GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

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AQUEOUS TWO PHASE SYSTEM BASED DOWNSTREAM PROCESSING OF *ENDO*-1, 4 B-D-XYLANSE FROM CULTURE FILTRATE OF *ASPERGILLUS NIGER*

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ABSTRACT
Aqueous two phase extraction system [ATPS], a partition based downstream process was used to purify *endo*-1, 4 β-D-xylanse, an industrial enzyme from culture filtrate of *Aspergillus niger*. The xylanase was extracted by partitioning in ATPS composed of polyethylene glycol (PEG) and ammonium sulphate $(NH_4)_2SO_4$ or phosphate salt was used. The effects of PEG conc., $(NH_4)_2SO_4$, K-phosphate, loading mass, system temperature and pH on xylanase partition in ATPs were assessed. Single step purification of xylanase by ATPS resulted in a high recovery of 87.70 % with a 4.42 fold purification factor. The optimal system consisted of 8.0 % (w/w) of PEG-6000 and 15.0 % (w/w) of $(NH_4)_2SO_4$ at pH 6.0 and temp of 50^0C, while 8.0 % (w/w) of PEG-6000 and 20.5 % (w/w) of phosphate salt at pH 7.0 and temp of 50^0C represents the most suitable combination in ATPS.

KEYWORDS: *Aspergillus niger*, xylanase, ATPS.

INTRODUCTION

Downstream processing is an integral part of any product development and the final cost of the product depends largely on the cost incurred during extraction and purification techniques. The conventional techniques used for product recovery, for example precipitation and column chromatography, are not only expensive but also result in lower yields. Recovery and Purification of biomolecules is a sophisticated, cost intensive process and can account for up to 70% of the product cost of biomolecules (Diamond and Hsu, 1989). There has been an increasing interest in the development of efficient and economical downstream processing methods for separation of enzymes or proteins. Aqueous two phase extraction has been an attractive alternative for recovery and purification of enzymes. The simplicity of the process and low cost of the phase forming materials make it feasible for large scale protein purification using appropriate scale up techniques. Aqueous two phase systems have various advantages such as high water content, biocompatibility, low interfacial tension, high capacity and yield, easy to scale up technique and the potential for polymer recycling (Hustedt *et al*., 1998). Generally, PEG/dextran systems are employed but it is expansive due to high cost of dextran though crude un-fractioned dextran used as a cost-effective substitute for dextran in enzyme extraction. However, a major drawback in the industrial application of dextran is its high molecular weight and high viscosity. Therefore, PEG/salt systems have been preferred for large-scale enzyme extraction. However, a number of inexpensive substitutes of dextran, like derivatives of starch cellulose, polyvinyl alcohol, hydroxypropyl starch (HPS), ethyl hydroxyl ethyl cellulose (EHEC) has also been used (Albertsson *et al*., 1990) and are not only inexpensive but can be used at lower concentrations as well.

Xylanase is a class of enzymes which degrade the linear polysaccharide β-1, 4-xylan into xylose, thus breaking down hemicelluloses, one of the major components of plant cell walls. Xylanases are extracellular enzymes produced by different types of microorganisms. A complete and efficient enzymatic hydrolysis of this complex polymer depends mainly on two types of enzymes; Endo-1, 4 -xylanases, which hydrolyse the xylanopyranose of the central chain and xylosidases, which hydrolyse xylobiose and other xylooligosaccharides resulting from the action of endoxylanases. Xylanases have considerable interest because of their applications in food processing, bleaching of pulp during paper industry, bio-conversion of biomass wastes to fermentable sugars and clarification of fruit juices. Some successful extraction by ATPs of xylanase has also been demonstrated (Garai and Kumar, 2013). The more advanced technique such as using metal ligands was applied for partitioning and purification of xylanase produced by *Aspergillus Niger* (Fakhari *et al*., 2017). In the present study, an aqueous two phase system composed of either PEG/$(NH_4)_2SO_4$ or PEG/phosphate salt was used to purify xylanase produced by *Aspergillus niger*, isolated from agricultural soil of Baruipur, West Bengal. The partitioning behaviour of the enzyme in respect of phase composition, system pH, and temperature was also evaluated.

