Chapter 1

Aqueous Two Phase Extraction of Alcohol Dehydrogenase from Baker’s Yeast
Chapter 1A

Partial Purification of Alcohol Dehydrogenase Employing Precipitation
1A.1 Introduction:

Alcohol dehydrogenase (ADH, EC: 1.1.1.1), part of the oxidoreductase family, catalyzes the oxidation of alcohols, using NAD\(^+\) or NADP\(^+\) as the electron acceptor (White and White 1997). The reaction is reversible and substrates can be a variety of primary or secondary alcohols, and hemiacetals. Alcohol dehydrogenases are present in most organisms, with yeast containing the most active form of the enzyme.

Alcohol dehydrogenases are useful in fuel cells to catalyze the breakdown of fuel for an ethanol fuel cell (Moore et al., 2005). In biotransformation, alcohol dehydrogenases are often used for the synthesis of enantiomerically pure stereoisomers of chiral alcohols. In contrast to the chemical process, the enzymes yield directly the desired enantiomer of the alcohol by reduction of the corresponding ketone.

1A.1.1 Precipitation

Protein precipitation achieves separation by the conversion of soluble proteins to an insoluble state, which subsequently can be removed by various means. Precipitation can be used to remove components in cell culture media that may interfere with downstream purification methods. Ideally, precipitation results in both concentration and purification thus, precipitation is often used early in the sequence of downstream processing, reducing the volume and increasing the purity of the protein prior to the application of any other purification steps.

The main advantage of precipitation is the relative ease of its use. In addition, precipitating agents can be chosen that provide a more stable product than found in soluble form. A protein is made insoluble by changing its surface, charge characteristics or changing the solvent characteristics; the latter being preferred. (Asenjo and Juan, 1990)
Greater the initial concentration of the desired protein, greater is the efficiency of precipitation. A protein is least soluble when present at its isoelectric point (pl) (Janson et al., 1989), therefore, selection of a buffer at or near the pl of the protein is recommended. However, some proteins may get denatured at their pl. Above the pl, solubility of a protein increases with the addition of salt and reaches a maximum after which there is a rapid linear decrease in solubility (Harrison and Roger, 1993).

**1A.1.2 Methods of precipitation**

There are several methods to reduce the solubility of proteins and some of which are: ionic salts (e.g. ammonium sulfate, sodium chloride), temperature, pH, metal ions (e.g. Cu$^{2+}$, Zn$^{2+}$ and Fe$^{2+}$), nonionic polymers [e.g. polyethylene glycol (PEG)], organic solvents (e.g. ethanol, acetone), tannic acids, heparin, dextran sulfates, cationic polyelectrolytes (e.g. protamines), short chain fatty acids (e.g. caprylic acid), trichloracetic acid (TCA), lectins (e.g. concanavalin A), group-specific dyes (e.g. Procion Blue) and ligand-antibody complexes (Harrison and Roger, 1993). Ionic precipitation, utilizing inorganic salts, is the most common precipitation method. Precipitation by various salts or nonionic polymers is the preferred method to utilize whenever possible. Such precipitation typically yields stable non-denatured products. The use of temperature, pH or organic solvents can lead to denaturation and should be performed with care to minimize any decrease in yield or activity. Other methods are typically more specific for certain proteins or a particular class of proteins.

**1A.1.2.1 Precipitation with inorganic salts**

Inorganic salts can be utilized for the precipitation of proteins, with ammonium sulfate being the most common. The advantages of ammonium sulfate are: (1) at saturation, it is of sufficiently high molarity that it causes the precipitation of most proteins; (2) it does not have
a large heat of solution, allowing heat generated to be easily dissipated; (3) its saturated solution (4.04 M at 2° C) has a density (1.23g cm\(^{-3}\)) that does not interfere with the sedimentation of most precipitated proteins by centrifugation; (4) its concentrated solutions are generally bacteriostatic; and (5) in solution it protects most proteins from denaturation. (Deutscher and Murray, 1990)

The concentration of ammonium sulfate required for precipitation varies from protein to protein and needs to be determined empirically. Typically, ammonium sulphate is used in a series of steps performed over the range 2 - 8° C. For example, ammonium sulfate is added in increments to a concentration of 20% saturation while gently stirring for allowing to dissolve and equilibrate, between two additions. Any precipitate formed is removed and discarded. This step typically yields macromolecules such as ribosomes, membrane fragments and even denatured proteins. This precipitation step is then followed by an increase in the ammonium sulfate concentration to 50% of saturation, in which the protein of interest is “salted-out” and collected via centrifugation. The remaining supernatant may contain “contaminating” proteins, which are then discarded. The collected precipitate can be resuspended in the minimal volume of buffer suitable for the next step in the purification process, typically via dialysis. Thus concentration, purification and buffer exchange are performed in one process.

