## 8.1. Overview

The recovery of biomolecules viz. proteins or enzymes from the PEG-rich top phase or the salt-rich bottom phase is a bottleneck in the aqueous two-phase extraction technology for the purification of enzymes and proteins. The PEG-rich phase is viscous and maintains a hydrophobic environment along with the aqueous rich milieu, due to which PEG removal is not easy through any membrane using ultra-filtration. However, some researchers have suggested the use of the back extraction technique (Rahimpour et al., 2016) for the recovery of enzyme protein after partitioning in the aqueous two-phase system. Some other approaches used for the removal of PEG contaminants from proteins and peptides have been documented viz. use of titanium dioxide (Zhao and O'Connor, 2007), gel filtration chromatography, ion-exchange chromatography, etc. All the above-mentioned techniques are multistep processes to reach up to a certain level of purity, as it involves the removal of undesirable substances to achieve the maximum yield of desired protein; which further adds cost to the downstream processing of the target protein/enzyme. Dialysis may also be one of the methods for the removal of PEG from the partitioned Chox, but one major problem is that the protein gets diluted in this process and where the protein again needs to be concentrated using certain membrane concentrators. So, despite removing undesirable substances, one possible approach is the recovery of desired target protein from the mixture. This can be achieved by immobilization of the partitioned enzyme onto some solid support directly from the top/bottom phase.

In this chapter, a novel approach for the recovery of enzyme Chox partitioned in an aqueous two-phase system (discussed in chapter 5) has been studied for its possible application in the determination of total serum cholesterol. In order to avoid the intricacy

of the process and cost added to recover the Chox after partitioning in PEG-Ammonium Sulfate-Water system (section 5.3.2), an easy one-step method of immobilization of Chox enzyme onto chitosan beads were developed. This strategy of Chox recovery from an aqueous two-phase system has two advantages; firstly, the inexpensive recovery of Chox from the two-phase by immobilization onto chitosan beads, and secondly, the Chox loaded on chitosan beads were evaluated for their suitability in the estimation of total serum cholesterol. This approach is novel because it needs no further purification of the Chox enzyme for any application study. This approach of Chox recovery from two-phase system is first time studied in this chapter and to the best of my knowledge, no reports on such study have been documented to date.

## 8.2. Experimental

### 8.2.1. Chemicals used

Cholesterol was purchased from Sigma Aldrich Pvt. Ltd. Chitosan (deacetylation degrees higher than 85%), Cholesterol esterase, Cholesterol oxidase, and Horseradish Peroxidase were purchased from Sisco Research Laboratories, Mumbai, India. All chemicals used were of analytical grade.

### 8.2.2. Preparation of glutaraldehyde treated chitosan beads

The Chox purified by PEG-Ammonium Sulfate-Water ATPS was recovered from the top phase through immobilization onto chitosan beads. The chitosan solution was prepared by dissolving chitosan (2% w/v) in acetic acid (1.5% w/v) under constant stirring overnight. Spherical beads of chitosan were prepared with the help of a syringe, by dropwise precipitation of chitosan solution into a beaker 500 ml containing 1M NaOH. The beads were allowed to stir with NaOH solution for at least 2 – 4 h for hardening of the

beads. The beads were washed with deionized water till neutral pH was obtained. The chitosan beads were transferred to glutaraldehyde solution (2.5% v/v) for crosslinking reaction and continued to incubate for 6 – 8 h. **Fig 8.1 (a)** displays the as prepared chitosan beads treated with glutaraldehyde. The glutaraldehyde treated chitosan beads were washed extensively with deionized water for the removal of excess glutaraldehyde.

### 8.2.3. Recovery of Cholesterol oxidase from top phase of ATPS through immobilization onto chitosan beads

In chapter 5, **Table 5.1** it was described that the partition coefficient of PEG-Ammonium sulfate-Water system was 1.32 ± 0.58, which means the partitioning of Chox showed more affinity towards the PEG-rich top phase and was accumulated more in the top phase **(Fig 5.3)**. Therefore, the as-prepared glutaraldehyde treated chitosan beads (20 mg) were added to 10 ml of the top phase and kept at stirrer overnight for the immobilization of Chox enzyme present in the ATPS onto the surface of glutaraldehyde activated chitosan beads. **Fig 8.1 (b)** displays the Chox immobilized chitosan beads. This method was eventually adapted for the recovery of Chox from the PEG-rich phase of the ATPS. The schematic representation of the steps followed for the recovery of Chox and its immobilization onto glutaraldehyde-activated chitosan beads is shown in **Fig. 8.2**. Chox immobilization onto chitosan beads was confirmed by the activity measurement of Chox using 4-aminoantipyrine, phenol, and peroxidase, and immobilization efficiency was calculated by eq.(8.1).

