

# Partitioning of Cholesterol Oxidase Produced by *Streptomyces olivaceus* MTCC 6820 in Different PEG-Salt-Water Aqueous Two Phase Systems

Shraddha Sahu<sup>1</sup>, Kshitij Agarwal<sup>1</sup>, Swati Vasisht<sup>2</sup>, Shailendra Singh Shera<sup>1</sup>, Rathindra Mohan Banik<sup>3\*</sup>  
Research Scholars, School of Biochemical Engineering, Indian Institute of Technology (Banaras Hindu University),  
Varanasi, India<sup>1</sup>

Student, Department of Bioscience and Biotechnology, Banasthali University, Jaipur, India<sup>2</sup>

Professor, School of Biochemical Engineering, Indian Institute of Technology (Banaras Hindu University),  
Varanasi, India<sup>3</sup>

**ABSTRACT:** Screening of aqueous two phase systems (ATPS) for partitioning of cholesterol oxidase (Chox) produced by *Streptomyces olivaceus* MTCC 6820 was done using eight different ATPS composed of two different molecular weight of polyethylene glycol (PEG)X (X= 4000 and 6000) with salts (ammonium sulphate, potassium phosphate, trisodium citrate and sodium sulphate) and water. PEG4000 [10.63% (w/w)] and ammonium sulphate [14.5% (w/w)] was selected as most suitable system components for Chox partitioning on the basis of maximum partition coefficient (K=1.32). 2.34 fold purification of Chox was obtained in the top phase with 75.85% yield and 65.21 U/mg specific activity of partitioned Chox.

**KEYWORDS:** Aqueous Two Phase Systems, Cholesterol oxidase, Partitioning, PEG-salt Two Phase Systems

## I. INTRODUCTION

Downstream processing is an integral part of any product development and plays a very crucial role in the commercial production of enzymes produced through submerged fermentation process. The conventional purification technique is a complex multi-step process which suffers the limitation of being time-consuming and labour intensive, also the loss of enzyme activity at each step reduces the recovery of desired enzyme resulting in lower yield. ATPS may provide an appropriate alternative to conventional method for purification of enzymes by preferentially partitioning the enzyme in one phase and interfering substances into other phase. The biocompatibility of ATPS provides a very low interfacial tension between the phases, which results in high mass transfer and ease of scale up. ATPS have been proved to be useful for the extraction and affinity purification of various intracellular and extracellular enzymes. PEG-salt-water system have been proved to be useful for their low cost and simplicity in handling for enzymes like alkaline phosphatase [Pandey and Banik, 2011], glucose oxidase [Singh and Verma 2010].

Chox (EC 1.1.3.6) is a FAD-dependent oxido-reductase enzyme that catalyzes the oxidation of cholesterol to form 4-cholesten-3-one and hydrogen peroxide [MacLachlan et al., 2000, Salva et al., 1999]. Chox is a key enzyme used for the detection and quantification of cholesterol in blood serum [Allain et al., 1974; Lolekha and Jantaveesirirat, 1992]. An effective downstream processing involving aqueous two phase extraction would increase the recovery and yield, possibly reducing the cost of the enzyme. *Streptomyces* Chox have been reported to be superior to other commercially available sources of Chox [Lolekha and Jantaveesirirat, 1992]. However, no literatures are available for Chox

# International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

production by *Streptomyces olivaceus* (*S. olivaceus*). The purification of Chox has been described by many conventional techniques like ion exchange chromatography [El Naggar et al., 2017], affinity chromatography [Rhee et al., 1991] and hydrophobic ionic chromatography [Sasaki et al., 1982]. Partitioning of Chox by ATPS has not been reported till now. In the present work, partitioning of Chox produced by *S. olivaceus* MTCC 6820 in different PEG-salt-water two phase systems was studied.