1A.1.2.2 Precipitation with nonionic polymers

The use of nonionic polymers for the precipitation is a method that can help to prevent protein denaturation and assist in removal of detergents. Typically, larger proteins precipitate at lower concentrations of nonionic polymers. Several water soluble uncharged polymers used for precipitation include dextrans, polyvinyl pyrrolidone, polypropylene glycols and polyethylene glycols. Polyethylene glycols (PEG) are the preferred non-ionic polymers for
protein precipitation because the viscosity of concentrated solutions is lower than that of other nonionic polymers (Harrison and Roger, 1993).

Polyethylene glycols are polymers of ethylene oxide typically ranging in size from 200 Da to 20 kDa. PEG is highly very soluble in water due to the ether oxygens spread along the length of the polymer, which are strong Lewis bases and form hydrogen bonds with water molecules. In addition, the formation and equilibration of precipitates take significantly less time with PEG as the precipitating agent than with ammonium sulfate or ethanol (Deutscher and Murray, 1990; Asenjo and Juan, 1990).

In the present work precipitation of alcohol dehydrogenase was carried out using ammonium sulphate and PEG before employing ATPE.

1A.2. Materials and Methods

1A.2.1. Materials

Polyethylene glycol (PEG Mol. wt 6000) was procured from Sisco Research Laboratories, Mumbai (India). Nicotinamide adenine dinucleotide (NAD⁺) from Himedia (India), ammonium sulphate ((NH₄)₂SO₄), magnesium sulphate (MgSO₄), sodium phosphate (Na₂HPO₄, NaH₂PO₄) were purchased from Ranbaxy Chemicals (India). Baker’s yeast was procured from AB Mauri, India Pvt Ltd. All the chemicals used were of analytical grade.

1A.2.2. Methods

1A.2.2.1. Preparation of crude extract

The Baker’s yeast (dry form) was suspended in 10 mM Sodium phosphate buffer (pH 7.5, 1:10 ratio (v/w)) and stirred for 1 h. Disruption of yeast suspension was carried out using Sonic homogenizer (F-873, Ultrasonics Ltd, England) for 5 min at 1000 rpm. The homogenate was centrifuged (MP 400R, Eltek) for about 10 min at 10,000 rpm and the clear
supernatant obtained (crude extract) was used for the experiments (Zhi-Guo-Su and Xiao-Li-Feng, 1999, Stenson, 2008).

1A.2.2.2 Precipitation:

A known amount (taken from standard chart for each fraction) of ammonium sulphate salt crystals are slowly added to the cold (4°C) crude extract with constant slow stirring till they reach complete dissolution. The solution was centrifuged at 10000 rpm for 10 min in cold centrifuge. Pellet was separated, redissolved in 10 mM sodium phosphate buffer (pH 7.5) and analysed for protein concentration and enzyme activity.

For PEG precipitation, a known amount of PEG 6000 was added to cold sodium phosphate buffer (10 mM, pH 7.5) followed by the yeast and the mixture was subjected to homogenization. The homogenate was kept for 20 min for protein precipitation, induced by PEG. On centrifugation, cell debris along with some protein precipitate settled down and clear supernatant was obtained which was analyzed for protein concentration and enzyme activity.

1A.2.2.3. Protein determination

Concentration of the protein was determined by Bradford method (Bradford, 1976) using coomassie blue G-250 dye as a reagent and bovine serum albumin (BSA) as standard, by measuring the absorbance at 595 nm at 25°C in UV Spectrophotometer (Spectronic UV-160A, Shimadzu, Japan).

1A.2.2.4. Enzyme assay

ADH assay was carried out using 1.3 ml of 50 mM sodium pyrophosphate buffer (pH 8.8), 1.5 ml 15 mM NAD⁺ solution, 0.1 ml 95% ethanol and 0.1 ml enzyme solution. The total reaction mixture contains 22 mM sodium pyrophosphate, 3.2 mM ethanol, 7.5 mM NAD⁺, 0.3 mM sodium phosphate, 0.03% BSA and final assay volume was 3 ml (Kagi and Vallee,
The absorbance, measured for 5 min at 340 nm, indicated the generation of NADH. One unit of ADH activity is defined as the amount of enzyme required for catalyzing the formation of 1.0 μmole acetaldehyde from ethanol per minute, at pH 8.8 at 25°C (Bergmeyer, 1974). ADH activity (U/ml) in the samples was calculated using the following equation.

\[
\text{Activity of ADH (U/ml)} = \frac{(A_{340} / \text{min})(3)(\text{DF})}{(6.22)(0.1)}
\]

---(3)

In the above equation, ‘3’ is the total volume (ml) of assay; DF, the dilution factor; 6.22, the millimolar extinction coefficient of NADH at 340 nm and 0.1, the volume (ml) of enzyme solution.