$$\text{Immobilization Efficiency (\%)} = \left(\frac{\text{Activity of Enzyme bead}}{\text{Initial Activity of the top phase}}\right) \times 100 \quad \ldots\ldots\ldots (8.1)$$

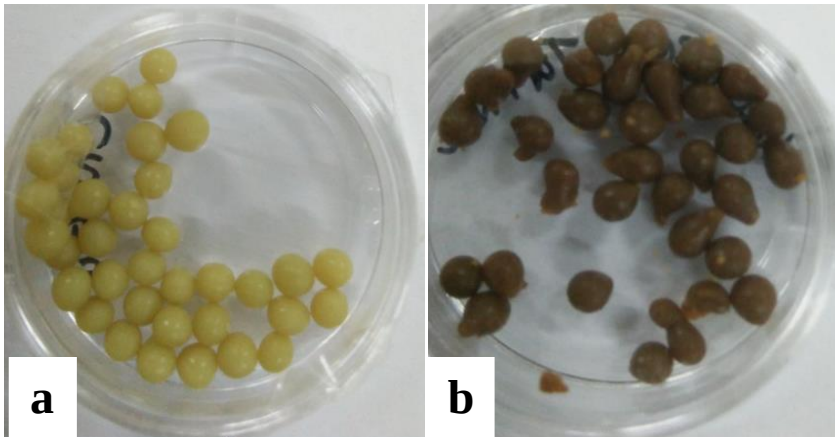



**Fig 8.1 Chitosan beads (a) Glutaraldehyde activated bare chitosan beads (b) Cholesterol oxidase immobilized from the top phase of ATPS**

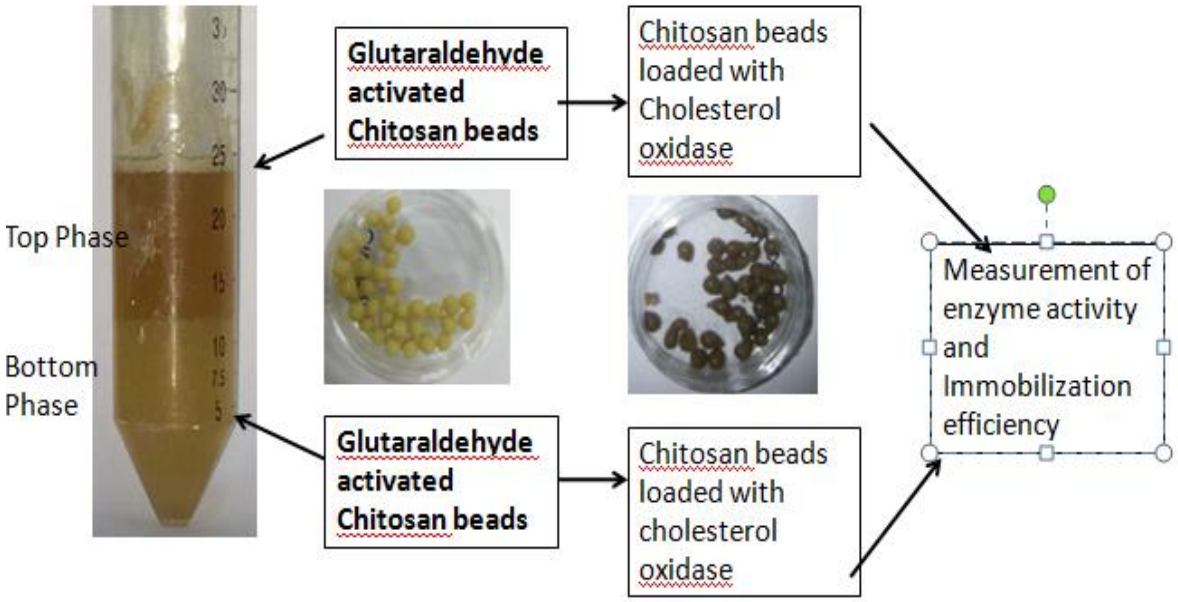



**Fig 8.2 Schematic representations of the steps followed for the recovery of Cholesterol oxidase and its immobilization onto glutaraldehyde activated chitosan beads**

### 8.2.4. Estimation of Cholesterol oxidase enzyme activity in Cholesterol oxidase immobilized chitosan beads

Chox activity in immobilized chitosan beads was estimated by the 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 (HRP) and sodium phosphate buffer (20 mM, pH 8.0) and pre-incubated at 30ºC for 5 min. 5mg of Chox loaded beads were added to the above-mentioned reaction mixture and incubation continued at 30ºC for 10 min. The reaction was terminated by removing the enzyme-loaded beads and 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 buffer in place of test samples.

