5.1. Overview

Downstream processing is an integral part in the commercial production of enzymes and plays a very crucial role in the development of fermentation based products through the submerged fermentation process. Around 70-80% of the overall cost of industrial enzymes is incurred in downstream processing alone. One of the underlying reasons is the complexity of the conventional purification techniques which are multi-step processes having the constraints of time-consuming and labor-intensive. They usually end-up with relatively lower enzyme yield due to the loss of enzyme activity at each subsequent step, thus causing reduced recovery of the desired enzyme. Aqueous Two-Phase System (ATPS) may provide an appropriate alternative to the conventional method for purification of enzymes by preferentially partitioning the enzyme in one phase and interfering substances into other phase. ATPS has emerged as an effective green extraction technique for purification and recovery of a wide range of biological products such as enzymes, proteins, nucleic acids, amino acids, cellular organelles, and microorganisms from their respective contaminants and impurities (Rahimpour et al., 2016, Souza et al., 2015, Xavier et al., 2015).

The biocompatibility of ATPS offers an aqueous-rich milieu and a non-denaturing environment for proteins, which overcomes the limitation of techniques like ion exchange and affinity chromatography. The very low interfacial tension between the phases in ATPS results in high mass transfer, which allows the biomolecule to partition from one phase to another. Polyethylene glycol (PEG)-Salt-Water ATPS are mostly preferred because of their low cost and simplicity in handling of enzymes like alkaline phosphatase (Pandey and Banik, 2011), glucose oxidase (Singh and Verma 2010), protease (Lee and Chang, 1990;

Hotha and Banik, 1997), α-amylase (Stredansky et al., 1993) and β-galactosidase (Kuboi et al., 1995), etc. There was no report on the purification of Chox from *S. olivaceus* MTCC 6820 using different ATPS.

ATPS offers a low-cost technology for the purification of proteins and enzymes with ease of scale-up. Chox is a bacterial enzyme produced exclusively through the microbial fermentation process. It is a high-value product with a low yield having enormous industrial importance and biotechnological applications. The production media for Chox contains a large amount of yeast extract, peptone, soyabean meal, and salt. The purification of Chox has been described by many conventional techniques like ion-exchange chromatography (IEX) (El Naggar et al., 2017), affinity chromatography (Rhee et al., 1991), and hydrophobic ionic chromatography (Sasaki et al., 1982). IEX is a sophisticated process involving precipitation, desalting, and protein binding to IEX resin in the column; elution and again desalting, which is often time-consuming and labor-intensive. 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 purification technology with reduced processing time and energy.

An effective downstream processing involving aqueous two-phase extraction would increase the recovery and yield, possibly reducing the cost of the enzyme. The objective of the present work is to study the partitioning of Chox produced by *S. olivaceus MTCC 6820* in different PEG-salt-water two-phase systems in order to find out the most suitable PEG-salt-water ATPS for Chox partitioning. ATPS was developed using PEGs of two different molecular weights (PEG-4000 and PEG-6000) and four different salts (dipotassium phosphate, sodium citrate, sodium sulfate and ammonium sulfate).

5.2. Experimental

5.2.1. Chemicals used

Polyethylene glycol (PEG) of molecular weights 4000 and 6000 were purchased from Sisco Research Laboratories, Mumbai, India. Ammonium sulfate, potassium phosphate, sodium sulfate, and tri-sodium citrate were purchased from Qualigens, Mumbai, India. All chemicals and media components used were of analytical grade.

5.2.2. Crude extract of Cholesterol oxidase

The supernatant obtained by centrifugation of the fermented broth at 12,000 rpm (4ºC) for 20 min was collected as crude Chox and it was concentrated by rotary vacuum evaporator (Vaiometra, RE 2000A, Shanghai Ya Rong, China) to remove the excess of water in the broth. This concentrated crude Chox was directly used for further purification using PEG-salt ATPS.

5.2.3. Enzyme 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 1.5 mM 4-aminoantipyrine, 5 mM phenol, 10 U/ml horseradish peroxidase and sodium phosphate buffer (20 mM, pH 8.0) and pre-incubated at 30ºC 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 30ºC 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 the discontinuous spectrophotometric method. Blank was prepared by taking the ATPS (top/bottom phase)

samples with no enzyme loaded on them. The enzyme activity in the top phase was measured against the enzyme blank of the 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 µmol of hydrogen peroxide (0.5 µmol of quinoneimine dye) per minute at 30ºC and pH 8.0.

