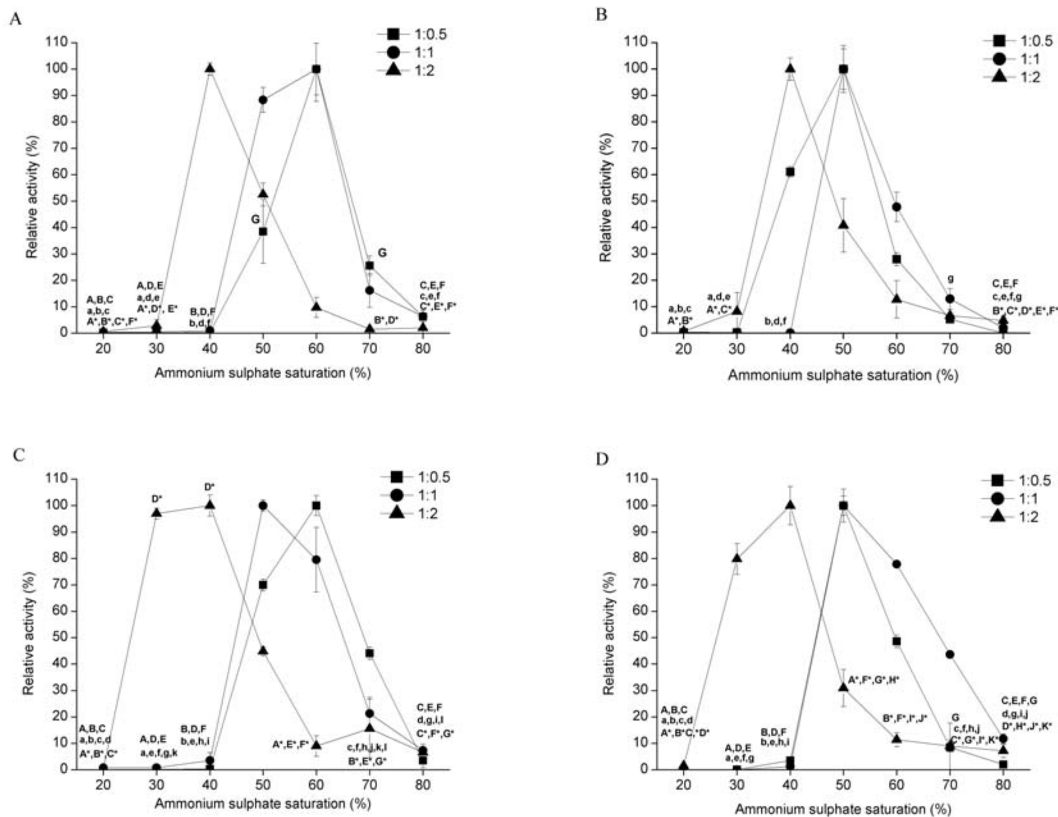

Figure 2. . Effect of different pH (A) 6.5, (B) 7.0, (C) 7.5 and (D) 8.0, aq/tb (v v^{-1}) ratio and saturation with ammonium sulphate (w v^{-1}) on the partitioning profiles of the laccase. The activity was expressed in relative terms, where 100% was the fraction of the highest activity. Symbols (■),(●),(▲) means different aq/tb ratios, 1:0,5; 1:1; 1:2, respectively. A, B, C,..., a, b, c,..., and A*, B*, C*, .., – means sharing the same superscript are not significantly different from each other. The samples were compared with each other within a given series

