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Three phase partitioning of *exo*-polygalacaturonase from the culture filtrate of *Pleurotusostreatus*

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Running title: three phase partitioning of *exo*-polygalacturonase

ABSTRACT

Three phase partitioning (TPP), an elegant bio-separation technique was used to investigate the partitioning behavior of *exo*-ploygalacturonase, obtained from the culture filtrate of *Pleurotusostreatus*in a system containing water, tert-butanol and an inorganic electrolyte, $(NH_4)_2SO_4$. The protein appears as an interfacial precipitate between upper organic solvent and lower aqueous phases. The various conditions, i.e., ammonium sulphate concentration, pH and amount of tert-butanol for purification of the enzyme were optimized. It was observed from the present set of researches that 50% ammonium sulphate saturated with 1:1 ratio of crude culture filtrate to tert-butanol at a system pH of 6.0 and 25^0C temperature gave 58.87% activity recovery yield with a 3.09 fold purification. This work shows that TPP is a simple, quick and economical downstream technique for purification of *exo*-ploygalacturonase, which has an immense industrial importance, particularly in food processing industries. As TPP is a scalable process, the process has the potential to be carried out at the industrial level.

KEY WORDS: three phase partitioning, *exo*-polygalacturonase, *Pleurotusostreatus*

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1.INTRODUCTION

The last decade has some serious rethinking on the strategies for downstream processing of enzymes. The conventional enzyme purification process involves ammonium sulphate precipitation, chromatography, affinity tagging in a sequential manner. However, an efficient purification protocol must minimize the number of steps in a unit process. Moreover, enzymes required in industries do not require a high purity at a large scale level and therefore, if the objective is to obtain industrial grade enzymes in reasonable amount, the non-chromatographic technique is the most suitable approach. Three phase partitioning is a simple but elegant non-chromatographic process used for purification and concentration of proteins. TPP uses *tert*-butanol and ammonium sulphate to precipitate proteins from aqueous medium. In TPP, proteins are excluded from two immiscible liquid phases into a middle zone that becomes concentrated into a thin disc. Both ammonium sulphate and *tert*-butanol reinforce each other's physicochemical effects, such as ionic strength effects, kosmotrophy, osmotic stress and exclusion-crowding effects to partitioning the protein as a mid-layer between aqueous and the organic phase. Some *tert*-butanol also binds to the protein and increases its buoyancy against gravity and makes it float between the aqueous and the organic layer as a gel-like disc.A more developed TPP technique called macro-affinity ligand facilitated three phase partitioning has all the advantages of TPP besides having the advantage of a more predictable design. A number of enzymes have already been purified by TPP such as xylanase[2], α-amylase[3], ficin[4], protease[5], α-galactosidase[6], laccase[7], catalase[8], peroxidase[9].Microbial pectinolytic enzymes of fungal origin have tremendous industrial potential particularly in food processing industries. Pectinases or pectinolyticernzymes are a group of enzymes that catalyze the degradation of pectic substances, present in plant cell wall and polygalacturonase (PG) that catalyze the hydrolytic cleavage of the polygalacturonic acid chain with water molecule. Filamentous fungi are the most frequently used microorganism in the enzyme industry and *Aspergillusniger* is the most commonly used fungal species for industrial production of pectinolytic enzymes. The edible mushroom, *Pleurotusostreatus*is non-sporogenous in mycellialculture and the enzymes produced by this organism is highly desirable from the food processing industrial point of view. Very few works have been reported on polygalacturonase production by *Pleurotus ostreatus*[10]and this study was focussedon three phase partitioning based purification technique of *exo*-polygalacturonase (*exo*-PG, EC 3.2.1.67), obtained from the culture filtrate of *Pleurotusostreatus*, a widely cultivated edible mushroom.

2.EXPERIMENTAL SECTION

2.1Micro-organism and fermentation system

The mycellial culture of mushroom *Pleurotusostreatus*wasgrown at $28^0 \pm 1^0$C for 5 days in a medium containing 1% glucose, 1% malt extract, 10% potato extract and 0.15 % KH_2PO_4. The enzyme production medium contained (g l^{-1}) $NH_4H_2PO_4$– 24, $MgSO_4.7H_2O$- 0.5, $CaCl_2.2H_2O$- 0.37, $FeSO_4.7H_2O$– 0.25, $MnCl_2$– 0.032, $NaMoO_4$– 0.032, KH_2PO_4- 1.5, in combination with a carbon source. The white portion of the peel of pomello (*Citrusmaxima*) was initially washed by water followed by dist. water and oven-dried at 50^0C. The dried mass was grounded into fine powder and screened through 200 meshes. The sieved mass was dried overnight at 50^0C and used as the carbon substrate. All other chemicals used were of analytical grade. Fermentation was carried out at 30 ± 1^0C for 7 days in shake flask assisted culture and culture filtrates were used as the source of enzymes.After the termination of the fermentation, the fermented broth containing extracellular enzymes was collected by centrifugation followed by vacuum filtration and the supernatant was assayed for the enzyme activities.