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.

### 8.2.5. Application of Cholesterol oxidase loaded chitosan beads for serum cholesterol assay

#### 8.2.5.1. Methodology

Determination of serum cholesterol by enzymatic endpoint method employs three enzymes:

- Cholesterol esterase (EC 3.1.1.13): hydrolyse cholesterol esters to free cholesterol
- Cholesterol oxidase (Chox) (EC 1.1.3.6): oxidizes free cholesterol to 4-cholesten-3-one and hydrogen peroxide

- Peroxidase (EC 1.11.1.7): catalyzes the oxidative coupling of hydrogen peroxide with 4-aminoantipyrine/phenol to form a quinoneimine dye, whose absorbance can be measured at 500 nm.

**8.2.5.2. Reagents used**

The stock reagent consisted of cholesterol esterase (from *Pseudomonas*) 0.4 U/ml, peroxidase 10 U/ml, 4-aminoantipyrine 1.5 mmol/l, phenol 10 mmol/l, potassium phosphate buffer pH 8.0; 0.1 mol/l and Triton X-100 2 ml/l.The concentration of Chox was optimized by preparing the working reagents with the addition of 0.1, 0.2, 0.3, 0.4, and 0.5 U/ml of Chox loaded beads in the above reagent base. For the optimization study, the stock standard of cholesterol (25.9 mmol/l) was prepared by dissolving 0.25g cholesterol in 25 ml of an ethanol diluent (absolute ethanol with Triton X-100, 10% v/v). The cholesterol working standards were prepared from the stock in the range of 2.6 to 25.9 mmol/l.

For reaction with human sera, cholesterol esterase (from *Pseudomonas*) 0.4 U/ml was added to the reagents containing optimal activity of *S. olivaceus* Chox.

**8.2.5.3. Sample collection**

4.5 ml blood samples (overnight fasting) were collected from the volunteer donors. Blood sample collection was performed by the paramedical technicians as permitted by the Chief Medical OfficerStudent's Health Care Centre, Banaras Hindu University, Varanasi (U.P). Serum was separated by centrifugation at 5000 rpm, 25°C for 15 min and stored at -20˚C. Serum samples were used as real-time samples for cholesterol detection by enzymatic endpoint assay method involving cholesterol esterase, Chox and peroxidase.

### 8.2.5.4. Optimization of Cholesterol oxidase concentration and linearity assessment

Chox concentration was optimized using cholesterol standard solutions in the range of 2.6 to 25.9 mmol/l. The absorbances versus cholesterol concentration obtained with increasing enzyme activities were plotted. The minimal Chox activity producing maximal cholesterol linearity was chosen.

For linearity assessment, a set of sera samples with cholesterol concentration ranging from 2.6 to 25 mmol/l was used and the absorbance versus cholesterol concentration was plotted. Linearity was evaluated as per the NCCSLS EPA-6 guideline and the linear region was reported as the reportable range (NCCLS, 2003).

## 8.3. Results and Discussion

### 8.3.1. Cholesterol oxidase recovery and immobilization efficiency

A novel method of recovery of the enzyme from the mixture of ATPS by the use of immobilization technique on a solid support was accounted. Although ATPS is a green technology for the purification and extraction of enzymes and proteins; still after partitioning, the removal of PEG from the top-phase of the ATPS for further purification of the enzyme is a challenging aspect. The high viscosity of PEG does not support further purification by ultra-filtration and the use of ion-exchange chromatography or back extraction of the enzymes from ATPS is a tedious and complicated process. In view of the above facts and observation, a novel method for the separation and recovery of the desired enzyme from the PEG-Salt mixture was employed, which not only would save energy and resources rather the enzyme is recovered in a more useful form.

The immobilization efficiency of immobilized Chox was found to be 60% and the residual activity in the top phase was again recovered by subjecting the bare

glutaraldehyde activated chitosan beads for immobilization of Chox. The SEM image of the chitosan beads before and after Chox immobilization is presented in **Fig 8.3 (a, b) and 8.4 (a, b).** Fig 8.3 (a) shows the diameter of the chitosan bead. Fig 8.3 (a) and Fig 8.4 (a) show the 100 X magnification of bare chitosan bead and Chox loaded chitosan bead with 1.7 mm and 1.362 mm diameter respectively. The reduction in bead size may be attributed to the hypertonic effect, which may have been caused due to the higher amount of PEG present in the PEG-rich top phase. **Fig 8.3 (b)** and **Fig 8.4 (b)** depicting the surface morphology of the chitosan bead before and after Chox immobilization respectively, also confirm the changes in the compactness of the bead surface after immobilization.