## II. LITERATURE REVIEW

ATPS have emerged as an effective green extraction technique for purification and recovery of a wide range of biological products such as enzymes, proteins, nucleic acids, aminoacids, cellular organelles and microorganisms from their respective contaminants and impurities [Rahimpour et al., 2016, Souza et al., 2015, Xavier et al., 2015]. ATPS being an uncomplicated procedure offers a low cost technology for purification of proteins and enzymes with ease of scale-up. It offers a non-denaturing environment which overcomes the limitation of conventional purification techniques like ion exchange and affinity chromatography. Chox is a bacterial enzyme produced exclusively through microbial fermentation process. It is a high value product and the augmented industrial importance owes to its usefulness in various biotechnological applications. Downstream processing of industrial enzymes cost around 70-80% of the overall cost of the final product. The production media for Chox contains a large amount of yeast extract, peptone and salt. Studies suggest that Chox has been largely purified by two conventional methods viz. ion exchange chromatography (IEX) and affinity chromatography. IEX is a sophisticated process involving precipitation, desalting, protein binding to IEX resin in column, elution and again desalting, which is often time consuming and labour intensive. Exposing proteins to high salt concentration increases the risk of protein denaturation and the multiple steps leads to the loss of enzyme activity at each step thus reduced recovery of the desired enzyme resulting in lower yield. ATPS can be used as a better alternative for purification of such proteins, which not only improves the final yield but also provides a low cost technology.

## III. MATERIALS AND METHODS

- a. **Materials Used:** *S. olivaceus* culture was procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Poly ethylene glycol (PEG) of molecular weights 4000 and 6000 were purchased from Sisco Research Laboratories, Mumbai, India. Ammonium sulphate, potassium phosphate, sodium sulphate and tri-sodium citrate were purchased from Qualigens, Mumbai, India. All other chemicals and media components used were of analytical grade.
- b. **Fermentative Production of Cholesterol Oxidase:** Chox was produced in the production medium consisting of (g/L): cholesterol- 2, glucose – 12, starch – 9, yeast extract -6, peptone – 4,  $(\text{NH}_4)_2\text{SO}_4$ – 7.5, cholesterol -2,  $\text{K}_2\text{HPO}_4$  – 1,  $\text{MgSO}_4$  – 0.5, NaCl – 1,  $\text{MnSO}_4$  – 0.008,  $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$  – 0.002,  $\text{ZnSO}_4$  – 0.002,  $\text{FeSO}_4$  – 0.02,  $\text{CaCl}_2$  – 0.0002 and Tween 80 – 10ml [Yazdi et al., 2001]. The composition of seed and production media remained the same. 48 h old seed culture of *S. olivaceus* was used for inoculation of the production medium. Chox fermentation was carried out under optimized shake flask conditions for 72h (32°C, 180 rev/min) in an orbital shaker. Crude Chox was collected as a supernatant obtained by centrifugation of the fermented broth at 12,000xg (4°C) for 20 min and concentrated by vacuum evaporator. The *S. olivaceus* culture was maintained in slants containing *Streptomyces* growth medium and sub-cultured at the interval of every 30 days.
- c. **Cholesterol Oxidase Assay:** Chox activity was estimated by modified method of Allain et al. [Allain et al., 1974; MacLachlan et al., 2000]. 50 µl of 0.6% (w/v) cholesterol was added to 1 ml of reaction mixture containing 1.5mM 4-aminoantipyrine, 5mM phenol, 10U/ml horseradish peroxidase and sodium phosphate buffer (20 mM, pH 8.0) and pre-incubated at 30°C for 5 min. 100 µl of partitioned enzyme sample (top / bottom phase) was taken and added to the pre-incubated reaction mixture to start the reaction and the incubation continued at 30°C for 10 min. The reaction was terminated by placing the samples in a boiling water bath for 2 min and then immediately placed in an ice-bath for 2 min for color development. Absorbance was recorded at 500 nm by discontinuous spectrophotometric method. Blank was prepared by taking the ATPS (top / bottom phase) samples instead of Chox samples. The enzyme activity in the

## International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

top phase was measured against the enzyme blank of same top phase; similarly the enzyme activity in the bottom phase was measured against the enzyme blank of the same bottom phase.

One unit of Chox activity was defined as the formation of 1 $\mu$ mol of hydrogen peroxide (0.5  $\mu$ mol of quinoneimine dye) per minute at 30°C with a pH of 8.0.

- d. Protein Determination:** Protein concentration was determined by Bradford's method using Coomassie Brilliant Blue G-250 dye. Blank was prepared by taking ATPS (top / bottom phase) samples containing no Chox.