5.2.4. 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 crude Chox. The standard curve of BSA was prepared in the concentration range 0.01 to 0.1 mg/ml and the absorbance was recorded at 595nm (Bradford, 1976). The standard curve of BSA using Bradford's reagent is shown in Appendix-1.

5.2.5. Preparation of Phase Diagram

ATPS comprising eight different compositions of PEG X (X = 4000, 6000) and salts (ammonium sulfate $(NH_4)_2SO_4$), dipotassium phosphate (K_2HPO_4), sodium sulfate (Na_2SO_4), and tri-sodium citrate ($Na_3C_6H_5O_7$) were formed and characterized with the help of binodal curve, tie-line and phase volume ratio (V_t/V_b). Phase diagram of ATPS was constructed by stepwise titration of salts (25%w/w) with fixed amounts of (35%w/w) PEG till turbid endpoint and diluted with water till the disappearance of turbidity.

Merchuk's equation was used for plotting the binodal curve for all the PEG-salt-water systems; equation. (5.1) (Merchuk et al., 1998).

$$Y = 71.1\exp\left(-0.62X^{0.5} - 0.00123X^{3}\right) \quad \text{..... (5.1)}$$

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 6.2 to 6.5) and four variables (Y_T, Y_B, X_T and X_B) (Merchuk et al., 1998) using the Solver tool in Microsoft Excel®.

$$Y_T = A\exp\left[\left(BX_T^{0.5}\right) - \left(CX_T^3\right)\right] \quad (5.2)$$

$$Y_B = A\exp\left[\left(BX_B^{0.5}\right) - \left(CX_B^3\right)\right] \quad (5.3)$$

$$Y_T = \left(\frac{Y_M}{\propto}\right) - \left[\left(\frac{1-\propto}{\propto}\right)\right] Y_F \quad (5.4)$$

$$X_T = \left(\frac{X_M}{\propto}\right) - \left[\left(\frac{1-\propto}{\propto}\right)\right] X_F \quad (5.5)$$

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 sulfate, and tri-sodium citrate), Y is the weight fraction of PEG X (X= 4000, 6000) and α is the ratio between mass of the top phase and mass of the mixture.

The tie lines were obtained by solving equations (5.2) to (5.5) and are given by equation (5.6) (Merchuk et al., 1998):

$$Y = Yo + SX \quad (5.6)$$

where, the slope S is given by equation (5.7):

$$STL = \frac{Y_T - Y_B}{X_T - X_B} \quad (5.7)$$

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 (5.7) (Merchuk et al., 1998).

5.2.6. Partitioning of Cholesterol oxidase in different PEG-Salt-Water ATPS

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 1 h leading to complete phase separation. The top and bottom phases were separated carefully using a Pasteur pippete, analyzed for the Chox activity (U/ml) and partition coefficients were determined.

5.2.6.1. Partition Coefficient (K)

Partition coefficient is the ratio of the concentration of Chox in the top and bottom phases and is given by equation (5.8).

$$K = \frac{C_T}{C_B} \qquad (5.8)$$

where, C_T is the activity of Chox in top phase and C_B is the activity of Chox in bottom phase.

5.2.6.2. 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 (5.9):

$$Y(\%) = \frac{\text{Chox activity in top phase} \times 100}{\text{Total Chox activity added to the system}} \qquad (5.9)$$

$$SA\ (\text{Units/mg}) = \frac{\text{Enzyme activity (Units/ml)}}{\text{Total Protein (mg/ml)}} \qquad (5.10)$$

$$\text{Purification Fold} = \frac{\text{SA of Partitioned enzyme}}{\text{SA of Crude extract}} \qquad (5.11)$$