1A.2.2.4.1. Enzyme activity recovery:

Enzyme activity recovery was calculated using the following equation.

\[
\text{Enzyme activity recovery (\%)} = \frac{(\text{Activity})(\text{Dilution factor})}{(\text{Initial activity})(\text{Volume of crude extract})} \times 100
\]

---(4)

1A.3. Results and Discussion

In order to know the relative performance, precipitation experiments were performed using inorganic salt (ammonium sulphate) and non-ionic polymer (PEG 6000). Precipitate from ammonium sulphate precipitation was redissolved in 10 mM sodium phosphate buffer (pH 7.5) and subjected to ATPE. In PEG precipitation the supernatant obtained was subjected to ATPE for further purification. The results obtained are discussed in the following sections.

1A.3.1 Ammonium sulphate precipitation
Ammonium sulphate is commonly used at different concentrations for the precipitation of the enzymes and proteins (Smith et al., 2002; Foster et al., 1971). ADH did not precipitate up to 40% saturation of ammonium sulphate, hence, the precipitate of other proteins obtained at this saturation could be discarded. Precipitate from the 50% saturation and above were analyzed for ADH activity. Results are shown in the Figure 1A.1. The specific activity of ADH in the precipitate increased with an increase in concentration of ammonium sulphate till 65% saturation. Further increase in ammonium sulphate concentration (>65%) resulted in a decrease in ADH specific activity, although the protein precipitated was increasing, indicating enzyme denaturation at high salt concentrations. Maximum specific enzyme activity 151 U/mg with 1.9 fold enrichment (specific activity of crude was 77 U/mg) was obtained at 65%. Hence, ammonium sulphate concentration of 65% (w/v) was selected for further experiments. The precipitate from this saturation was dissolved in a small volume of 10 mM sodium phosphate buffer (pH 7.5), dialyzed against 10 mM sodium phosphate buffer and the solution was subjected to ATPE in subsequent experiments (Chapter 1B).

1A.3.2. PEG precipitation

For optimization of PEG concentration, cell disruption followed by precipitation was carried out at different concentrations of PEG 6000 (5 to 50% w/v) in buffer. At each concentration, the supernatant was assayed for enzyme activity and protein concentration. The obtained results were shown in Figure 1A.2. It can be observed from the figure that, ADH specific activity in supernatant increased with an increase in the concentration of PEG till 15% and above it rapidly decreased. However, the protein concentration in the supernatant gradually decreased with an increase in PEG concentration. It can be inferred that till 15% PEG contaminant proteins precipitated and above it, ADH also started precipitating. Similar results were reported by Zhi-Guo-Su and Feng (1999). Maximum specific enzyme activity 141.2 U/mg with 2.4 fold enrichment (specific activity of crude was 58 U/mg) was obtained at
15% PEG. The supernatant obtained was subjected to ATPE for further purification in subsequent experiments (Chapter 1B).

1A.4. Conclusions:

Precipitation could be successfully demonstrated for the primary purification of ADH from baker’s yeast. It enabled quick removal of large amount of contaminant proteins that interferes with the subsequent purification methods. In the present study among precipitating agents employed (ammonium sulphate and PEG), PEG was found to result in higher purification.
Figure 1A.1: Effect of Ammonium sulphate concentration upon precipitation of proteins and specific activity of ADH.
Figure 1A. 2: Effect of PEG 6000 concentration on precipitation of proteins and specific activity of ADH
Chapter 1 B

Effect of Process Parameters on Partitioning of Alcohol Dehydrogenase from Baker’s Yeast
1B.1. Introduction

Alcohol dehydrogenase (ADH, E.C. No. 1.1.1.1) is an enzyme that occurs in large amounts in the microorganisms and liver of the animals. It plays an important role in several physiological functions including metabolism of alcohol. Alcohol dehydrogenase (141 kDa) enzyme is widely used in biochemical, forensic science for estimating the concentration of primary alcohols, NAD⁺, ethylene glycol (John et al., 1980) numerous aldehydes and enzymatic catalysis of organic solvents and also in biosensors (Gonchar et al., 2002). Generally Baker’s yeast (Saccharomyces cerevisiae) is a good source for this enzyme. Various methods such as chromatography, electrophoretic techniques, crystallization etc., (Willoughby et al., 1999; Hidayat., et al., 2004; Negoro and Wakabayashi, 2004) were developed for the separation and purification of alcohol dehydrogenase (ADH). However, most of them involve a number of steps and furthermore the scale up of these methods is difficult and expensive on large scale. In view of this, an attempt is made in the present work to develop a simple and more efficient method for separation and purification of enzymes. An integrated process involving precipitation followed by aqueous two phase extraction (ATPE) for the ADH recovery from crude extract of baker’s yeast (Saccharomyces cerevisiae). Precipitation is a conventional primary purification step in downstream processing of proteins.