The standard curve of bovine serum albumin (BSA) with concentrations ranging from 0.01 to 0.2 mg/ml was prepared taking absorbance at 595nm [Bradford, 1976].

- e. Phase Diagram:** ATPS comprising eight different compositions of PEG X (X = 4000, 6000) and salts (ammonium sulphate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], potassium phosphate (K<sub>2</sub>HPO<sub>4</sub>), sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) and tri-sodium citrate (Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>) were formed and characterized with the help of binodial curve, tie-line and phase volume ratio (V<sub>t</sub>/V<sub>b</sub>). Phase diagram of ATPS was constructed by stepwise titration of salts (25%w/w) with fixed amounts of (35%w/w) PEG till turbid end point and diluted with water till the disappearance of turbidity.

A binodial curve was plotted for all the PEG-salt-water systems using the Merchuk's equation eq (1) [Merchuk et al., 1998].

$$Y = 71.1 \exp(-0.62X^{0.5} - 0.00123X^3) \quad (1)$$

where Y and X, are PEG and salt weight percentages, respectively.

The tie lines (TL) were obtained by solving a system of four equations (equations (2) to (5)) and four variables (Y<sub>T</sub>, Y<sub>B</sub>, X<sub>T</sub> and X<sub>B</sub>) [Merchuk et al., 1998] using the Solver tool in Microsoft Excel®.

$$Y_T = A \exp[(BX_T^{0.5}) - (CX_T^3)] \quad (2)$$

$$Y_B = A \exp[(BX_B^{0.5}) - (CX_B^3)] \quad (3)$$

$$Y_T = \left(\frac{Y_M}{\alpha}\right) - \left[\left(\frac{1-\alpha}{\alpha}\right)\right] Y_F \quad (4)$$

$$X_T = \left(\frac{X_M}{\alpha}\right) - \left[\left(\frac{1-\alpha}{\alpha}\right)\right] X_F \quad (5)$$

where subscripts T, B and M refers respectively to the top phase, bottom phase and the mixture X is the weight fraction of different salts (ammonium sulfate, potassium phosphate, sodium sulphate, and tri-sodium citrate), Y is the weight fraction of PEG X (X= 4000, 6000) and  $\alpha$  is the ratio between mass of the top phase and mass of the mixture.

The tie lines were obtained by solving equations (2) to (5) and are given by equation (6) [Merchuk et al., 1998]:

$$Y = Y_0 + SX \quad (6)$$

where, the slope S is given by equation (7):

$$STL = \frac{Y_T - Y_B}{X_T - X_B} \quad (7)$$

Slope of the tie line (STL) of different ATPS was determined by the ratio between mass fraction difference of PEG and different salts in the top and bottom phases using equation (7) [Merchuk et al., 1998].

- f. Partitioning of Cholesterol Oxidase in Different PEG-Salt-Water Two Phase Systems:**

The partitioning studies of crude Chox were done in graduated centrifuge tubes (15ml) involving eight different PEG-salt-water ATPS. The concentrated crude Chox was homogeneously mixed with the PEG-salt mixture on a magnetic stirrer for at least 45 min and allowed to stand for a maximum of 1h leading to complete phase separation. The top and bottom phases were separated carefully using a Pasteur pipette, analyzed for the Chox activity (U/ml) and partition coefficients were determined.

**Partition Coefficient (K):** Partition coefficient is the ratio of concentration of Chox in the top and bottom phases and is given by equation (8).

$$K = \frac{C_T}{C_B} \quad (8)$$

where, C<sub>T</sub> is the activity of Chox in top phase and C<sub>B</sub> is the activity of Chox in bottom phase.

**Yield (Y%) and Specific Activity (SA):** Yield (Y%) is given by the ratio of Chox activity in the top phase to initial activity in the original sample before partitioning, equation (9):

$$Y(\%) = \frac{\text{Chox activity in top phase} \times 100}{\text{Total Chox activity added to the system}} \quad (9)$$

# International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

$$SA \text{ (Units/mg)} = \frac{\text{Enzyme activity (Units/ml)}}{\text{Total Protein (mg/ml)}} \quad (10)$$

## IV. EXPERIMENTAL RESULTS

ATPS were developed using PEG-salt-water systems, the top phase is continuous and is rich in PEG while the bottom phase is rich in salt. The enzymes and proteins get easily separated on the top PEG rich phase. ATPS composed of two different molecular weights of PEG-X (X= 4000 and 6000) with four different inorganic salts (ammonium sulphate, potassium phosphate, tri-sodium citrate and sodium sulphate) were used for partitioning of Chox (Table 1 and 2). PEG 4000-ammonium sulphate-water system was found to be the most suitable ATPS for Chox partitioning.