5.2.7 Determination of molecular weight by gel electrophoresis

The enzyme purity and molecular weight of the purified Chox were determined by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method described by Laemmli (Laemmli, 1970). The purified enzyme was mixed in a

solution containing SDS 2% (w/v), β-mercaptoethanol 4% (v/v), glycerol 20% (v/v), and Tris-HCl (10mM, pH – 7.2), and heated in a boiling water bath for 3 – 4 min. Electrophoresis was carried out in the acrylamide gel (resolving gel 12% and stacking gel 5% with 0.1% SDS) at 25mA current and 25˚C. After electrophoresis, the gel was carefully removed from the gel plates and the proteins were stained with Coomassie Brilliant Blue R-250. Destaining was carried out with a solution of methanol, acetic acid, water (2:1:7) till the protein bands were visible. The molecular weight of the enzyme was determined by referring to the Midrange 3 protein marker (14 - 95 kDa) obtained from BioLit, SRL, Mumbai, India.

5.3 Results and Discussion

ATPS was 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 sulfate, di-potassium phosphate, tri-sodium citrate, and sodium sulfate) were used for partitioning of Chox (**Table 5.1 and 5.2)**. PEG 4000-ammonium sulfate-water system was found to be the most suitable ATPS for Chox partitioning.

5.3.1. Binodal Curve

The binodal curve of eight different ATPS using PEG X (X = 4000 and 6000) and four inorganic salts (ammonium sulfate, potassium phosphate, tri-sodium citrate, and sodium sulfate) have been presented in **Fig 5.1 (A)** and **Fig 5.1 (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.