2.2Exo-polygalacturonase activity assay

Exo-polygalacturonase (*exo*-PG) activity was assayed by incubating the enzyme for 10 min at 50^0 C with 0.5 % (w/v) polygalacturonic acid in 50 mM citrate buffer (pH 6.0). The released reducing groups were determined by DNS reagent[11]. The enzyme activity was expressed as Uml^{-1} in which, one enzyme unit is defined as the amount of enzyme required for releasing of 1µmol of reducing groups per minute under the assay condition.

2.3Three phase partitioning of exo-polygalacturonase

Three phase partitioning of *exo*-polygalacturonase is an one step purification technique, based on a modified procedure reported by Sharma and Gupta[12]. The crude enzyme solution was brought to varying concentration of ammonium sulphate saturation, followed by a different ratio of *tert*-butanol. The interfacial precipitate containing *exo*-polygalacturonase was collected assayed for enzyme activity and protein content.

2.4Statistical analysis and data presentation

The five set of each of the fermentation and partitioning experiment was carried out under identical condition and the results were presented as the mean values of data with SE of ±5.

3.RESULTS AND DISCUSSION

In order to evaluate the best three-phase partitioning system for the purification of *exo*-polygalacturonase from culture filtrate of *Pleurotusostreatus*, various process parameters including

percent saturation of ammonium sulfate, crude extract to *tert*-butanol ratio and also pH and temperature of the system were analyzed. Figure 1 shows the partitioning of exo-polygalacturonaseinto the interfacial phase with different percent saturation of ammonium sulphate with *tert*-butanol equal in volume to the starting crude enzyme solution. The maximum exo-polygalacturonaseactivity recovery in interfacial phase was obtained as 58.21% with fold purification of 3.21in a system comprised with 50 % ammonium sulphate saturation.

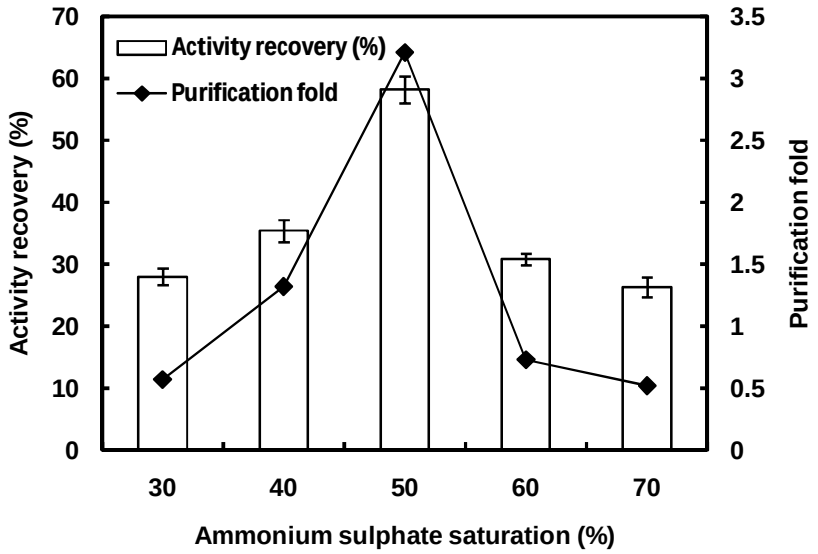

Figure 1: Effect of varying concentration of ammonium sulphate on the recovery of exo-polygalacturonase from the culture filtrate of *Pleurotusostreatus*. The mixtures containing of different concentration of $(NH_4)_2SO_4$ and culture filtrate before addition of *tert*-butanol and culture filtrate (1:1), kept at 25^0C at pH 6.0 for three phase partitioning for 1 h. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content. Data are presented here the mean value of five identical set of experiments with standard error bar.