MATERIAL & METHODS

Microorganism and culture medium

The fungus *Aspergillus niger*, isolated from agricultural soil sample, collected from Baruipur, West Bengal was used in the present study. Fungal inoculum was prepared in medium containing (gl-1) of glucose-10, malt extract-

10, potato extract-100, KH_2PO_4-1.5 with an adjusted pH of 5.0. The synthetic medium for submerged fermentation was prepared containing (g/l) $NH_4H_2PO_4$-24, $MgSO_4$. $7H_2O$-0.5, $CaCl_2.2H_2O$-0.37, H_3PO_4-0.57, $FeSO_4.7H_2O$-0.25, $MnCl_2$-0.032, $NaMoO_4$-0.032, yeast extract-5 in combination with a carbon source at different concentration and pH of medium was adjusted to 4.5. Tamarind kernel powder was used as the carbon source for the enzyme production (Chatterjee *et al*., 2010). Fermentation was carried out at 30 $\pm 1^0$C for 7 days in shake flask and culture filtrates were used as the source of enzyme.

Aqueous two-phase system preparation

In all the experiments, the phase system was prepared by mixing the required quantities of phase forming solutes *i.e*., polyethylene glycol with combination of $(NH_4)_2SO_4$ or KH_2PO_4 in crude enzyme extract on a w/w% basis, prepared according to the modified method of Vaidya *et al*. (2006). After equilibrium, the individual phases were collected and aliquots of the phases were analyzed for enzyme activity and protein concentration.

Xylanase activity assay

Xylanase was assayed by measuring the reducing sugars liberated from Xylan (Sigma Chemicals) dissolved in 0.1M acetate buffer (pH 5). The reaction mixture contains 1.0% (w/v) xylan, enzyme and 0.1M acetate buffer (pH 5), incubated for 10 min at 50ºC and the released reducing groups were measured by DNSA (Chatterjee *et al*., 2010). Assays were performed in triplicate for each of the treatment and the averages were used in calculations.

Protein concentration determination

Protein concentration was determined with a coomassie protein assay reagent, a method based on the dye-binding technique (Bradford, 1976). Assays were performed in triplicate and the averages were used in calculations.

Partition parameters

The partition co-efficient (*Ke*) for xylanase activity and protein in the aqueous two phase systems was defined as the ratio of the volumetric enzyme activity the top phase over that in the bottom phase:

$$Ke = \frac{enzyme\ activity\ (top\ phase)}{enzyme\ activity\ (bottom\ phase)}$$

Purification factor (Pf) was calculated as the ratio of the specific activity in the top phase over the specific activity of the crude enzyme before the partitioning as:

$$Pf_{top} = \frac{specific\ activity\ of\ enzyme\ (top\ phase)}{specific\ activity\ (crude\ enzyme)}$$

Yield ($Y_{top\ phase}$) of enzyme was defined as the ratio of total activity of enzyme in the top phase over that in initial crude extract, expressed as percentage as:

$$Y_{top\ phase} = \frac{enzyme\ in\ top\ phase\ (U/ml)}{total\ amount\ of\ enzyme}\ x\ 100$$

RESULTS AND DISCUSSION

As ammonium sulphate is commonly used for protein precipitation techniques, a two-phase system composed of PEG ammonium sulphate was evaluated for partitioning of xylanase. According to the phase diagram of PEG-$(NH_4)_2SO_4$ system by Saltabat, (2001), the system comprising 12% (w/w) PEG-6000-15% (w/w) $(NH_4)_2SO_4$ provides two separated phases. The system containing more than 12% (w/w) PEG resulted in a highly viscous mixture, whereas the system consisting of more than 15% ammonium sulphate provoked the precipitation of proteins. Table 1 shows the partitioning behavior of xylanase in PEG-$(NH_4)_2SO_4$ system.