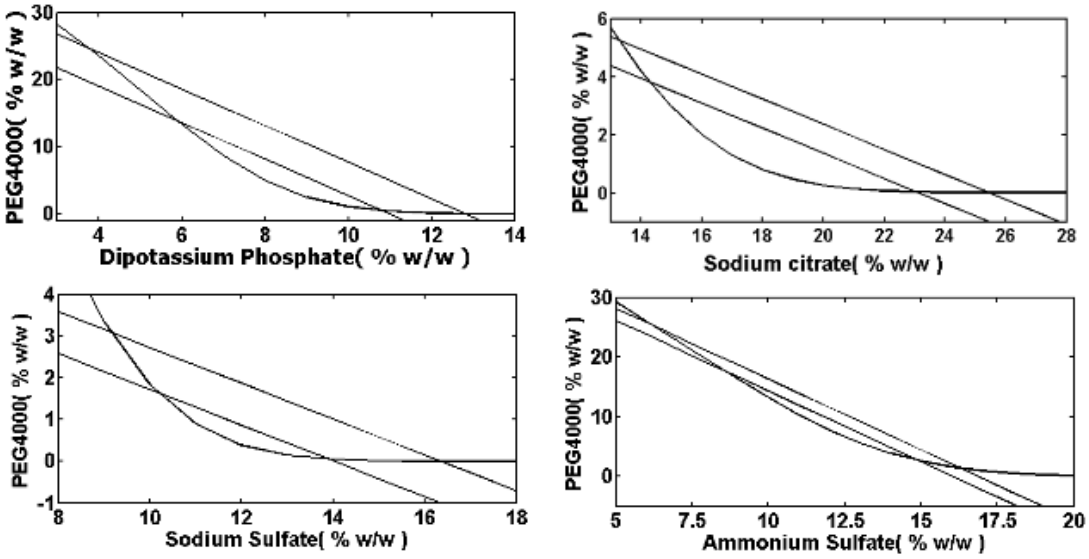

Fig 5.1 (A) Binodal Curve of PEG 4000-salt-water ATPS

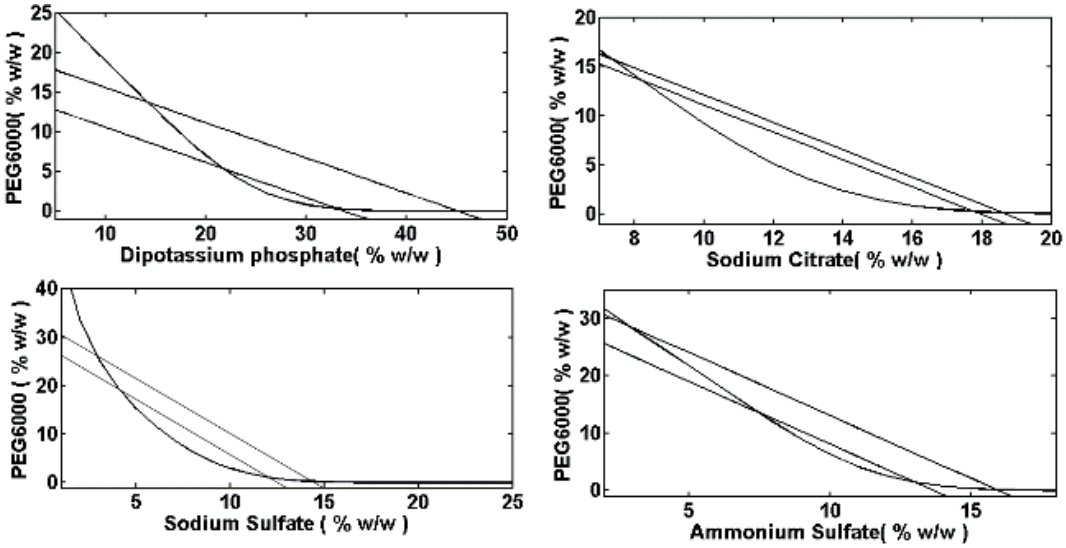

Fig 5.1 (B) Binodal Curve of PEG 6000-salt-water ATPS

5.3.2. 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 5.1 and 5.2**. PEG 4000 [10.63% (w/w)]-ammonium sulfate [14.5% (w/w)]-water was found to be the most suitable system component for partitioning of Chox with highest partition coefficient 1.32 ± 0.58 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, labor intensive, and costlier conventional technique.

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, polyvinyl 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 sulfate system, at a much lower cost than the PEG - polymer system. The binodal curve of PEG 4000-ammonium sulphate-water system has been displayed in **Fig 5.2**.

Table 5.1 Partitioning of Cholesterol oxidase from *Streptomyces olivaceus* using PEG 4000-salt-water ATPS

System	% PEG	Salt	V_t/V_b	Chox Activity (U/ml)		K
				Top (C_t)	Bottom (C_b)	
PEG4000 - $(NH_4)_2SO_4$	10.63	14.5	0.99	0.3 ± 0.03	0.23 ± 0.04	1.32 ± 0.58
PEG4000 - K_2HPO_4	10	10	0.28	0.31 ± 0.17	0.73 ± 0.35	0.43 ± 0.34
PEG4000 - $Na_3C_6H_5O_7$	14.45	36	0.48	0.27 ± 0.02	0.33 ± 0.1	0.84 ± 0.30
PEG4000 - Na_2SO_4	3.15	16	0.13	0.66 ± 0.30	0.67 ± 0.03	0.88 ± 0.36

All the experiments were performed in triplicate and the results were presented in terms of mean and standard deviation.

Table 5.2 Partitioning of Cholesterol oxidase from *Streptomyces olivaceus* using PEG 6000-salt-water ATPS

All the experiments were performed in triplicate and the results were presented in terms of mean and standard deviation.

System	% PEG	% Salt	V_t/V_b	Chox Activity (U/ml)		K
				Top (C_t)	Bottom (C_b)	
PEG6000- $(NH_4)_2SO_4$	14.1	9.5	0.79	0.52 ±0.04	0.41±0.05	14±0.050
PEG6000-K_2HPO_4	20	15	0.2	0.22±0.04	0.94±0.04	0.24±0.45
PEG6000- $Na_3C_6H_5O_7$	11.93	13	0.45	0.36±0.05	0.45±0.05	0.76±0.29
PEG6000- Na_2SO_4	25	4	3.22	0.43±0.06	0.48±0.07	0.89±0.33

5.3.3. Effect of PEG molecular weight on partitioning of Cholesterol oxidase

PEG 4000-salt-water systems showed greater partition coefficients as compared to PEG 6000-salt-water systems **(Table 5.1 and 5.2)**. 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 sulfate, dipotassium phosphate, and trisodium citrate were 0.99, 0.28, and 0.48 respectively while it was 0.79, 0.2, and 0.45 in case of PEG 6000-ammonium sulfate, dipotassium phosphate and tri-sodium citrate systems respectively. While exceptionally, the phase volume ratios of PEG 4000 - sodium sulfate system and PEG 6000 – sodium sulfate 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. Therefore, low molecular weight PEG resulting in higher partition coefficient and phase volume ratio was preferred for partitioning of Chox.