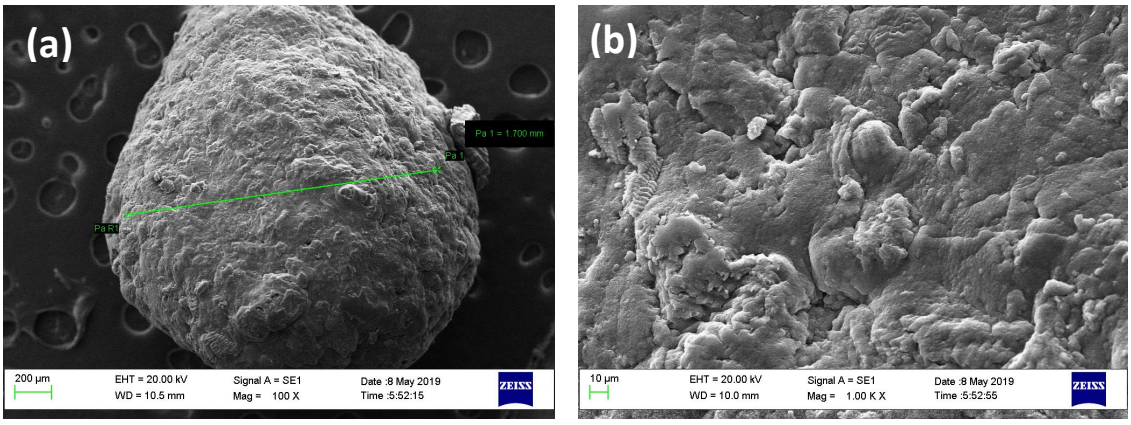
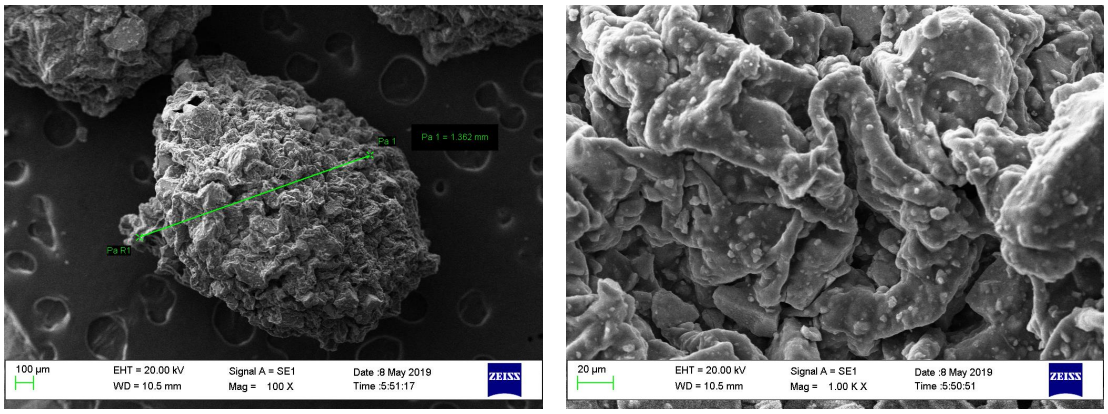



**Fig 8.3 (a) 100 X magnification of bare chitosan bead showing diameter of 1.7 mm before Cholesterol oxidase immobilization (b) 500 X magnification of bare chitosan bead showing surface morphology before Cholesterol oxidase immobilization**




**(a)** **(b)**

**Fig 8.4 (a) 100 X magnification of Cholesterol oxidase loaded bead showing the diameter of 1.362 mm after Cholesterol oxidase immobilization (b) 500 X magnification of Cholesterol oxidase loaded chitosan bead showing surface morphology after Cholesterol oxidase immobilization**

### 8.3.2. Serum assay

The Chox immobilized beads were used for the estimation of total cholesterol in human serum. The serum samples were used in order to assure the suitability of the Chox (purified from *S. olivaceus MTCC 6820*) for serum cholesterol assay. It was found that the reactions catalyzed by the Chox loaded chitosan beads were positive and the color of the test samples turned pink.