- a. **Binodial Curve:** The binodial curve of eight different ATPS using PEG X (X = 4000 and 6000) and four inorganic salts (ammonium sulphate, potassium phosphate, trisodium citrate and sodium sulphate) have been presented in Fig 1(A) and Fig 1 (B). The nature of curve largely depends upon the type of salt used. The phase formation rate was also different with respect to different salts used to form the ATPS. It was observed that PEG molecular weight has no significant effect on the nature of curve.

**Table 1 Partitioning of cholesterol oxidase from *Streptomyces olivaceus* using PEG 4000-salt-water aqueous two phase systems**

System	% PEG	% Salt	V <sub>t</sub> /V <sub>b</sub>	Chox activity (U/ml)		K
				Top (C <sub>t</sub> )	Bottom (C <sub>b</sub> )	
PEG4000-(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	10.63	14.5	0.99	0.31	0.23	1.32
PEG4000-K <sub>2</sub> HPO <sub>4</sub>	10	10	0.28	0.3	0.7	0.43
PEG4000-Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub>	14.45	36	0.48	0.28	0.33	0.84
PEG4000-Na <sub>2</sub> SO <sub>4</sub>	3.15	16	0.13	0.60	0.67	0.88

**Table 2 Partitioning of cholesterol oxidase from *Streptomyces olivaceus* using PEG 6000-salt-water aqueous two phase systems**

System	% PEG	% Salt	V <sub>t</sub> /V <sub>b</sub>	Chox activity (U/ml)		K
				Top (C <sub>t</sub> )	Bottom (C <sub>b</sub> )	
PEG6000-(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	14.1	9.5	0.79	0.52	0.41	1.25
PEG6000-K <sub>2</sub> HPO <sub>4</sub>	20	15	0.2	0.23	0.94	0.24
PEG6000-Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub>	11.93	13	0.45	0.36	0.46	0.77
PEG6000-Na <sub>2</sub> SO <sub>4</sub>	25	4	3.22	0.44	0.49	0.89

- b. **Partition coefficient (K):** The most suitable ATPS for partitioning of Chox was selected on the basis of higher partition coefficient (K); the results of the partitioning experiments with eight different PEG-salt-water systems are presented in Table 1 and 2. PEG 4000 [10.63% (w/w)] - ammonium sulphate [14.5% (w/w)]-water was found to be most suitable system component for partitioning of Chox with highest partition coefficient 1.32 amongst the eight different PEG-salt-water ATPS studied. Similar, result was found in case of PEG 4000- dextran T500 ATPS having partition coefficient of 1.31 [Singh and Banik, 2012]. The partitioning of Chox more towards the top phase (PEG-rich) suggests the hydrophobic nature of the partitioned protein, such proteins need more than ion exchange chromatography for purification, which is a complex multistep, labour intensive and costlier conventional technique.

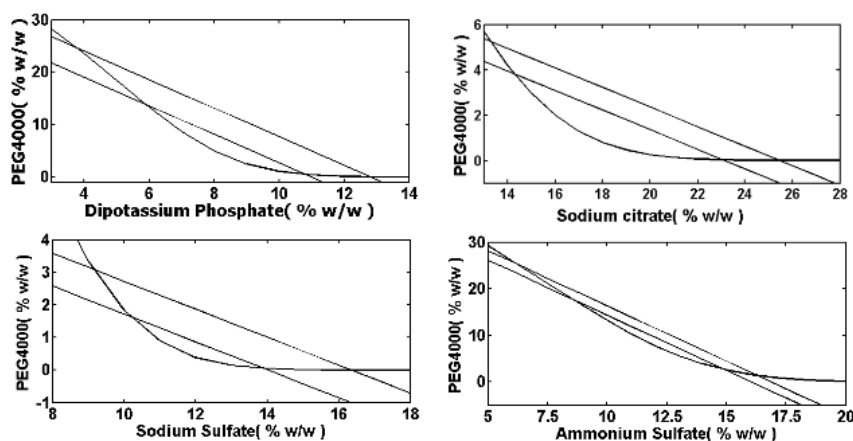
## International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