TABLE 1: Composition of PEG 6000-$(NH_4)_2SO_4$ in aqueous two phase system on partitioning of xylanase [phase system pH 6.0 at 40^0C]

PEG 6000 (w/w%)	$(NH_4)_2SO_4$ (w/w%)	$Ke_{xylanase}$	$Ke_{protein}$	Pf_{top}	$Y_{top\ phase}$
5.0	15.0	0.98	1.0	1.05	41.67
8.0	15.0	0.82	0.97	2.30	58.39
12.0	15.0	0.80	1.66	4.94	69.68
15.0	15.0	0.82	2.51	3.96	59.99

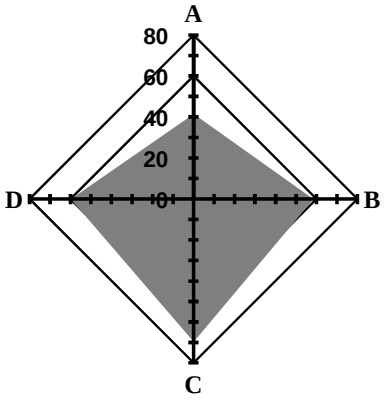

FIGURE 1: Composition of PEG 6000-$(NH_4)_2SO_4$ in aqueous two phase system on partitioning of xylanase % yield [phase system pH 6.0 at 40^0C]. Composition of PEG 6000-$(NH_4)_2SO_4$ (w/w%] are as follows: A= 5.0-15.0, B= 8.0-15.0, C= 12.0-15.0, D= 15.0-15.0

The most appropriate condition for selective partitioning of the proteins, corresponding to xylanase activities were a combination of 12% PEG-6000 with 15% $(NH_4)_2SO_4$ under which the highest purification fold of 4.94 with a yield of 69.68% of xylanase were obtained, when the extraction system equilibrated with a constant temperature of 40^0C and a pH of 6.0 (Figure 1).

An increase in the tie line length causes an increase in the protein partition coefficient that in turn increases the yield of proteins in the top phase due to reduction of the bottom phase. Increasing tie line length in the PEG-salt system causes the salting out more effectively, leading to shift of the proteins in the top-rich phase (De Silva and Franco, 2000). Xylanase obtained from solid state cultivation of *Polyporus squamosus* (Antov *et al*., 2006) was partitioned in PEG 1500-20% (w/w) ammonium suphate (70%) w/w at pH 5.1 with a yield of 85.6% and partition coefficient of 17.37, which supports our present set of research. In the present study, the effect of volume exclusion did not influence the partitioning of xylanase. The enzyme partitioning to the top phase was observed when a high PEG molar mass (6000g/mol) was used. The possible cause could have been an electrostatic extraction between the xylanase protein molecules, with a negatively charged surface lower than the pH used in present study and positively charged PEG molecules (Maciel *et al*., 2014). The low molecular weight (>80 KDa) of the xylanase may also have influenced the partitioning for the PEG-rich top phase. Hence in the top phase, there was enough space for the accommodation of the enzyme when using PEG with high molecular weight (6000 g/mol).

Among the different composition studied, a phase system having a composition of 8% (w/w) PEG 6000 and 20.5% phosphate (w/w) salt gave the best overall results in terms of xylanase activity recovery (70.18%) as well as purification factor at pH 6.0 with the system temperature of 40^0C (figure 2). However if the hydrophobicity and molecular weight of the target protein and major contaminants are known, the selection of the most suitable ATPs for separation and purification is simpler.

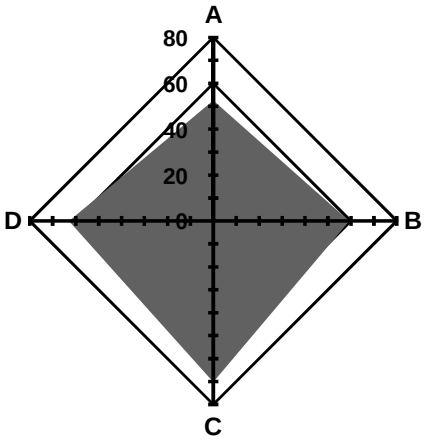

FIGURE 2: Composition of PEG 6000-phosphate salt in aqueous two phase system on partitioning of xylanase % yield [phase system pH 6.0 at 40^0C]. Composition of PEG 6000-phosphate (w/w%] are as follows: A= 3.0-30.5, B= 5.0-25.5, C= 8.0-20.50, D= 12.0-10.5