The amount of *tert*-butanol to enzyme solution is also an important factor in protein separation by TPP. The amount of ammonium sulphate was fixed to 50 % (w/v) and the ratio of enzyme solution to *tert*-butanolwasvaried from 1.0:0.5 to 1.0: 2.0 (v/v). Based on the experimental findings, best results were obtained at 50% saturation (w/v) with ammonium sulfate and 1:1 (v/v) ratio of crude extract to *tert*-butanol as 59.0% recovery with 2.85 fold purification of enzyme activity (Figure 2). The experimental results are in also concordance with the study reported by Dogan and Tari[13], which reveals that crude *exo*-polygalacturonase of *Aspergillussojae*was purified using three-phase partitioning (TPP) saturated to 30% (w/v) with ammonium sulphate and with a crude extract to *tert*-butanol ratio of 1:1 (v/v) at 25 °C resulted in 25.5% recovery with a 6.7-fold purification. It was also reported by Calci*et. al*[14]that 50% of ammonium sulphate saturation with 1:1 ratio of crude extract to *tert*-butanol at pH 4.5 gave 4.3 fold purification with 80% activity recovery of α-galactosidase of tomato (*Lycopersiconesculantum*).

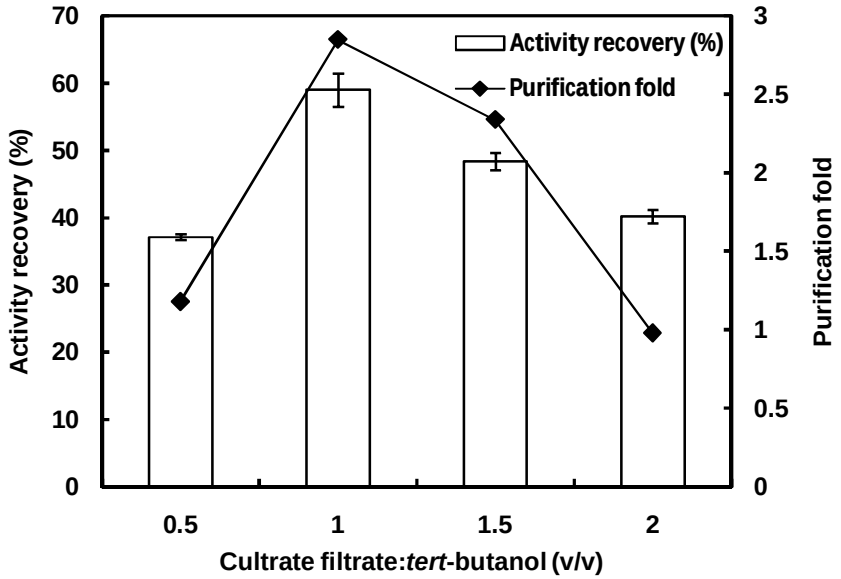

Figure 2: Effect of varying ratios of culture filtrate to *tert*-butanol on the recovery of exo-polygalacturonase from the culture filtrate of *Pleurotusostreatus*. The mixtures containing of 50 % saturation of $(NH_4)_2SO_4$ and before addition of *tert*-butanol and culture filtrate, kept at 25^0C at pH of 6.0 for three phase partitioning for 1 h. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content. Data are presented here the mean value of five identical set of experiments with standard error bar.

Bayraktar and Onal[15]observed that α-galactosidase of watermelon (*Citrullus vulgaris)* was purified with a yield of 76.7% and 2.7-fold purification at the interphase of the TPP system, which consisted of the crude extract to *ter*t-butanol ratio of 1:1 (v/v) in the presence of 50% (w/v) $(NH_4)_2SO_4$ at pH 5.5. The optimum enzyme activity recovery therefore depends upon the two factors: the conc. of ammonium sulfate and ratio of crude culture filtrate to *tert*-butanol. This experiment also reveals that with an increase of conc. of ammonium sulfate, the degree of activity recovery value was also increased significantly. However, the enzyme activity yield was reduced after 50 % of ammonium saturation to enzyme soln. The high concentration of ammonium sulfate may reduces the selectivity of extraction also, thus reducing the degree of purification[14].

Ammonium sulphate is frequently used in protein salting-out as it is readily available, high degree of solubility and protein stabilizing property. Moreover, NH_4^+ and SO_4^{2-} are at the ends of their respective Hofmeister series and have been shown to stabilize intermolecular interaction in protein structure. Therefore $(NH_4)_2SO_4$ saturation is of critical importance and plays a major role in TPP as it is responsible for protein-protein interaction and precipitation[16]. The use of *tert*-butanol in TPP technique is of highly significant since *tert*-butanol is a C_4 non-ionic kosmotrope that is very soluble and miscible in water, but after the addition of solid salt, becomes hydrated and acts as a differentiating solvent. It does not cause denaturation of the partitioned enzymes as it is unable to

permeate inside the folded three dimensional structure of protein due to its larger molecular molecular size and branched structure. Therefore, sulfate ion and *tert*-butanol are known to be excellent protein structure markers or kosmotrophs[16].