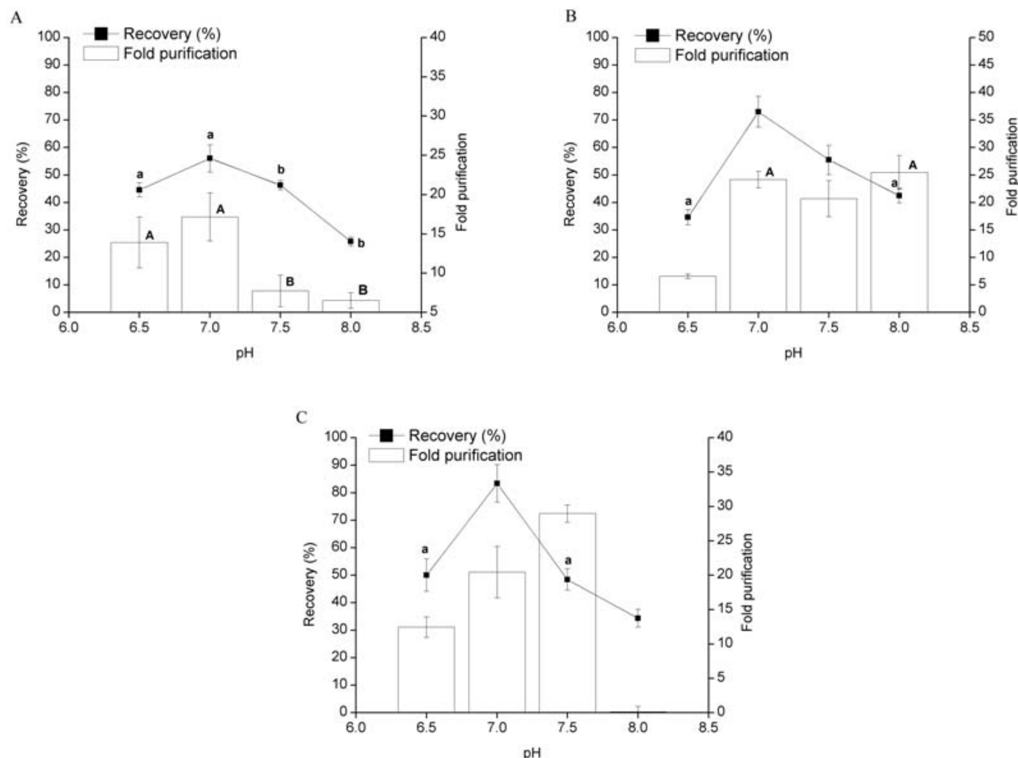

Figure 3. . Effect of pH and aq/tb ratios (v v^{-1}) A) 1:0.5, B) 1:1 and C) 1:2, on the degree of purification fold and recovery (%) of laccase. Graphs show samples with the highest recovery and purification fold at given pH and aq/tb ratios (v v^{-1}). A, B, C,..., and a, b, c,..., means sharing the same superscript are not significantly different from each other. The samples were compared with each other within a given series