Andrews *et al*. (2005) have studied partition behavior of 12 selected proteins (both hydrophilic and hydrophobic) in ATPs regarding the effects of surface hydrophobicity and changes and conducted that in the PEG-salt system, the hydrophobicity of the protein plays a very important role in the purification of the biomolecules. Among twelve proteins studied, the enzyme, amyloglucosidase from *Aspergillus niger*, which is more hydrophilic tended to partition into salt rich bottom phase. Similarly, Su and Chiang (2006) have studied the partitioning and purification of hydrophobic lysozyme by using PEG-salt ATPs and observed that maximum lysozyme partitioning was towards the PEG rich top phase. The partition coefficient for enzyme (k_e) protein (k_p) fold of purification (P_f) and yield (Y%) of xylanase of *A. niger* are shown in table 2, which illustrates the most suitable phase system was of 8% (w/w) PEG 6000 and 20.50 % (w/w) phosphate salt, give the purification fold of 2.06 and 70.18% activity recovery of enzyme yield.

TABLE 2: Composition of PEG 6000-phosphate salt in aqueous two phase system on partitioning of xylanase [phase system pH 6.0 at 40^0C]

PEG 6000 (w/w%)	Phosphate (w/w%)	$Ke_{xylanase}$	$Ke_{protein}$	Pf_{top}	$Y_{top\ phase}$
3.0	30.5	0.79	0.88	0.50	52.64
5.0	25.5	0.85	1.07	0.88	59.29
8.0	20.50	0.82	1.21	2.06	70.18
12.0	10.5	0.57	1.32	2.10	63.04

Garai and Kumar, 2013 reported that using PEG 4000/Na_2HPO_4 system, xylanase was purified with partitioning coefficient of 8.41 and 88.10% enzyme yield at top phase was attained. Kumar et al (2013) reported that enzyme purification factor was found maximum in presence of low molecular weight of PEG-4000 at a system containing 8.66% (w/w) PEG with a high salt concentration of 22.4% (w/w). Under optimized condition

threefold increase of purification factor with partition coefficient 8.41% and 88.10% enzyme yield at top phase was attained, which are in accordance with our experimental findings. Loureiro *et al* (2017) reported that PEG/sodium citrate is the best system to partition xylanase with $k_p$value of 17.7 ±0.3 and also concluded that different molar mass of PEG slightly altered the native structure of xylanase without altering the catalytic activity in aqueous two phase system. Fakhari *et al.* (2017) reported that PEG 6000 can be activated using epichlorohydrin, covalently linked to aminodiacetic acid, which was used in partitioning system of xylanase of *A. niger*. Malhotra *et al.* (2016) reported the specific activity of xylanase was obtained as 6.27 mu/mg of protein when the ATPs were used as phosphate salt and polyethylene glycol. Xylanase of *A. niger* partitioning was favorable to the polymer rich phase in the PEG 1000/sodium citrate system (Romanini *et al*., 2016).

The present state of research shows the feasibility of using ATPs as a first step downstream processing of xylanase from a fungal crude extract, considering this conclusion, the next experiment was conducted to examine the integration of one kind of aqueous two phase extraction using the system of polyethylene glycol 6000-phosphate salt, which is also reported for separation and extraction for a number of enzyme downstream processing. The concentration of PEG (MW 6000) and phosphate salt were selected in this experimental study according to the method of Vaidya *et al.* (2006) to ensure every system could lie above the bimodal curve in the respective phase diagram to ensure phase separation. The phase composition greatly influences the partitioning behavior of xylanase and total protein and also influences the purification factor. It was interestingly observed that for all phase compositions, the enzymes partitioned preferentially towards the top phase.

Aqueous two phase system with PEG molecular weights of 4000 and 6000 were generally employed because the partition effect was more pronounced than the PEG molecular weight of less than 6000 (Naganagouda and Mulimani (2008). However, in this present study, all the experiments were done using only PEG-6000 molecular weight, therefore it is difficult to comment upon the molecular weight on the partitioning behavior of xylanase. It should be considered that biomolecule partitioning in ATPs is a complex function of a variety of parameters including the biomolecule size, its surface properties, net charge, system pH, system temperature and the polymer molecular weight (Baskir *et al*., 1989) usually the partition coefficient decreases as the PEG chain length increases, as the size of biomolecule increases, preference for partitioning into once phase also tends to increase. The partitioning behavior is also influenced by the net charge of protein. Although the experimental findings of the present study for the xylanase partitioning are not so good, the purification fold and activity recovery of xylanase clearly indicates that aqueous two phase extraction system could be a viable alternative for xylanase purification and future research would elucidate the responsible factors for optimized yield of xylanase, an industrially important enzyme.