A variety of precipitating agents such as inorganic salts, organic solvents, polyelectrolytes and nonionic polymers are used in this unit operation and ammonium sulphate is most commonly used salt. Precipitation by polyethylene glycol (PEG) is advantageous over precipitation by salt because it can be carried out at ambient temperature and the polymer does not significantly interact with the protein. Further, comparatively low concentration of polymer (5-20%) is sufficient to precipitate most of the proteins (Mahadevan and Hall, 1992). PEG has been used in the isolation and concentration of biological components for the last two decades (Atha and Ingham, 1992).
ATPE is gaining more importance in the purification of proteins, because of the low cost of the system and biocompatible environment. It has been exploited for the recovery of biological products for more than four decades (Albertsson, 1985; Raghavarao et al., 1995; Tanuja et al., 1997; Zhi-Guo-Su and Xiao-Li Feng, 1999). Hustedt and coworkers developed ATPE at large scale for removal of cell debris from microbial cells in separation of fumarase, aspertase and formate dehydrogenase (Hustedt et al., 1985). In ATPE, the selective partitioning of the desired enzymes or proteins to one phase and contaminant proteins to the other phase, not only purifies the enzymes but also concentrates them into one of the phases. Precipitation was used for the partial purification of ADH from crude extract. Except the target proteins other contaminants were removed from the crude extract by this process, which enhances the purity of the ADH. Further the process integration with ATPE helps in the recovery of the ADH with high purity in a concentrated form. Influence of process parameters like pH, molecular weight of PEG, tie line length (TLL), volume ratio and neutral salt on ADH partitioning is studied in order to enhance the enzyme activity recovery.

1B.2. Materials and Methods

1B.2.1. Materials

Polyethylene glycol (PEG Mol. wt 600, 1000, 1500, 4000, 6000, 20,000) was procured from Sisco Research Laboratories, Mumbai (India). Nicotinamide adenine dinucleotide (NAD\(^+\)) from Himedia (India), potassium phosphate salts (KH\(_2\)PO\(_4\), K\(_2\)HPO\(_4\)), ammonium sulphate ((NH\(_4\))\(_2\)SO\(_4\)), magnesium sulphate (MgSO\(_4\)), sodium phosphate (Na\(_2\)HPO\(_4\), NaH\(_2\)PO\(_4\)) were purchased from Ranbaxy Chemicals (India). Baker’s yeast was procured from AB Mauri, India Pvt Ltd. All the chemicals used were of analytical grade.
1.B.2.2. Methods

1.B.2.2.1. Preparation of crude extract

The baker’s yeast (dry form) was suspended in 10 mM sodium phosphate buffer (pH 7.5, 1:10 ratio (v/w)) and stirred for 1 h. Disruption of yeast suspension was carried out using Sonic homogenizer (F-873, Ultrasonics Ltd, England) for 5 min at 1000 rpm. The homogenate was centrifuged (MP 400R, Eltek) for about 10 min at 10,000 rpm and the clear supernatant obtained (crude extract) was used for the experiments.

1.B.2.2.2. Aqueous Two Phase Extraction

Predetermined quantities of polymers and salts from the phase diagrams (Albertsson, 1985; Zaslavasky, 1995) were weighed and added to crude extract making the total weight of the system 100% on w/w basis. The contents were mixed thoroughly for 1 h using a magnetic stirrer and were allowed to separate for about 8 h in a separating funnel. After clear separation, the top and bottom phases were collected and analyzed for protein and enzyme activity. An average of three replicates was considered. The error in the analysis was within ±1%.

1.B.2.2.2.1. Tie Line Length:

The tie line length (TLL) of the aqueous two phase system was calculated from its reported phase diagram (Albertsson, 1985; Zaslavasky, 1995) according to the following equation.

\[
TLL(\%) = \sqrt{(C_{pt} - C_{pb})^2 + (C_{sb} - C_{st})^2}
\]  

----- (1B.1)

where, \( C_{pt} \) and \( C_{pb} \), are PEG concentrations (\%, w/w) in the top and bottom phases, respectively and \( C_{st} \) and \( C_{sb} \), are salt concentrations (\%, w/w) in top and bottom phases, respectively.
1.B.2.2.2. Phase Volume Ratio:

The phase volume ratio ($V_r$) is defined as the ratio of volume of the top phase to that of the bottom phase.

$$\text{Volume Ratio (} V_r \text{)} = \frac{V_t}{V_b}$$  \hspace{1cm} -----(1B.2)

where, $V_t$ and $V_b$, are volumes of the top and bottom phases, respectively. Volume ratio is varied by considering different total compositions along the selected tie line.

1.B.2.2.3. Protein determination

Concentration of the protein was determined by Bradford method (Bradford, 1976) using coomassie blue G-250 dye as a reagent and bovine serum albumin (BSA) as standard, by measuring the absorbance at 595 nm at 25°C in UV Spectrophotometer (Spectronic UV-160A, Shimadzu, Japan).

