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Purification of lipase enzyme by using 4-vinyl pyridine based crosslinked poly(ionic liquid) networks

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Abstract: *Lipases are a class of water-soluble enzymes that act on water insoluble substrates and are stable in both polar and non-polar environments. Lipases catalyzed the ester hydrolysis, ester synthesis and transesterifications reactions. These have been used in the hydrolysis of oils and fats in an aqueous phase, or in an emulsion, or two separate phases these also used in esterifying various alcohols with fatty acids. Due to the effective catalytic activities lipases are used in different types of industries applications such as chemical, bakery, fats and oils, cosmetic, dairy, food dressing and cleaning industries. The purification process of lipases is very important and generally carried by using ion exchange chromatography (IEC) in which the column has many charged molecules that are securely bound to it by covalent bonds. In principal, one could use either a cation or an anion exchanger to bind to the target protein. The quaternized nitrogen containing polymers with alkylating agents increase their spectrum in many advanced applications and the preparation of poly (ionic liquids) [PILs] is one of such advancement. PILs are very attractive materials, and have largely used as anion exchangers. As the PILs are made of ions, therefore, these can also be used as packing material in IEC for the separation of products such as enzymes, nutritional proteins, and certain biopharmaceuticals. In PILs positively charged beads associate with, and therefore, exchange with negatively charged counter ions and act as anion exchangers, therefore, these can be used for the purification of enzyme such as lipase.*

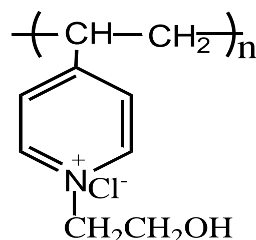
Key Words: *Lipase, purification, poly (ionic liquids), anion exchangers.*

1. INTRODUCTION:

Lipases are a class of water-soluble enzymes that act on water insoluble substrates and are stable in both polar and nonpolar environments. Lipases catalyzed the ester hydrolysis, ester synthesis and transesterifications reactions. These have been used in the hydrolysis of oils and fats in an aqueous phase, or in an emulsion, or two separate phases (1, 2). Lipases have been reported in esterifying various alcohols with fatty acids (3). Due to the catalysis of these reactions, lipases are used in different types of industries applications such as chemical, bakery, fats and oils, cosmetic, dairy, food dressing and cleaning industries.

Lipase has a large numbers of applications in almost all fields. Therefore, its purification is very important and generally carried by using ion exchange chromatography (IEC) In IEC the packing material (resin) of the column has many charged molecules that are securely bound to it by covalent bonds. IEC is a high resolution technique for separating proteins according to their charge. The most weakly charged compounds will elute first, followed by those with successively stronger charges. Because of the nature of the separating mechanism, pH, type of buffer, buffer concentration, and temperature all play important rolls in controlling the separation (4). The way that a protein interacts with the packing material depends on its overall charge and on the distribution of that charge over the protein surface. The net charge on a given protein will depend on the composition of amino acids in the protein and on the pH of the buffering solution. The charge distribution will depend on how the charges are distributed on the folded protein (5-7). In principal, one could use either a cation or an anion exchanger to bind to the target protein by selecting the appropriate pH. The quaternization of nitrogen containing polymers with alkylating agents increase their spectrum in many advanced applications and the preparation of poly (ionic liquids) [PILs] is one of such advancement. PILs are very attractive materials, and have largely used as surfactants (8,9), antimicrobial agents (10–13) CO_2 adsorbents (14,15) and anion exchangers (16).

As the PILs are made of ions, therefore, these can also be used as packing material in IEC for the separation of products such as enzymes, nutritional proteins, and certain biopharmaceuticals. In PILs positively charged beads associate with, and therefore, exchange with negatively charged counter ions and act as anion exchangers, therefore, these can be used for the purification of enzyme such as lipase. In this an attempt has been made to study the purification of lipase by using reported crosslinked 4-vinyl pyridine (4-VP) based PILs having bioactive choline analogous group shown below (17).



To have a series of crosslinked PILs the counter anion (Cl^-) of the prepared PIL was replaced with Br^-, OH^-, SH^-, NO_3, BF_4^- and CF_3COO^- by the simple metathesis reaction (17). The networks have positively charged beads that associated with exchangeable anion and therefore act as anion exchangers in lipase purification.

2. EXPERIMENTAL:

Lipase was obtained from the Department of Biotechnology, Himachal Pradesh University, Shimla, India, which was extracted from *Bacillus coagulans* BST-1 as follows. The isolate was screened earlier from the kitchen waste of a sweet shop and characterized as *Bacillus coagulans* BST-1. Seed culture was prepared by inoculating 100 mL of broth with loop full of culture. The culture was allowed to grow for 36h at 45°C under shaking condition at 180 *rpm* (Orbitik, shaking incubator). Thereafter, 36h old seed culture was used to inoculate 50 mL of the production medium taken in a 250 mL flask in replicates. The seeded broth was incubated at 45°C and 180 *rpm* for 48 h. The culture broth was centrifuged at 10,000 g for 5 minutes at 4°C. The cell pellet was discarded and the supernatant was retained.

The cell free production broth obtained after 48 h post inoculation was used for the purification of lipase. The required amount of ammonium sulfate was added to the cell free broth to achieve 60% (w/v) saturation. The content were mixed thoroughly and kept over night at 4°C. The supernatant was discarded and precipitates reconstituted in minimum volume of Tris buffer (pH 8.5). These were extensively dialyzed against this buffer to remove ammonium sulfate. The lipase activity was assayed and the concentrated lipase preparation was stored at low temperature. This concentrated fraction was labeled as crude lipase.