Effect of system pH and temperature on xylanase partitioning and purification

The culture filtrate was partitioned in ATPS at pH values of 6.0 and 7.0 at different temperatures of 40ºC, 50ºC and 60ºC for the selection of a suitable pH for xylanase single step purification. In all cases, pH 6.0 gives the best result for partitioning of xylanase in ATPS system of PEG-6000/ $(NH_4)_2SO_4$. In system having increased pH, increased enzyme partition coefficient ($k_{xylanase}$) were observed. The change in partition coefficient can be explained by considering the change in the net charge of the enzyme surface compared to their isoelectric points. The negatively charged protein concentrates in the top phase and therefore the partition coefficient increases. The maximum recovery of 83.36% and purification fold 5.03 were observed at pH 6.0 at 50ºC (Table 3).

TABLE 3: Effect of pH and temperature in aqueous two phase system as composition of PEG 6000-$(NH_4)_2SO_4$ on partitioning of xylanase

PEG 6000 (w/w%)	$(NH_4)_2SO_4$ (w/w%)	pH	Temp (^{0}C)	$Ke_{xylanase}$	$Ke_{protein}$	Pf_{top}	$Y_{top\ phase}$
8.0	15.0	6.0	40	0.80	1.66	4.94	69.68
8.0	15.0	7.0	40	0.64	1.31	3.57	54.01
8.0	15.0	6.0	50	0.91	1.78	5.03	83.36
8.0	15.0	7.0	50	0.83	1.62	4.81	78.02
8.0	15.0	6.0	60	0.72	1.11	3.58	67.54
8.0	15.0	7.0	60	0.68	0.83	2.13	57.93

Most of the biomolecules, especially proteins and enzymes are stable at neutral pH 7.0. In case of PEG 6000/phosphate salt system, maximum recovery was obtained at pH 7.0 as compared to pH 6.0, when the system temperature was at 50ºC. Goja *et al.* (2013) also reported that a pH value below 6.5 is compatible with the PEG 6000/sulphate system whereas a pH value of above 7.0 is most suitable for PEG 6000/phosphate aqueous two phase partitioning system, which is in accordance with our experimental results. If we compare the % yield of xylanase in ATP system, then the experimental findings clearly revealed that at 40ºC, the yield recovery were highest in the combination of 12% PEG-6000 and 15% $(NH_4)_2SO_4$ at pH 6.0, whereas highest (Table 4) yield of xylanase was found at pH 7.0 with a combination of 8% phosphate salt and 20.5% PEG-6000 system. Naganagouda and Mulimani (2008) reported that α-galactosidase was maximally recovered at pH 7.0 with a slight increase at pH 8.0 as compared to a low pH profile. To select the optimal temperature for xylanase partitioning, different temperatures of 40^{0}C, 50^{0}C and 60ºC at constant pH 7.0 were studied and the partitioning coefficient of xylanase and total protein with respect to temperature are shown in table 3 and 4. From the table it is

evident that there is monotonic increase in xylanase and protein partition coefficients, when the temperature is raised from 40ºC to 50ºC but starts decreasing above 50ºC of system temperature.