1.B.2.2.4. Enzyme assay

ADH assay was carried out using 1.3 ml of 50 mM sodium pyrophosphate buffer (pH 8.8), 1.5 ml 15 mM NAD$^+$ solution, 0.1 ml 95 % ethanol and 0.1 ml enzyme solution. The total reaction mixture contains 22 mM sodium pyrophosphate, 3.2 mM ethanol, 7.5 mM NAD$^+$, 0.3 mM sodium phosphate, 0.03% BSA and final assay volume was 3 ml (Kagi and Vallee, 1960). The absorbance, measured for 5 min at 340 nm, indicated the generation of NADH. One unit of ADH activity is defined as the amount of enzyme required for catalyzing the formation of 1.0 μmole acetaldehyde from ethanol per minute, at pH 8.8 at 25°C (Bergmeyer, 1974). ADH activity (U/ml) in the samples was calculated using the following equation.

$$\text{Activity of ADH (U/ml)} = \frac{(A_{340/\text{min}})(3)(DF)}{(6.22)(0.1)}$$ \hspace{1cm} -----(1B.3)
In the above equation, ‘3’ is the total volume (ml) of assay; DF, the dilution factor; 6.22, the millimolar extinction coefficient of NADH at 340 nm and 0.1, the volume (ml) of enzyme solution.

1.B.2.2.4.1. Enzyme activity recovery:

Enzyme activity recovery was calculated using the following equation based on bottom phase for which the enzyme has shown preference.

\[
\text{Enzyme activity recovery (\%)} = \frac{(\text{Activity}) \cdot (\text{Volume of bottom phase})}{(\text{Initial activity}) \cdot (\text{Volume of crude extract})} \times 100
\]

---(1B.4)

1.B.2.2.5. Gel Electrophoresis:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% gel was performed using electrophoresis unit (Bangalore Genei) and standard molecular weight markers. Electrophoresis was run at 50 V, 12.5 mA for about 3 - 4 h. The gel was stained with coomassie brilliant blue R-250 of 0.05% (w/v), 50% (v/v) methanol and 12% (v/v) acetic acid. The gel was destained using the same buffer without coomassie brilliant blue (Deuscher, 1990).

1.B.3. Results and Discussion

ATPE experiments were carried out using crude yeast extract. The selection of the suitable phase system is very important for the efficacy of ATPE. Since, multiple factors affect the recovery of the desired product (ADH in the present case), standardization of process parameters is essential. Hence, the influence of process parameters such as pH, molecular weight of PEG, tie line length (TLL), phase volume ratio and concentration of the neutral salt on ADH activity recovery was studied. The results are discussed in the following sections.
1.B.3.1. Selection of phase forming salt

In order to select the most suitable phase forming salt, partition studies were carried out using different phase forming salts with PEG 6000 in aqueous two phase extraction. The results are shown in Table 1B.1. Among the salts, namely, sodium phosphate, sodium sulphate, magnesium sulphate, potassium phosphate and ammonium sulphate, the best activity recovery was observed in case of potassium phosphate (Table 1B.1). ADH stability depends on the pH of the system (which in turn affects its activity recovery) and highest stability was observed at pH 7.0 (Zanon et al., 2007). Thus the variation in the activity recovery can be attributed to the pH of the system with respect to the phase forming salt (PEG being nonionic will not contribute to pH) and the potassium phosphate resulted in the system pH of 7±0.2. Hence, the PEG/potassium phosphate system was selected for further study.

1.B.3.2. Selection of PEG molecular weight

In order to select the most suitable molecular weight, PEG of different molecular weights (600, 1000, 1500, 4000, 6000 and 20,000) and potassium phosphate salt (keeping its concentration constant) were used in the partition experiments. The results are shown in the Figure 1B.1. It can be seen that specific activity of ADH in bottom phase was observed to increase with an increase in molecular weight of PEG. The free volume in the polymer (top) phase significantly decreases with an increase in the molecular weight of the polymer (PEG) and as a result the biomolecules selectively partition to the bottom phase due to the volume exclusion effect (Almedia et al., 1998; Rabelo et al., 2004). Maximum increase in specific activity (1.5 fold) was observed in case of PEG 20,000, with negligible activity of ADH in the top phase. Further, recovering the target biomolecule from salt phase is easier than from the PEG phase. Hence, PEG 20,000/potassium phosphate system was selected for further studies.
After selecting the phase forming salt and molecular weight of the polymer, it was attempted to integrate the precipitation with ATPE to increase the overall productivity of the process. In order to enhance performance, experiments were performed in two schemes. In the first scheme, ammonium sulphate precipitation was carried out and by redissolving the pellet in 10 mM sodium phosphate buffer (pH 7.5), ATPE was carried out employing PEG 20000/potassium phosphate system. The second scheme involves precipitation using PEG, and the supernatant obtained was subjected to ATPE employing the same phase system.