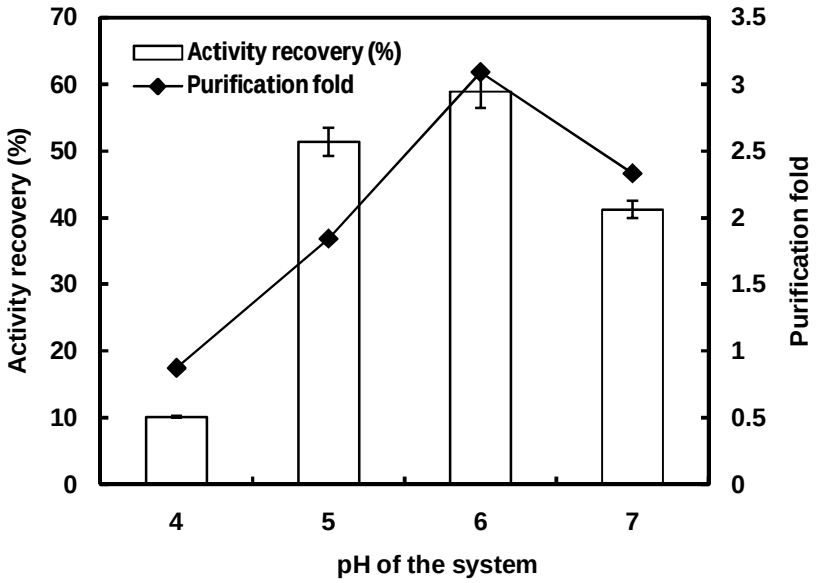

Figure 3: Effect of system pH on the recovery of exo-polygalacturonase from the culture filtrate of *Pleurotusostreatus*. The mixtures containing of 50 % saturation of $(NH_4)_2SO_4$, *tert*-butanol and culture filtrate (1:1), kept at 25^0C at different system pH for three phase partitioning for 1 h. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content. Data are presented here the mean value of five identical set of experiments with standard error bar.

As the enzyme shows different behavior in TPP system, depending upon their molecular weight and pI values, the effect of pH on the partitioning of the *exo*-polygalacturonase was also studied (Figure 3). The efficiency of the salting out of proteins will depend on sulphate concentration and also on the net charge of the proteins which is highly pH dependent. The crude culture filtrate of *exo*-polygalacturonase was brought to 50 % ammonium sulphate saturation and the pH was adjusted to different pH values in the range of 4.0 to 7.0, before addition of *tert*-butanol in the ratio of 1:1 (v/v). It can be seen from the figure 3, that the best results are obtained at pH 6.0 with a 3.09 fold purification and 58.87 % of activity recovery. If proteins are damaged by moderate or severe pH i.e., acidic pH below 4.0, exposure before addition of sulfate or *tert*-butanol may denature them. They are likely to partition out in a denatured state but may lose activity[17]. Behavior of TPP system usually sharply changes around the pI of proteins because of the electrostatic component of the reactions when sulfate anion binding to cationic proteins is involved[18].

In order to determine the best TPP system, temperature of the partitioning system was also evaluated. From the experimental findings, it was observed that, TPP system temperature at 25^0C yielded the highest activity recovery of 57.31 % with a fold purification value of 3.12 (Figure 4),

when culture filtrate was at 50 % saturation before addition of *tert*-butanol in the ratio of 1:1 (v/v) at pH 6.0. Similar findings were also reported by Dogan and Tari[13], which states that optimum purification level was obtained at a TPP system temperature of 25^0C. However, in case of TPP based peroxidase purification, optimum result was found at 30^0C, at a system comprising of 50 % $(NH_4)_2SO_4$ saturation with a ratio of 1:1.5 of broth to *tert*-butanol solvent[9].