5.3.4. Effect of phase-volume ratio on the partitioning of Cholesterol oxidase in PEG 4000-ammonium sulfate-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 sulfate at point A was found to have an optimum phase volume ratio of 0.99, as displayed in **Table 5.3 and Fig 5.2**.

Fig 5.3 displays the picture of equilibrated PEG 4000-ammonium sulfate system with phase volume ratio (V_t/V_b) 0.99.

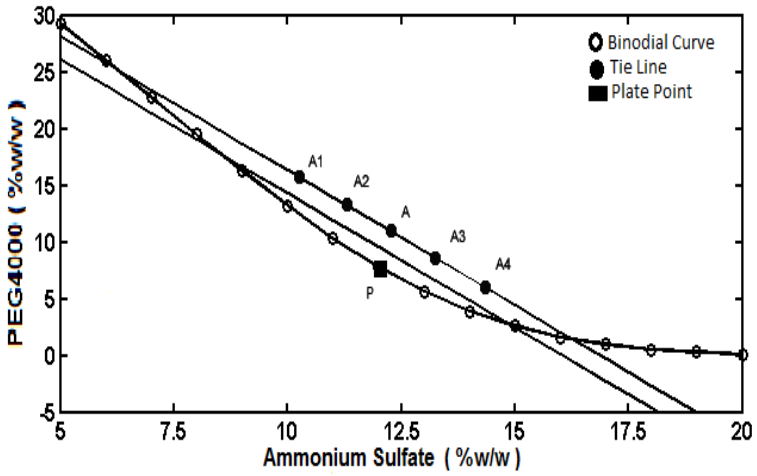

Fig 5.2: Binodal curve of PEG 4000-ammonium sulfate-water system

Table 5.3 Effect of phase volume ratio on Cholesterol oxidase partitioning in PEG 4000-ammonium sulfate-water system

System	%PEG 4000	$\%NH_4(SO_4)_2$	V_t/V_b	C_t	C_b	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

All the experiments were performed in triplicate and the results were presented in terms of mean and standard deviation.

Figure 5.3: PEG 4000-Ammonium Sulfate ATPS: equilibrated phase volume ratio

5.3.5. Yield, Specific Activity and Purification Fold

The specific activity of Chox obtained in the top phase of PEG 4000-ammonium sulfate-water system with the highest partition coefficient (1.32) was 65.21 U/mg with 2.34 fold purification and 75.85% yield **(Table 5.4)**. The specific activity and the yield so obtained clearly indicate the successful accomplishment of the PEG-salt two-phase extraction technique by obtaining 2.34 fold purification in a single step.

Table 5.4 Purification fold, yield and specific activity of partitioned Cholesterol oxidase in PEG 4000-ammonium sulfate-water system

Purification steps	**Total Recovery**		**Specific Activity (U/mg)**	**Fold Purification**	**% Yield**
	Protein[a] (mg)	**Enzyme[b] (Units)**			
Crude extract [c]	0.148	4.13	27.91±0.35	1	100
Partitioning	0.048	3.13	65.21±0.29	2.34±0.07	75.85±0.04

[a] Protein was determined by Bradford assay
[b] Cholesterol oxidase activity was assayed by measuring the H_2O_2 generated
[c] Crude extract was obtained from the culture supernatant of *S. olivaceus MTCC 6820*
five replicates of the each partitioning experiment were carried out independently and the results are displayed in terms of mean and standard deviation.

5.3.6 Determination of molecular weight by gel electrophoresis

The molecular weight of the purified Chox was determined by the SDS-PAGE as per the method described by Laemmli (Laemmli, 1970). **Figure 5.4 (a) and (b)** shows the enzyme purity achieved from the purification of Chox with the PEG-Ammonium sulfate-Water ATPS partitioning. The electrophoretic mobility of proteins and the molecular weights of the standard protein marker having molecular weight (MW) range 14- 95 kDa were photographed.**Figure 5.4(a)** displays the SDS-PAGE of the crude extract (concentrated by rotatory vacuum evaporator) and **Figure 5.4 (b)** displays the SDS-PAGE of Chox obtained through ATPS partitioning in the top-phase of the PEG- Ammonium sulfate-Water ATPS (lanes 1- 4). Lanes 1, 2, 3, and 4 (5µg, 10µg, 15µg, and 20µg protein were loaded in each well, respectively) revealed a single sharp protein band of the purified Chox, which signifies the purity of the protein. The purified Chox enzyme has an apparent molecular mass ~ 60 kDa, portrayed in **Figure 5.4 (b)**. The molecular weights of most of the Chox have been reported in the range of 47- 61 kDa (Doukyu, 2009; Yehia et al, 2015; El-Naggar et al, 2017).