1B.3.3. Scheme 1: Ammonium sulphate precipitation followed by ATPE

Ammonium sulphate is commonly used at different concentrations for the precipitation of the enzymes and proteins (Foster et al., 1971; Smith et al., 2002). Ammonium sulphate concentration of 65% (w/v) was selected for precipitation of the enzyme (Section 1A.3.1). The precipitate from the this saturation was dissolved in a small volume of 10 mM sodium phosphate buffer (pH 7.5), dialyzed against 10 mM sodium phosphate buffer and the solution was subjected to ATPE.

ATPE experiments were carried out for at three different tie line lengths. The results are shown in Table 1B.2. Specific activity of ADH in bottom phase was found to increase with an increase in TLL. However, above 12% TLL, precipitation was observed at the interface due to high concentrations of polymer and salt, resulting in the highest specific activity (391.6 U/mg) of ADH at 12% TLL, with 4 fold increase (Specific activity of crude was 93.8 U/mg).

In general, salting out effect was observed in PEG/salt systems with increasing TLL, resulting in a shift in the partitioning of proteins from salt-rich to polymer-rich phase (Rito-Palomares, 2005). However, in the present study, the volume exclusion effect due to high molecular weight PEG being more dominant, the ADH partitioned preferentially to the bottom phase. Since, decrease in activity recovery as well as specific activity was observed due to
precipitation near the interface at higher TLL (Table 1B.2), middle TLL was selected for further study.

The effect of volume ratio was studied at a TLL 12% and the results are shown in the Table 1B.3. ADH specific activity increased with an increase in volume ratio and the highest specific activity was observed at the volume ratio of 2.6. It may noted that on the same TLL when volume ratio is varied by changing the total composition, the composition of the PEG (top) and salt (bottom) phases remains the same. At higher volume ratios, ADH by virtue of its large size and high molecular weight of PEG, preferentially partitioned to the bottom phase due to the volume exclusion effect. However, with a decrease in volume ratio, the contaminant proteins partitioned to the bottom phase mainly due to increase in its volume. Thus, in this scheme, the maximum ADH specific activity obtained was 409.68 U/mg (4.4 fold increase over the crude extract of 93.8 U/mg) at volume ratio of 2.6.

1B.3.4. Scheme 2: PEG precipitation followed by ATPE

PEG precipitation was carried out by addition of 15% (optimized in the previous section: 1A.3.2) of PEG 6000 which has resulted in 1.7 fold enrichment. The supernatant obtained was subjected to ATPE, employing PEG 20,000/potassium phosphate system at three TLLs and results are shown in Table 1B.4. With an increase in the TLL, the concentration of the polymer and salt in the individual phases increases, which in turn causes precipitation of protein/enzyme at the interface (Rito-Palomares and Lyddiatt, 2002). Hence, specific activity of ADH decreased with an increase in the TLL and the best results (specific activity 415.6 U/mg with 4.5 fold increase over that of crude extract) was observed at the lowest TLL of 6.22%.

In order to examine the effect of volume ratio, four different volume ratios were considered on the tie line with TLL 6.22%. Results are shown in Table 1B.5. At higher volume ratios there was an increased in concentration of both total protein and ADH in the
bottom phase due to the reduction in its volume. Specific activity of ADH increased with an increase in volume ratio similar to that of scheme 1. However, the specific activity of ADH was higher (431.5 U/mg) with a 5 fold increase in specific activity at volume ratio of 2.6. The same volume ratio was used to study the effect of neutral salt on partitioning of the ADH.

Effect of neutral salt (NaCl) was studied at different concentrations (0.2 -1.0 %) employing 12/7.33 %, w/w total phase composition of PEG/salt with a volume ratio of 2.6. The results are shown in Table 1B.6. A gradual increase in the specific activity was observed with an increase in salt concentration. Maximum increase in enrichment (6.6 fold) was observed at 1% NaCl with 447.3 U/mg specific activity over the crude extract of 68.4 U/mg.

The results from both the schemes are shown in the Table 1B.7. Precipitation with PEG followed by ATPE resulted in higher degree of purification (6.6 fold) with 90% enzyme activity recovery. It may be noted that the degree of enrichment of ADH from yeast obtained in the present process was higher than the reported values (Zhang et al., 2000; Chang et al., 2006; Bansal-Muthalik and Gaiker, 2006).

1B.3.5. Recovery of ADH from bottom phase

Separated bottom phase after ATPE was dialyzed against 10 mM sodium phosphate buffer (pH 7.5) for the removal of salt. The obtained solution was analyzed for enzyme activity and found that there was no loss in enzyme activity.