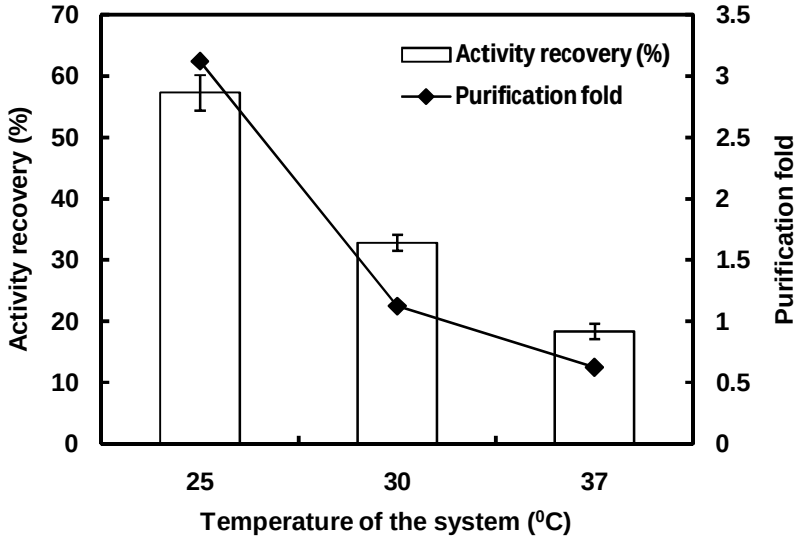

Figure 4: Effect of system temperature on the recovery of exo-polygalacturonase from the culture filtrate of *Pleurotusostreatus*. The mixtures containing of 50 % saturation of $(NH_4)_2SO_4$, *tert*-butanol and culture filtrate (1:1), kept at pH 6.0 with a varying temperature of the system for three phase partitioning for 1 h. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content. Data are presented here the mean value of five identical set of experiments with standard error bar.

The analysis of enzyme behavior in anhydrous media has an important implication in biotechnology and bioorganic chemistry. At present, the interaction between protein and organic solvent are inadequately understood. A clear understanding will facilitate the use of a non-aqueous system for many applications such as the organic synthesis, biosensor and bioseparation[19]. Three phase partitioning, therefore, a novel separation strategy in a single step purification technique. The addition of *tert*-butanol in the presence of ammonium sulfate pushes the protein out of the solution and *tert*-butanol adheres to the protein and the latter forms an interfacial precipitate between the lower aqueous and upper organic layers, although the physico-chemical basis of separation is not clearly understood but it is believed that the process is a combination of kosmotropic, conventional salting out, conformational protein tightening and protein hydration shift. An important finding of this partitioning experiment is that the interfacial layer is a gel, rather than a precipitate and the appearance of protein in this layer is a partition process and not a precipitation process[20].

In three phase partitioning of enzymes, the enzyme protein precipitate in the interfacial phase, pigments, lipids are concentrated in the upper phase, while polar compounds, cell debris are enriched

in the lower aqueous phase[21]. However, protein show different behavior in this condition, depending upon their source, molecular weight, pI etc. It is not necessary that all the proteins originally present in the aqueous solution would from the interfacial precipitate, such as protease of *Calotropisprocera* was concentrated at aqueous phase rather than interfacial precipitate in TPP of 65 % saturation of $(NH_4)_2SO_4$ with a ratio of crude extract to tert-butanol of 1:0.5[5]. Pike and Dennison[17]have shown that partitioning is affected by the hydrophilicity and molecular weight of the protein as well as the physical conditions (like temperature and pH) under which partitioning is carried out. By varying the concentration of ammonium sulphate, ratio of crude enzyme to *tert*-butanol, pH and temperature of TPP system, a remarkable selectivity can be obtained. This study shows that a high concentration of *exo*-polygalacturonase partitioned at the interface with 50% ammonium sulfate saturation, 1:1 (v/v) ratio of crude culture filtrate and *tert*-butanol at pH 6.0 and 25^0C. It has therefore, been possible to purify a number of proteins or enzymes using this single one-step process. Three phase partitioning is a relatively recent but fast developing and also non-chromatographic separation technique, which is simple, quick and economical technique and easily scalable at the industrial level.

4.CONCLUSION

Three phase partitioning has so far been shown to be useful for downstream operation in protein recovery. It is a simple, quick and economical technique and scaling up is convenient. It may be concluded from the present set of researches that with necessary optimization, the combination of 50 %(w/v) ammonium sulphate saturation with 1:1 ratio of culture filtrate to *tert*-butanol (v/v) at pH 6.0 and 25^0C temperature was optimal for attaining the best recovery of exo-polygalacturonase from *Pleurotusostreatus*. As TPP is a scalable process, the process described here has the potential to be carried out at the industrial level and data given in the experiments also shows the efficiency of TPP as an initial step for bioseparation of *exo*-polygalacturonase, which have various applications especially in food and fruit processing industries.

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