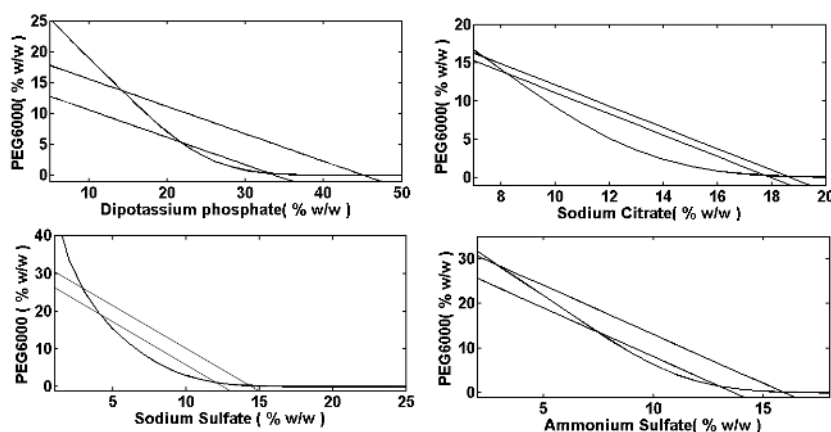
Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

It is worth mentioning here, that PEG 4000-dextran T500 system is an example of PEG-polymer system and its cost is much higher than the PEG-salt system. Although, PEG-polymer systems provide biocompatibility to the proteins and enzymes for partitioning, but are less practical due to higher cost of the polymers (eg. dextran, pullulan, poly vinyl alcohol) used. Here, it is clear from the above result that PEG-salt system is providing better partitioning in case of Chox partitioned in PEG 4000- ammonium sulphate system, at much lower cost than the PEG-polymer system. The binodial curve of PEG4000-ammonium sulphate-water system has been displayed in Fig 2.



**Fig 1 (A) Binodial Curve of PEG 4000-salt-water ATPS**



**Fig 1 (B) Binodial Curve of PEG 6000-salt-water ATPS**

- c. Effect of PEG molecular weight on Chox partitioning:** PEG 4000-salt-water systems showed greater partition coefficients as compared to PEG 6000-salt-water systems (Table 1 and 2). It shows that low molecular weight PEG favours higher partition coefficient for Chox. This may be due to the volume-exclusion effect caused due to higher molecular weight of the PEG 6000. The phase volume ratios of PEG 4000 with ammonium sulphate, potassium phosphate, and tri-sodium citrate were 0.99, 0.28, and 0.48 respectively while it was 0.79, 0.2, and 0.45 in case of PEG6000-ammonium sulphate, potassium phosphate and tri-sodium citrate systems respectively. While exceptionally, the phase volume ratios of PEG 4000-sodium sulphate system and PEG6000 – sodium sulphate system remained 0.13 and 3.22 respectively, on the other hand their partition coefficients remained less affected. It shows that with the increasing molecular weight of PEG, the phase volume ratio decreased in each of the PEG-salt-water systems studied.

## International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

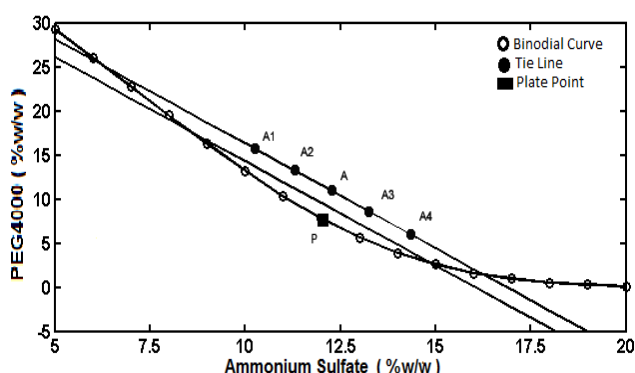
Therefore, low molecular weight PEG resulting in higher partition coefficient and phase volume ratio was preferred for partitioning of Chox.