TABLE 4: Effect of pH and temperature in aqueous two phase system as composition of PEG 6000-phosphate salt partitioning of xylanase

PEG 6000 (w/w%)	Phosphate (w/w%)	pH	Temp (^{0}C)	$Ke_{xylanase}$	$Ke_{protein}$	Pf_{top}	$Y_{top\ phase}$
8.0	20.50	6.0	40	0.82	1.21	2.06	70.18
8.0	20.50	7.0	40	0.87	1.34	2.51	72.60
8.0	20.50	6.0	50	0.83	1.42	3.97	79.94
8.0	20.50	7.0	50	1.42	1.93	4.42	87.70
8.0	20.50	6.0	60	0.73	0.98	1.67	70.59
8.0	20.50	7.0	60	0.87	1.08	1.14	63.47

The observed mono-ionic increase of both enzyme and total protein partition in both of the system *i.e*., PEG/ $(NH_4)_2SO_4$ and PEG/ phosphate salt system is due to the effect of temperature on the bimodal curve for PEG/ phosphate salt system. With the increased temperature, the bimodal curve moved down and the larger two-phase region above the binodal curve resulted in increased differences in the phase compositions. The change in the partitioning coefficients of the biomolecules with temperature maybe attributed to this variation in the phase composition. As increase in temperature was also accompanied by an increase in the phosphate salt concentration in the bottom phase. As a result, the number of water molecules available for solute solvation in the salt phase should decrease, which also reduces the solubility of xylanase and this process increased the affinity of the biomolecules for the top phase, as is evident from the enhanced partition coefficient. From figure 3 and 4, it is clearly observed that % yield of xylanase was highest in 50^0C as compared to 40^0C or 60^0C of ATP system temperature.

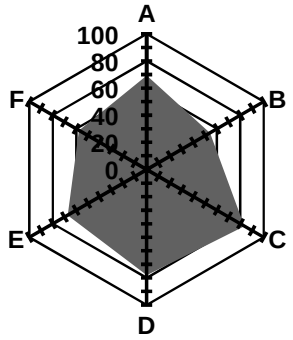

FIGURE 3: Effect of pH and temperature in aqueous two phase system as composition of PEG 6000-$(NH_4)_2SO_4$ on partitioning of xylanase [A= 6.0 pH, temp 40^0C]; [B= 7.0 pH, temp 40^0C]; [C= 6.0 pH, temp 50^0C]; [D= 7.0 pH, temp 50^0C]; [E= 6.0 pH, temp 60^0C]; [F= 7.0 pH, temp 60^0C]

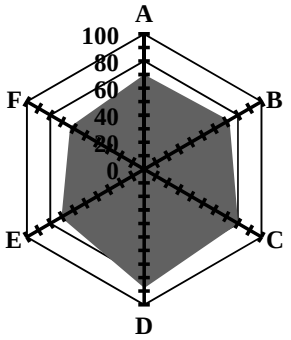

FIGURE 4: Effect of pH and temperature in aqueous two phase system as composition of PEG 6000-phosphate salt on partitioning of xylanase [A= 6.0 pH, temp 40^0C]; [B= 7.0 pH, temp 40^0C]; [C= 6.0 pH, temp 50^0C]; [D= 7.0 pH, temp 50^0C]; [E= 6.0 pH, temp 60^0C]; [F= 7.0 pH, temp 60^0C]

Gautam and Simon (2006) have made similar observation for β-galactosidase extraction in PEG/phosphate salt system. Furthermore, increasing temperature can destroy the bonds of biomolecules. As these bonds are weakened and broken, biomolecules become more flexible structure. Water in two phase system can interact and form new hydrogen bonds with the functional groups of the biomolecules. The presence of water molecules further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the structure is broken, hydrophobic groups are exposed to the solution. As a consequent, loss in solubility of molecules is observed.

CONCLUSION

In conclusion, aqueous two-phase system is a fast, simple, sensitive, and selective method which could be successfully employed for xylanase extraction from the culture filtrate of *Aspergillus niger*. Purification of xylanase by ATP resulted a high recovery of 83.36 % and 87.70 % in PEG 6000-$(NH_4)_2SO_4$ or PEG 6000-phosphate salt respectively. The optimum system pH is 6.0 and 7.0 in PEG 6000-$(NH_4)_2SO_4$ and PEG 6000-phosphate salt respectively although in both system, the optimal temperature for highest recovery is 50^0C. The partitioning of biomolecules in ATPs is influenced by pH, temperature, surface properties, size and concentration of the biomolecules and the types of employed polymers and salts. Future research would be necessary to elucidate the efficiency of aqueous two phase system for protein separation using copolymer, affinity ligand or substitute of polymers.

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