SDS-PAGE profiles of ADH samples and standard molecular weight markers were shown in the Figure 1B. 2. The crude yeast extract (supernatant) has a large number of bands (line 2), the standard ADH showing a single band (line 3) and the sample taken from the bottom phase of the ATPE showing a prominent dark band with a few small bands (Line 4).
1B.4. Conclusions

Aqueous two-phase extraction could be effectively used for the extraction and purification of ADH from the crude yeast extract. Precipitation was carried out using inorganic salt and nonionic polymer followed by ATPE. In the schemes employed in the present work, scheme 2, which involved precipitation with PEG followed by ATPE, showed better results (specific activity of 449.6 U/mg with 6.6 fold purification) when compared to scheme 1 as well as the conventional method. Overall results demonstrated that process integration (precipitation followed by ATPE) has enabled to develop a more efficient method for extraction and purification of alcohol dehydrogenase.
Table 1B.1: Recovery of ADH in PEG 6000/salt systems

<table>
<thead>
<tr>
<th>Phase forming Salt</th>
<th>Activity recovery of ADH (%)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate (Dibasic) (Na₂HPO₄)</td>
<td>80</td>
<td>9.2±0.2</td>
</tr>
<tr>
<td>Sodium phosphate (NaH₂PO₄)</td>
<td>83</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>Sodium sulphate (Na₂SO₄)</td>
<td>75</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>Magnesium sulphate (Mg₂SO₄)</td>
<td>78</td>
<td>5.7±0.2</td>
</tr>
<tr>
<td>Potassium phosphate (K₂HPO₄ and KH₂PO₄)</td>
<td>89</td>
<td>7.0±0.2</td>
</tr>
<tr>
<td>Ammonium sulphate ((NH₄)₂SO₄)</td>
<td>85</td>
<td>5.0±0.2</td>
</tr>
</tbody>
</table>
Table 1B.2: Effect of TLL on partitioning of ADH to bottom phase

<table>
<thead>
<tr>
<th>System</th>
<th>Total phase composition PEG/salt (w/w)</th>
<th>TLL (%)</th>
<th>Protein concentration (µg/ml)</th>
<th>Enzyme Activity (U/ml)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System 1</td>
<td>10.04/7.92</td>
<td>6.2</td>
<td>123.5</td>
<td>47.0</td>
<td>380.6</td>
</tr>
<tr>
<td><strong>System 2</strong></td>
<td><strong>8.91/8.36</strong></td>
<td><strong>12.0</strong></td>
<td><strong>127.3</strong></td>
<td><strong>49.8</strong></td>
<td><strong>391.6</strong></td>
</tr>
<tr>
<td>System 3</td>
<td>9.69/8.94</td>
<td>16.3</td>
<td>126.0</td>
<td>46.5</td>
<td>369.2</td>
</tr>
<tr>
<td>Crude extract</td>
<td>-</td>
<td>-</td>
<td>63.8</td>
<td>5.9</td>
<td>93.8</td>
</tr>
<tr>
<td>After Ammonium sulphate precipitation</td>
<td>-</td>
<td>-</td>
<td>106.3</td>
<td>16.7</td>
<td>157.5</td>
</tr>
</tbody>
</table>

**Phase system:** PEG 20,000/potassium phosphate system
Table 1B.3: Effect of Volume ratio on partitioning of ADH to bottom phase

<table>
<thead>
<tr>
<th>System</th>
<th>Total phase composition PEG/Salt (w/w)</th>
<th>Volume ratio</th>
<th>Protein concentration (µg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System 1</td>
<td>14/7</td>
<td>2.6</td>
<td>125.4</td>
<td>51.4</td>
<td>409.7</td>
</tr>
<tr>
<td>System 2</td>
<td>11/8</td>
<td>1.7</td>
<td>118.8</td>
<td>46.4</td>
<td>391.2</td>
</tr>
<tr>
<td>System 3</td>
<td>8/9.25</td>
<td>1.0</td>
<td>110.4</td>
<td>40.7</td>
<td>368.9</td>
</tr>
<tr>
<td>System 4</td>
<td>5.58/10</td>
<td>0.4</td>
<td>106.5</td>
<td>30.8</td>
<td>283.8</td>
</tr>
<tr>
<td>Crude extract</td>
<td>-</td>
<td>-</td>
<td>63.8</td>
<td>5.9</td>
<td>93.8</td>
</tr>
</tbody>
</table>

*Phase system:* PEG 20,000/potassium phosphate system (12% TLL)
Table 1B.4: Effect of TLL on partitioning of ADH to bottom phase