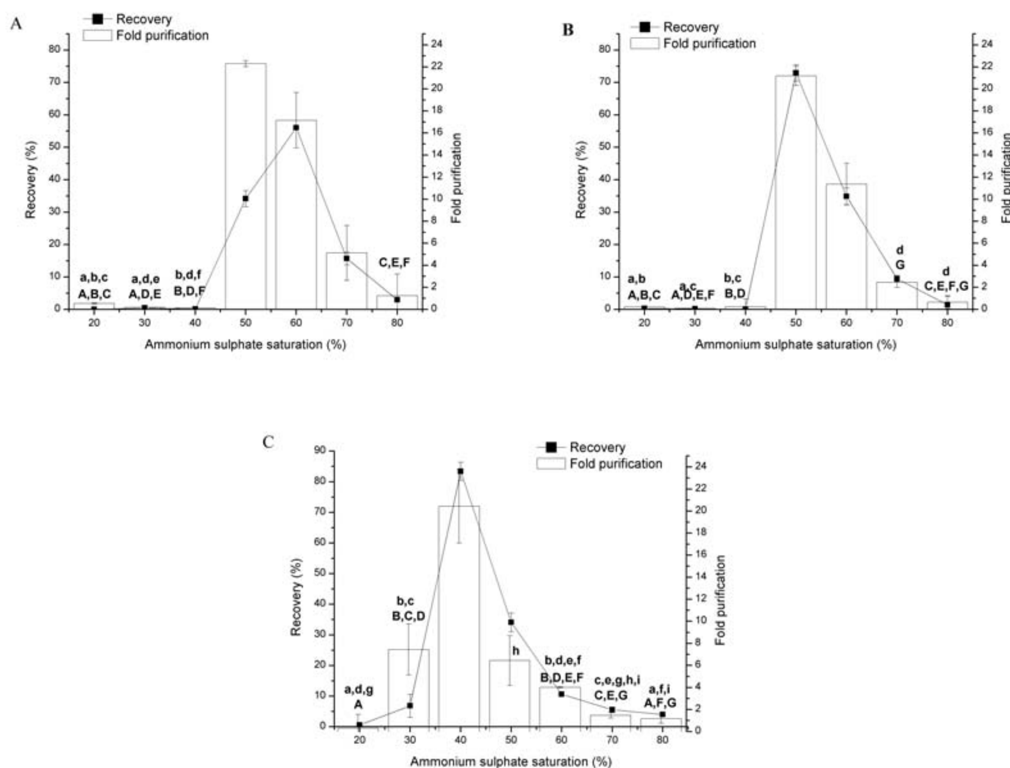

Figure 4. Effect of varying ammonium sulphate saturation (w v^{-1}) and aq/tb ratios (v v^{-1}) on the degree of purification fold and activity recovery (%) of laccase at pH 7.0. The crude extract (4 mL) was brought to different levels of ammonium sulphate saturation (20%, 30%, 40%, 50%, 60%, 70% and 80%) (w v^{-1}) and t-butanol was added in the ratios of A) 1:0.5; B) 1:1 and C) 1:2 (v v^{-1}) with respect to the volumes of crude extract. A, B, C,..., and a, b, c,..., means sharing the same superscript are not significantly different from each other. The samples were compared with each other within a given series

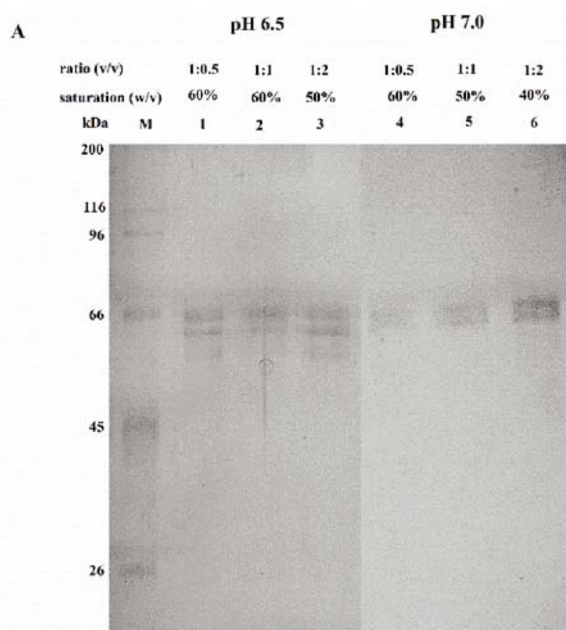

Figure 5. . SDS-PAGE analysis of purified laccase using TPP technique comparison fractions with the highest activity at pH 6.5 (line:1, 2 and 3) and 7.0 (line: 4, 5 and 6) in an aq/tb ratio (v v^{-1})

DISCUSSION

The efficient extraction of proteins without impurities from the aqueous phase and its purification by TPP depends on many factors. This is related mainly to protein's physicochemical properties and parameters of the process itself, i.e. type and concentration of salt, aqueous phase to solvent ratio, pH, and largely pH-dependent protein charge[4, 10, 23]. Kumat et al.[6] through optimisation of TPP process using statistical approach called Response Surface Methodology (RSM), were able to determine the interaction between operating variables: ammonium sulphate saturation, ratio of crude extract to *t*-butanol and temperature. The study upon purified laccase from *Pleurotus ostreatus* obtained 7.22 fold with 184% yield. Despite efficient optimization methods, a precise mechanism of TPP has not been explained yet. Available reports confirm that protein precipitation at the interphase occurs due to the combined effect of isoionic/isotonic precipitation, kosmotrophy, isoionic/isotonic salting out, osmolysis, electrostatic forces, co-solvent precipitation, hydration shifts and change molecular conformations of proteins[25]. Proteins below their isoelectric point are positively charged. Therefore, the sulphate anion (SO_4^{2-}) binds to them, resulting in protein precipitation. For example, precipitation of albumin does not take place above pI when the protein is negatively charged[10]. The isoelectric point of fungal laccases is in the acidic pH range, e.g. 3.5.

In the initial phase of fractionation, we used the lowest ammonium sulphate saturation, i.e. 20% (w v^{-1}), which was then increased in steps of 10 percentage points to find the level resulting in the highest yield of the target protein in the interphase. According to Rajeeva and Lele[3], the best effective laccase separation technique is