- d. Effect of phase-volume ratio on Chox partitioning in PEG4000-ammonium sulphate-water system:** The phase volume ratio (volume of top phase/ volume of bottom phase) decreased with increasing PEG molecular weight. The effect of phase-volume ratio ( $V_t/V_b$ ) on Chox partitioning was studied by taking five different points on the tie line viz. A1, A2, A, A3, A4 which corresponds to five different phase compositions. The phase volume ratio increased starting from point A1 through A2, reached its optimum at point A and then decreased subsequently at points A3 and A4. The phase composition of 10.63% PEG and 14.5% ammonium sulphate at point A was found to have optimum phase volume ratio of 0.99, as displayed in Table 3 and Fig 2.

Fig 3 displays the picture of equilibrated PEG4000-ammonium sulphate system with phase volume ratio ( $V_t/V_b$ ) 0.99.

**Table 3 Effect of phase volume ratio on cholesterol oxidase partitioning in PEG 4000-ammonium sulfate-water system**

System	%PEG 4000	%NH <sub>4</sub> (S O <sub>4</sub> ) <sub>2</sub>	V <sub>t</sub> /V <sub>b</sub>	C <sub>t</sub>	C <sub>b</sub>	K	%Y
A1	11.46	11.2	0.58	0.38	0.42	0.89	92.00
A2	10.98	11.4	0.67	0.3	0.37	0.81	72.64
A	10.63	14.5	0.99	0.31	0.23	1.32	75.85
A3	10.27	11.7	0.54	0.31	0.23	1.32	75.64
A4	8.84	12.3	0.44	0.33	0.37	0.91	90.01



**Figure 2: Binodial Curve of PEG 4000-ammonium sulphate-water system**

- e. Yield, Specific Activity and Purification Fold:** The specific activity of Chox obtained in the top phase of PEG 4000-ammonium sulphate-water system with highest partition coefficient (1.32) was 65.21 U/mg with 2.34 fold purification and 75.85% yield (Table 4). The specific activity and the yield so obtained clearly indicates the successful accomplishment of the PEG-salt two phase extraction technique by obtaining 2.34 fold purification in a single step.

## International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

**Table 4 Purification fold, yield and specific activity of partitioned cholesterol oxidase in PEG 4000-ammonium sulphate-water system**

Purification steps	Total Recovery		Specific Activity (U/mg)	Fold Purification	% Yield
	Protein (mg)	Enzyme (Units)			
Crude	0.148	4.13	27.91	-	-
Partitioning	0.048	3.13	65.21	2.34	75.85



**Figure 3: PEG 4000-Ammonium Sulphate ATPS: equilibrated phase volume ratio**

### V. CONCLUSION

ATPS composed of 10.63% (w/w) PEG 4000 and 14.5% (w/w) ammonium sulphate is the most suitable two phase system for the partitioning of Chox among the eight different ATPS studied. A low cost purification technique was developed for the Chox obtained from *S. olivaceus* MTCC 6820. PEG-ammonium sulphate-water two phase can be scaled-up effectively because of its biocompatibility for proteins due to aqueous rich milieu, low cost of the salt used and easy handling. Partitioning studies for Chox in PEG-salt-water system proved to be a cost effective method of downstream processing as compared to the PEG-dextran-water two phase systems.

### ACKNOWLEDGEMENT

Authors are thankful to the School of Biochemical Engineering, Indian Institute of Technology (Banaras Hindu University), Varanasi, India for providing the facilities to carry out this research work. Mrs. Shraddha Sahu is also thankful to the MHRD, Govt. of India for providing financial assistance in the form of Teaching Assistant.