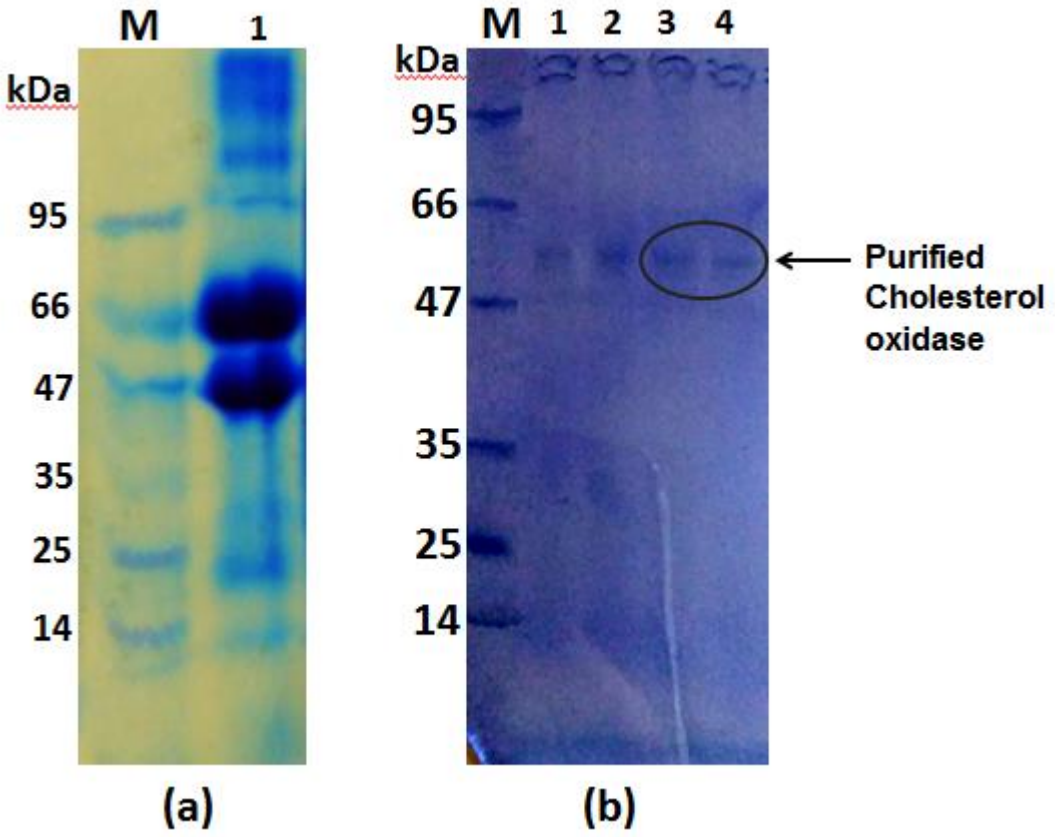

Figure 5.4 SDS-PAGE of cholesterol oxidase (a) Lane M: Standard Protein Marker (MW range 14-95 kDa); Lane 1: Crude extract (50µg) (concentrated by rotatory vacuum evaporator) **(b)** Lane M: Standard Protein Marker (MW range 14-95 kDa); Lane 1-4 (5µg, 10µg, 15µg, and 20µg respectively) samples of cholesterol oxidase purified by PEG - Ammonium sulfate - Water ATPS. The gel was stained with Coomassie brilliant blue R-250.