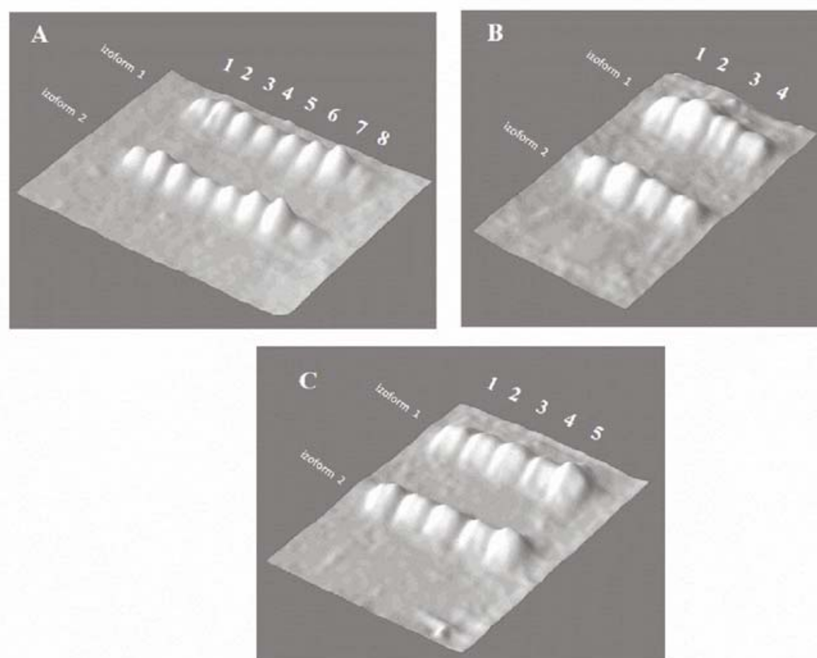

Figure 6. Densitometric analysis of zymograms for the TPP fractions with the highest activity at pH 7.0, A) line 1: 70% ammonium sulphate saturation, repetition 3 (3); line 2: 70% (2); line 3: 60% (3); line 4: 60% (2); line 5: 60% (1); line 6: 50% (3); line 7: 50% (20); line 8: lyophilizate, B) line 1: 50% (2); line 2: 50% (3); line 3: 60% (1); line 4: 60% (3), C) line 1: 40% (1); line 2: 40% (2); line 3: 40% (3); line 4: 50% (1); line 5: 50% (3)

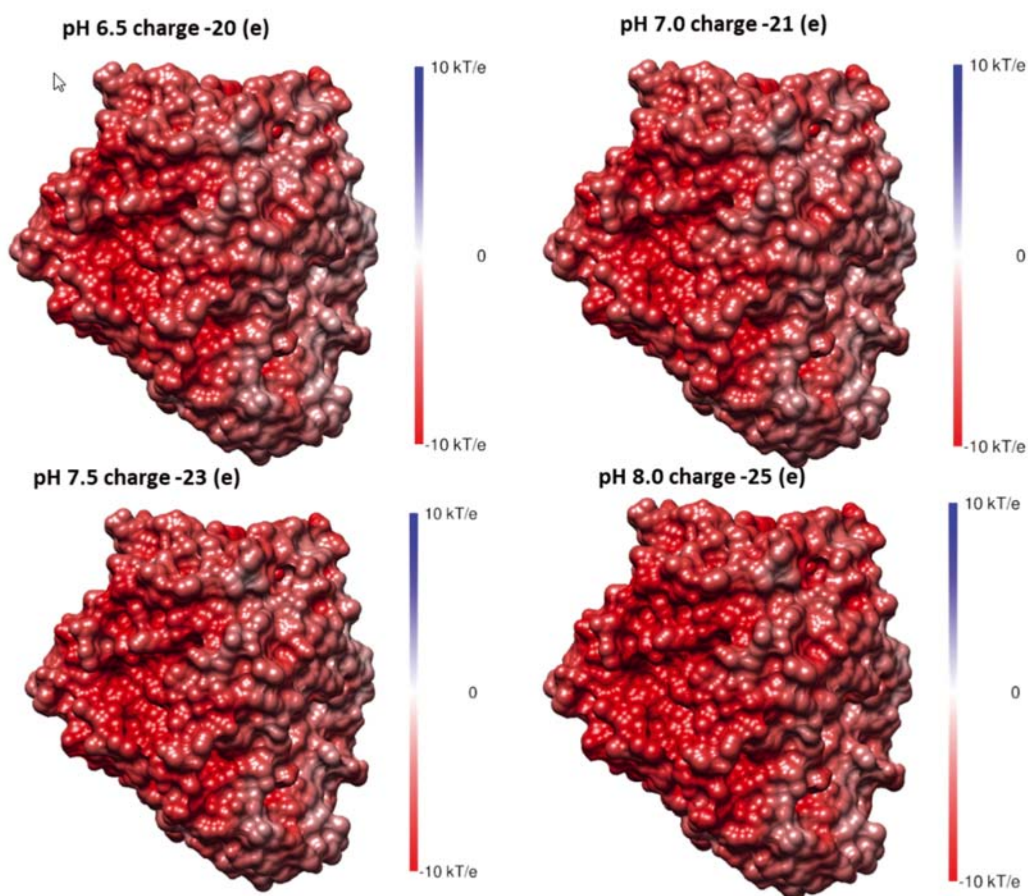

Figure 7. Predicted charge of laccase molecule at different pH. Structure of laccase from PDB with code 1KYA which shares 99.0% of structural similarity with laccase from the analysed strain (see supplementary materials), was taken for visualization purposes