Lipase assay was performed by a colorimetric method reported by Winkler and Stuckmann (18). The stock solutions (20 mM, micromoles) of p-nitrophenol palmitate (p-NPP) was prepared in HPLC grade iso-propanol. The reaction mixture comprised of 75 μL of p-NPP stock solution, 2 to 10 μL of each fractions and Tris-buffer (0.05 M, pH 8.5) to make final volume 3 mL. Appropriate control with a heat inactivated enzyme (5 min in boiling water bath) or SDS treated enzyme (in duplicate) was included with each assay. The absorbance of p-nitrophenol released was measured at 410 nm (Shimadzu UV/visible spectrophotometer, Japan). The unknown concentration of p-nitrophenol released was determined from a reference curve of p-nitrophenol. Lipase activity was defined as mM of p-nitrophenol released by 1 mL of enzyme i.e. activity/mL at 45°C.

The purification of lipase was carried out with the prepared PILs. A column packed with pre-swollen PIL was activated sequentially with 0.1M NaOH and 0.1N HCl. The column was equilibrated with 0.1 M phosphate buffer (pH 8.5) containing 1.0 M KCl. The column was washed thoroughly with 0.1 M phosphate buffer (pH 8.5) and the dialyzed enzyme (200 μL) was loaded on PIL column. The column was developed for 10 min with running buffer. Fifteen fractions were eluted using 0.1 M phosphate buffer containing 1.0 M KCl (fraction volume 3 mL). The fractions were analyzed for the lipase activity.

3. RESULTS AND DISCUSSION:

The enzyme purification is an important field of chemical research. The aim of enzyme purification procedure is to isolate the given enzyme with the maximum possible yield and the percentage activity of the recovered enzyme should be higher than the original extract. In addition, the purified enzyme should possess the maximum catalytic activity, i.e., there should be no degraded or inactivated enzyme present, and it should be of the maximum possible purity, i.e., it should contain no other enzymes or large molecules. There are number of methods available for the

purification of enzyme such as centrifugation, gel filtration, dialysis, ion exchange chromatography, electrophoresis, isoelectric focusing. Lipase has net negative charge and it can be purified by using the anion exchangers.

In the present study, we tried purification of lipase with the quaternary crosslinked polymers. But in these networks the liquid flow from the column was very low. However, the results were significant for the PIL, which have Cl^- as counter anion. These results are to the inactivation of lipase by the counter anions of the PILs. Therefore the PIL, which have Cl^- as counter anion was used for the lipase purification process. The results of lipase purification by the PIL are given in the Figure 3.1. The PIL purified the lipase, as the activity of the purified lipase was more than the crude. The activity of the first two fractions was 0.45 and for the third fraction it was near to 0.6 and after that activity remain constant upto 9th fractions. After that there was a regular decrease in the activity of enzyme. From these results, it follows that these polymers act as anion exchangers and partition the negative charged lipase to the positive centers on the polymers. The increase of the lipase activity with the progressive increase in the number of fractions suggests that there is a strong interaction between the lipase and the polymer support. Hence, more the fractions more lipase is leached out. However, it decreased after the maximum lipase was extracted. As this polymer purifies the enzyme, therefore, it can be used as packing material of the column in ion exchange chromatography for the purification and the bio-separation processes involving protein and enzyme.

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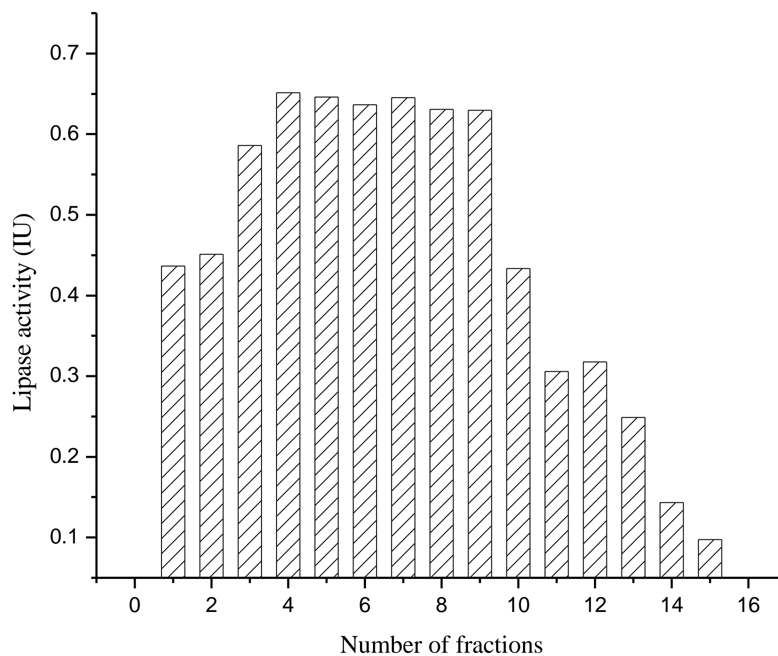

Figure 3.1. Purification of lipase by using [Poly(4-VP-*cl*-EGDMA)-$CH_2CH_2OH]^+$ Cl^-