5.3.7 Comparison of aqueous two-phase technology with other methods of Cholesterol oxidase purification

A number of different techniques have been used for the purification of Chox from different microbial sources. These techniques include ion-exchange chromatography, affinity chromatography, gel-filtration chromatography, the combination of ammonium sulfate precipitation with the ion-exchange or gel-filtration chromatography, and the combination of ion-exchange with affinity chromatography. An effective comparison between the different techniques can be made by using specific activity and yield (%). In many literatures, the cost-effectiveness of the purification process on large scale has also been effectively measured based on the number of steps involved in purification; the greater the number of steps greater will be the cost and time required for purification (Jiang, 2017; Naganagouda and Mulimani, 2008). A comparison of ATPS technology for Chox purification with the other techniques with respect to enzyme yield, specific activity, and purification fold has been tabulated in **Table 5.5**. In addition, fewer steps enable easy handling and scale-up. A comparative flowchart illustrating the number of steps involved in different purification techniques and ATPE of Chox has been outlined in **Figure 5.5**. In the present study, in case of aqueous two-phase extraction, the number of steps involved in achieving the final purification of Chox is three whereas other methods of purification involve a minimum of six steps. It is an established fact that in the scaling-up of any purification process, the cost is directly proportional to the number of purification steps involved. On the basis of these observations, ATPS might be a cost-effective alternative that also offers ease of handling and scale-up.

Table 5.5 Comparative study of Aqueous Two-Phase Extraction with other methods of Cholesterol oxidase purification

S. No	Organism name	Method of purification of Chox	Steps involved	Specific activity (U/mg)	Purification fold	Yield (%)	Reference
1.	*Pseudomonas sp. strain ST-200*	Ion-exchange chromatography and Gel filtration	Supernatant Ammonium Sulfate precipitation DEAE-cellulose Butyl-Toyopearl Sephadex G-100	0.42 0.40 5.9 11.1 **15.2**	1 1 14 26 **36**	100 64 55 33 **20**	Doukyu and Aono, 1998
2.	*Streptomyces fradiae*	Ion-exchange chromatography and Gel-filtration	Culture filtrate Ammonium sulfate precipitation DEAE-cellulose column Sephadex G-200	0.20 1.78 3.06 11.21	- - - -	100 74.5 50.3 27.5	Ouf et al, 2012
3.	*Streptomyces sp.*	Affinity Chromatography	Fermented broth supernatant Ammonium sulphate ppt Affinity Chromatography	1.54 6.50 **21.8**	1 4.22 **14.3**	100 78.0 **31.2**	Niwas et al., 2013
4.	*Enterococcus hirae*	Gel filtration Chromatophy	Crude Ammonium sulfate precipitation Sephadex G-100	53.30 94.40 **124.87**	1 1.8 **2.3**	100 105.70 **79**	Yehia et al., 2015
5.	*Streptomy Ces aegyptia NEAE 102*	Ion Exchange Chromatography	Culture filterate Ammonium sulphate DEAE Sepharose CL-6B	5.12 20.11 **16.08**	1 3.92 **3.14**	100 6.23 **2.56**	El-Naggar et al., 2017
6.	*Chromobacterium sp. DS1*	Affinity Chromatography	Crude extract Ni-CAM Affinity chromatography	4.6 **14.3**	1 **3.1**	100 **82.5**	Fazaeli etal., 2019
7.	***S. olivaceus MTCC 6820***	**Aqueous Two Phase System**	**Crude PEG 4000 - Ammonium sulfate aqueous two phase system**	27.91 **65.21**	1 **2.34**	100 **75.85**	**Present study**