### REFERENCES

1. S. K. Pandey and R. M. Banik, "Extractive fermentation for enhanced production of alkaline phosphatase from *Bacillus licheniformis* MTCC 1483 using aqueous two phase systems", *Bioresource Technology*, vol. 102, pp. 4226-4231, 2011.
2. J. Singh and N. Verma, "Partition of glucose oxidase from *Aspergillus niger* in aqueous two phase systems based on salt and polyethylene glycol", *Brazilian Archives of Biology and Technology*, vol. 53, pp. 1051-1056, 2010.
3. P. Singh and R. M. Banik, "Partitioning studies of L-glutaminase production by *Bacillus cereus* MTCC 1305 in different PEG-salt/dextran", *Bioresource Technology*, vol. 114, pp. 730-734, 2012.
4. M. T. Yazdi, M. Zahraei, K. Aghaepour, N. Kamranpour, "Purification and partial characterization of a cholesterol oxidase from *Streptomyces fradiae*", *Enzyme and Microbial Technology*, vol. 28, pp. 410 - 414, 2001.
5. C. C. Allain, L. S. Poon, C. S. G. Chan, W. Richmond and P.C. Fu, "Enzymatic determination of total serum cholesterol", *Clinical Chemistry*, vol. 20, pp. 470-475, 1974.
6. J. MacLachlan, A. T. L. Wotherspoon, R. O. Ansell and C. J. W. Brooks, "Cholesterol oxidase: sources, physical properties and analytical applications", *Journal of Steroidal Biochemistry and Molecular Biology*, vol. 72, pp. 169-195, 2000.

# International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: [www.ijirset.com](http://www.ijirset.com)

Vol. 8, Issue 2, February 2019

7. M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Analytical Biochemistry*, vol. 72, pp. 248-54, 1976.
8. J. C. Merchuk, B. A. Andrews, J. A. Asenjo, "Aqueous two-phase systems for protein separation: Studies on phase inversion", *Journal of Chromatography B*, vol. 711, pp. 285-293, 1998.
9. J. MacLachlan, A. T. L. Wotherspoon, R. O. Ansell and C. J. W. Brooks, "Cholesterol oxidase: sources, physical properties and analytical applications", *Journal of Steroidal Biochemistry and Molecular Biology*, vol. 72, pp. 169-195, 2000.
10. J. G. T. Salva, A. M. Liserre, A. L. Moretto, M. A. T. Zullo, G. Ventrucci, and T. J. B. Menezes, "Some enzymatic properties of cholesterol oxidase produced by *Brevibacterium sp.*", *Revista de Microbiologia*, vol. 30, pp. 315-323, 1999.
11. P. H. Lolekha and Y. Jantaveesirirat, "*Streptomyces*: a superior source for cholesterol oxidase used in serum cholesterol assay", *Journal of Clinical Laboratory Analysis*, vol. 6, pp. 405-409, 1992.
12. N. E. A. El. Naggar, S. F. Deraz, H. M. Soliman, N. M. El. Deeb and N. M. El. Shweihy, "Purification, characterization and amino acid content of cholesterol oxidase produced by *Streptomyces aegyptia NEAE 102*", *BMC Microbiology*, vol. 17, pp. 76, 2017.
13. H. I. Rhee, K. J. Jeong, B. K. Park, Y. S. Choi and S. Y. Lee, "One-step purification of cholesterol oxidase from culture broth of a *Pseudomonas sp.* using a novel affinity chromatography method", *Journal of General Microbiology*, vol. 137, pp. 1213-1214, 1991.
14. I. Sasaki, H. Gotoh, R. Yamamoto, H. Tanak, K. Takami, K. Yamashita, J. Yamashita and T. Horio, "Hydrophobic-ionic chromatography: its application to microbial glucose oxidase, hyaluronidase, cholesterol oxidase and cholesterol esterase", *Journal of Biochemistry*, vol. 91, pp. 1555-61, 1982.
15. F. Rahimpour, R. Hatti-Kaul, G. Mamo, "Response surface methodology and artificial neural network modelling of an aqueous two-phase system for purification of a recombinant alkaline active xylanase", *Process Biochemistry*, vol. 51, pp. 452-462, 2016.
16. R. L. Souza, S. P. Ventura, C. M. Soares, J. A. Coutinho, Á. S. Lima, "Lipase purification using ionic liquids as adjuvants in aqueous two-phase systems", *Green Chemistry*, vol. 17, pp. 3026-3034, 2015.
17. L. Xavier, M. S. Freire, I. Vidal-Tato, J. González-Álvarez, "Application of aqueous two phase systems based on polyethylene glycol and sodium citrate for the recovery of phenolic compounds from *Eucalyptus wood*", *Maderas. Ciencia Y Tecnología*, vol. 17, pp. 345-354, 2015.