#### 8.3.2.1 Optimization of Cholesterol oxidase and linearity assessment

**Fig 8.5 (a)** shows the optimal activity of Chox obtained from *S. olivaceus MTCC 6820*. The reaction pattern of *S. olivaceus* Chox shows the hyperbolic curve. With the increasing Chox activities, the cholesterol reaction increased slightly. The maximal cholesterol linearity of Chox purified from *S. olivaceus* was 13mmol/l at 0.2 U/ml enzyme activity. Fig 8.5 (b) depicts the linearity of serum cholesterol obtained from the reagents

containing the optimal activity of *S. olivaceus* Chox immobilized onto chitosan beads. The linear range for cholesterol reagents containing *S. olivaceus* Chox was 2.6 to 15.5 mmol/l.

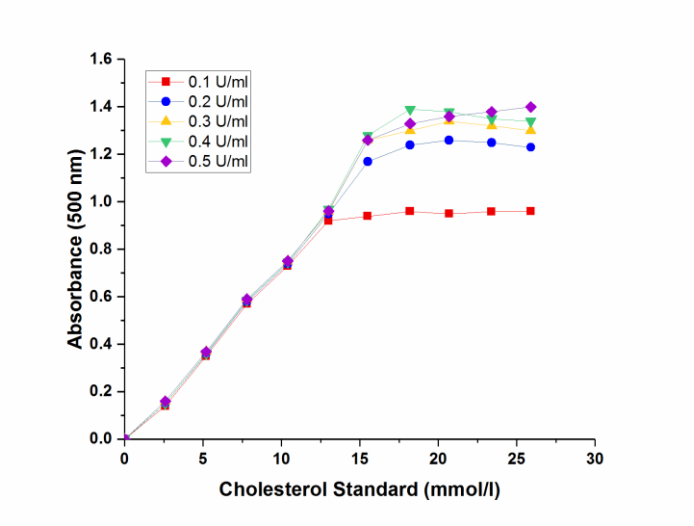



**Fig 8.5(a) Optimization of Cholesterol oxidase concentration obtained from *S. olivaceus MTCC 6820***

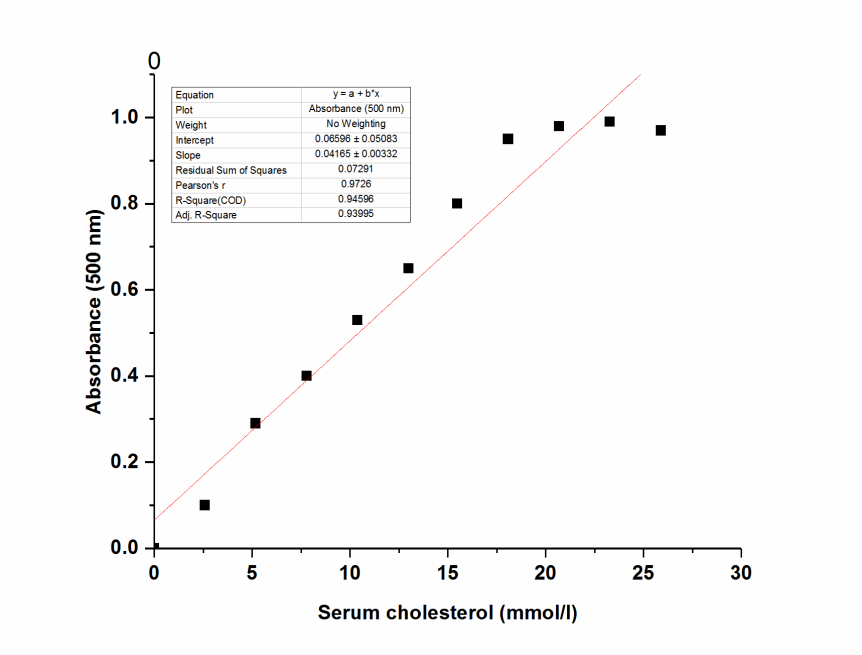



**Fig 8.5(b) Linearity of serum cholesterol obtained from *S.olivaceus MTCC 6820* immobilized onto chitosan beads**

## 8.4. CONCLUSION

A novel method for the extraction and recovery of Chox from the PEG-Ammonium Sulfate-Water ATPS was employed. No reports are available for such type of extraction and recovery methodology of enzyme from ATPS. Chox was successfully immobilized onto chitosan beads with the immobilization efficiency of 60% in the first batch of extraction. The application of Chox loaded chitosan beads for the estimation of serum cholesterol was successful, which proves that the Chox purified from *S. olivaceus MTCC 6820* is an analytically important enzyme suitable for estimation of total cholesterol in human serum with the linearity range of 2.6 – 15.5 mmol/l.