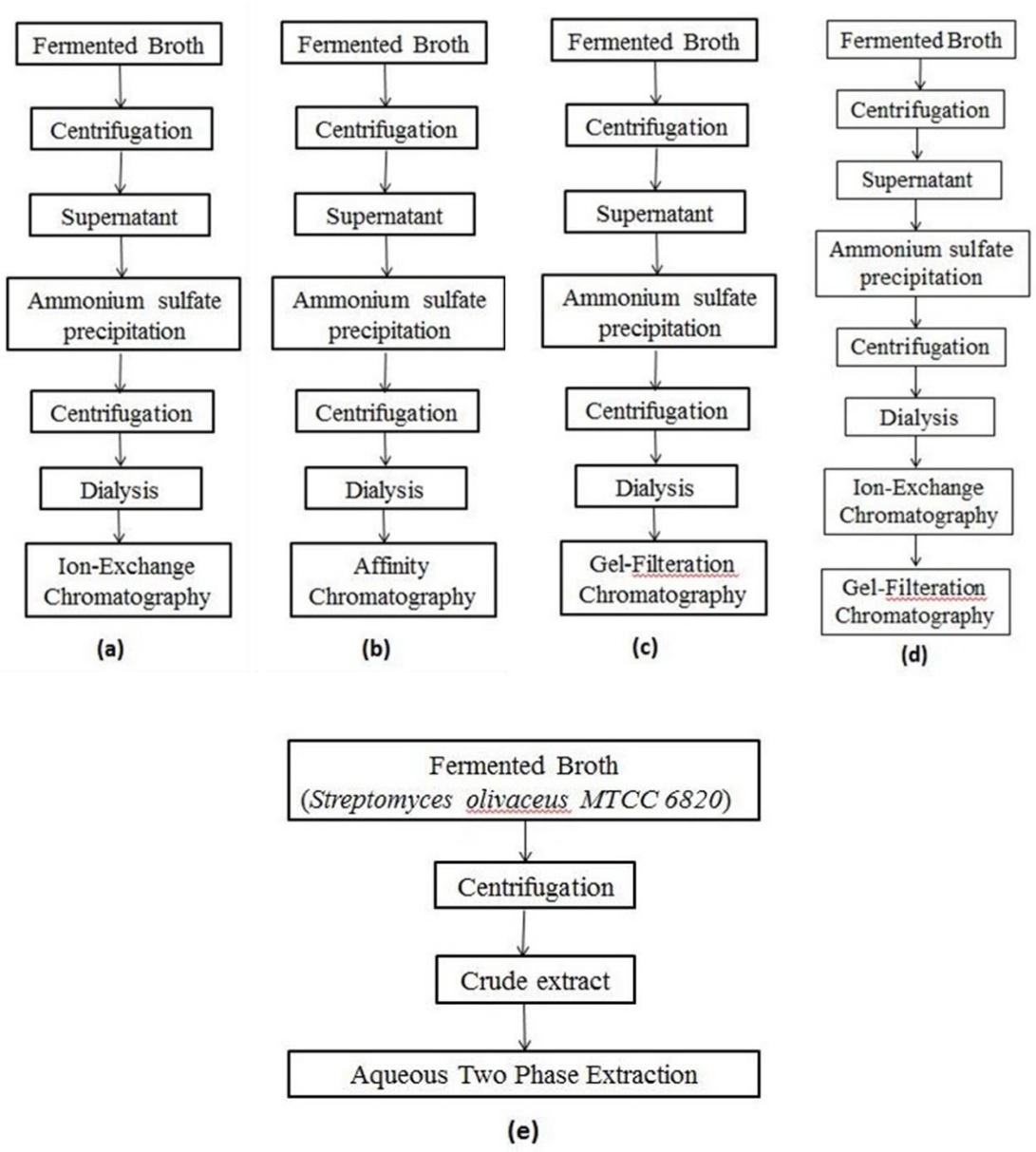

Figure 5.5 A comparison of the steps involved in different techniques used for Cholesterol oxidase purification (a) Ion-exchange Chromatography (b) Affinity Chromatography (c) Gel-Filteration Chromatography (d) Ion-exchange chromatography in combination with Gel-filteration chromatography (e) Aqueous Two Phase Extraction of Cholesterol oxidase

5.4. CONCLUSION

ATPS composed of 10.63% (w/w) PEG 4000 and 14.5% (w/w) ammonium sulfate 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 sulfate-water two-phase system 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 might be a cost-effective alternative method of downstream processing as compared to the PEG-dextran-water two-phase systems and other conventional techniques of Chox purification. Thus, it may be concluded that PEG-salt-water ATPS can be applied as an alternative bio-separation technique for the purification of Chox from microbial sources (such as *Streptomyces sp.* and other *actinomycetes*) and/or can be integrated with bioprocessing units such as fermentation as well as other purification methods. The only shortcoming about ATPS may be the PEG removal from the partitioned enzyme, which might be overcome by integrating ATPS with other techniques like enzyme immobilization for the recovery of biomolecules viz. enzymes.