a two-step TPP procedure. In step 1 (at 20%–80% saturation; w v^{-1} and aq/tb ratios 1:0.5, 1:1, 1:2; v v^{-1}), most of protein impurities are removed to the interphase. In the second stage (50%–90% saturation; w v^{-1} and aq/tb ratios 1:0.5; 1:0.75, 1:1, 1:1.5, 1:2; v v^{-1}), the aqueous phase laccase precipitates to the interphase. With this two-step procedure, a 60% recovery rate and 13.2-fold purification for laccase can be obtained.

T-butanol, the solvent we included in TPP, has been frequently used as an organic solvent producing the best

results during the fractionation of many other enzymatic proteins[9]. In addition to this solvent, protein fractionation may use other solvents, such as isopropanol, n-propanol, n-butanol, methyl *tert*-butyl ether (MTBE), t-amyl alcohol, dimethylformamide, tetrahydrofuran, 1,4-dioxane and dimethylsulphoxide, e.g. in the fractionation of α-galactosidase[5, 25]. Pravin and Ganapati[25] reported that n-propanol and MTBE, compared with *tert*-butanol showed low amount of precipitation at the interphase due to their limited hydrophobic interaction with protein that can cause its denaturation. Dhananjay and Mulimani[5] in optimization of TPP for the α-galactosidase purification also confirmed that *t*-butanol was the best organic solvent, yielding 92% activity recovery and 12-fold purification. This solvent can be used for fractionating the proteins from crude extract without prior sample preparation, removing low molecular weight compounds such as lipids and detergents during initial extraction[10]. In addition, the size of the molecule and branched structure of *t*-butanol prevents it from binding to the precipitated protein, also preventing its inactivation by denaturation[4]. In laccase fractionation with *t*-butanol, optimum ratio (aq/tb) was 1:1, but 1:2 (v v^{-1}) also gave very similar results. In our study, 1:1 aq/tb ratio (v v^{-1}) resulted in 73% recovery and 24-fold purification (Fig. 4B), while 1:2 ratio (v v^{-1}) gave 40% recovery and 21-fold purification (Fig. 4C). Therefore, there was only a slight increase in recovery (1:1 vs 1:2) (v v^{-1}) (Fig. 4C), but significant increase in the purity of the protein formulation (more than 3 units of fold) (Fig. 4B).

In conclusion, at the aq/tb ratio 1:1 (v v^{-1}), we received slightly lower activity of the protein preparation than for 1:2 (v v^{-1}), but with higher purity. Considering the economic aspect of the process, for which it is more advantageous to consume more relatively cheap salt such as ammonium sulphate than buying expensive, hazardous and flammable *t*-butanol, the results obtained are satisfactory.

On the presented zymograms, we observed two different laccase isozymes (Figure 6) that could not be isolated using TPP. The SDS-PAGE analysis of partitioned laccase showed that its molecular weight was approximately 66 kDa and the most homogeneous sample was obtained at pH 7.0 (Fig. 5). Previous works show that the typical molecular weight of laccase oscillates in the range of 50–110 kDa[31]. Taking into account the results of Liu et al.[4] who used TPP to purify 38 kDa laccase, it can be argued that TPP is suitable for the fractionation and purification of laccase.

CONCLUSIONS

After optimization of the three-phase partitioning conditions, we obtained a purification fold and recovery of up to 24 times and 73%, respectively. From the results, it can be concluded that the optimal conditions for partitioning, purification and recovery of laccase from *Trametes versicolor* are: 50% ammonium sulphate saturation (w v^{-1}), 1:1 the crude extract to the *t*-butanol ratio (v v^{-1}) and pH 7.0. Based on our outcome, we confirm that the three-phase partitioning is an adequate, very efficient technique for extraction and recovery of laccase from liquid media of *Trametes versicolor.*

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COMPLIANCE WITH ETHICAL STANDARDS

Conflicts of Interest The authors declare that they have no conflicts of interest.

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