<table>
<thead>
<tr>
<th>System</th>
<th>Total phase composition</th>
<th>TLL (%)</th>
<th>Protein concentration (µg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System 1</td>
<td>10.04/7.92</td>
<td>6.2</td>
<td>44.2</td>
<td>18.4</td>
<td>415.6</td>
</tr>
<tr>
<td>System 2</td>
<td>9.91/8.36</td>
<td>12.0</td>
<td>43.1</td>
<td>16.1</td>
<td>374.3</td>
</tr>
<tr>
<td>System 3</td>
<td>9.69/8.94</td>
<td>16.3</td>
<td>40.0</td>
<td>14.4</td>
<td>359.0</td>
</tr>
<tr>
<td>Crude extract</td>
<td>-</td>
<td>-</td>
<td>80.4</td>
<td>6.7</td>
<td>83.17</td>
</tr>
<tr>
<td>After PEG precipitation</td>
<td>-</td>
<td>-</td>
<td>33.4</td>
<td>5.4</td>
<td>123.4</td>
</tr>
</tbody>
</table>

**Phase system:** PEG 20,000/potassium phosphate system
Table 1B.5: Effect of Volume ratio on partitioning of ADH to bottom phase

<table>
<thead>
<tr>
<th>System</th>
<th>Total phase composition PEG/salt (w/w)</th>
<th>Volume ratio</th>
<th>Protein concentration (µg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System 1</td>
<td>12/7.33</td>
<td>2.6</td>
<td>44.2</td>
<td>19.1</td>
<td>431.5</td>
</tr>
<tr>
<td>System 2</td>
<td>11/7.62</td>
<td>2.3</td>
<td>44.0</td>
<td>17.0</td>
<td>386.5</td>
</tr>
<tr>
<td>System 3</td>
<td>9/8.33</td>
<td>2.1</td>
<td>36.6</td>
<td>13.9</td>
<td>382.1</td>
</tr>
<tr>
<td>System 4</td>
<td>8/8.68</td>
<td>2.0</td>
<td>37.2</td>
<td>12.7</td>
<td>340.7</td>
</tr>
<tr>
<td>Crude extract</td>
<td>-</td>
<td>-</td>
<td>80.4</td>
<td>6.7</td>
<td>83.17</td>
</tr>
</tbody>
</table>

**Phase system:** PEG 20,000/potassium phosphate system (6.2% TLL)
Table 1B.6: Effect of neutral salt (NaCl) on ADH partitioning to bottom phase.

<table>
<thead>
<tr>
<th>Concentration of NaCl (%)</th>
<th>Protein Concentration (µg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>61.6</td>
<td>20.3</td>
<td>329.7</td>
</tr>
<tr>
<td>0.2</td>
<td>54.9</td>
<td>20.1</td>
<td>366.6</td>
</tr>
<tr>
<td>0.4</td>
<td>52.0</td>
<td>19.3</td>
<td>371.2</td>
</tr>
<tr>
<td>0.6</td>
<td>46.4</td>
<td>19.7</td>
<td>424.9</td>
</tr>
<tr>
<td>0.8</td>
<td>45.2</td>
<td>19.3</td>
<td>426.5</td>
</tr>
<tr>
<td>1.0</td>
<td>43.2</td>
<td>19.3</td>
<td>447.3</td>
</tr>
<tr>
<td>Crude extract</td>
<td>33.4</td>
<td>5.4</td>
<td>123.4</td>
</tr>
</tbody>
</table>

Phase system: PEG 20,000/potassium phosphate system (phase composition 12/7.33 at volume ratio 2.6)
### Table 1B.7: Specific activity of ADH from scheme 1 and scheme 2.

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Sample</th>
<th>Protein Concentration (μg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Purification factor</th>
<th>Enzyme activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract</td>
<td>95.3</td>
<td>6.5</td>
<td>68.4</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After Ammonium sulphate precipitation</td>
<td>67.7</td>
<td>6.7</td>
<td>99.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Scheme 1</td>
<td>ATPE Top phase</td>
<td>9.0</td>
<td>0.4</td>
<td>43.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATPE Bottom phase</td>
<td>96.1</td>
<td>26.4</td>
<td>277.9</td>
<td>4.0</td>
<td>85</td>
</tr>
<tr>
<td>Scheme 2</td>
<td>After PEG precipitation</td>
<td>36.0</td>
<td>4.3</td>
<td>118.8</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATPE Top phase</td>
<td>10.2</td>
<td>0.4</td>
<td>39.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATPE Bottom phase</td>
<td>45.3</td>
<td>20.4</td>
<td>449.6</td>
<td>6.6</td>
<td>93</td>
</tr>
</tbody>
</table>

**Phase system:** PEG 20,000/potassium phosphate system (phase composition of 14/7 and 12/7.33 for scheme 1 and 2 respectively)
Figure 1B.1: Variation of specific activity with molecular weight of PEG
Figure 1B.2: SDS-PAGE. Line 1: Protein molecular markers, Line 2: Crude yeast extract, Line 3: Standard ADH sample (sigma), Line 4: ATPE bottom phase.