Chapter 3

Integration of Aqueous Two Phase Extraction with Membrane Processing
Chapter 3A

Process Integration for Downstream Processing of Alcohol Dehydrogenase from Baker’s Yeast.
3A.1. Introduction

Recent advances in biotechnology have focused on downstream processing, which constitute a major part of production costs, which has received lesser attention before. Thus, there is a strong demand for bioprocess, especially for downstream processing, which increases the yield, reduces the process time and capital expenditure (Schugerl and Hubbuch, 2005). Process integration, wherein two unit operations are combined into one in order to achieve specific goals which are not effectively met by discrete processes, offers considerable potential benefit for the recovery and purification of biological products (Rito-Palomares, 2004). It also could be integration of extraction with membrane processes or different membrane processes with each other for achieving desired selectivity and purity of the biomolecule (Ganapathi et al., 2007; Azevedoa et al., 2008). Several integrated approaches have been developed to optimize productivity and cost effectiveness of different bioprocesses (Farruggia et al., 2003). A number of recent studies have demonstrated the feasibility of aqueous two-phase extraction and membrane processes for the separation and purification of proteins (Srinivas et al., 2002a). Microfiltration and ultrafiltration have been widely used as a preferred methods for protein concentration, purification, buffer exchange and replaced size exclusion chromatography in these applications (Cheang and Zydney, 2003; Schugerl and Hubbuch, 2005; Madhusudhan et al., 2008; Saxena et al., 2009). Many investigations have reported successful purification of the ADH enzyme by immobilized dye-metal expanded bed affinity chromatography and other methods from the same source
with purification factors ranging from 6.5 to 8.8 and recoveries ranging from 40.7 to 93.5% (Willoughby et al., 1999, Smith et al., 2002; Hidayat et al., 2003; 2004).

The separation involving membrane processing and ATPE can be performed at ambient temperature with less energy consumption in comparison to other separation processes. Hence, the integration of aqueous two-phase extraction and membrane process (ultrafiltration/microfiltration) was attempted in the present study for the concentration and purification of alcohol dehydrogenase (ADH) from baker’s yeast to achieve higher purity without losing recovery.

The majority of the commercially available microfiltration/ultrafiltration membranes are inherently inhomogeneous (nonuniform in mass and thickness). This affects the operational performance. Nanofibrous microfiltration/ultrafiltration membranes offer unique properties for filtration and adsorption based separations including high specific surface area, good interconnectivity of pores and the potential to incorporate active chemistry on a nanoscale. Hence, nanofibrous microfiltration was attempted along with ATPE for the recovery of ADH. Different combinations of ATPE and membrane process were used for downstream processing of this enzyme. Three different sets of experiments were carried out by employing combination of these purification methods (ATPE followed by UF, UF followed by ATPE, MF followed by ATPE). ATPE experiments were carried out by selecting the standardized phase compositions from previous section (section 1B.3).
3A.2. Materials and Methods

3A.2.1. Materials

Polyethylene glycol (PEG, mol. wt, 6000, and 20,000), were procured from Sigma Aldrich, MO, USA. Nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) from Himedia (India), sodium phosphate (Na\textsubscript{2}HPO\textsubscript{4}, NaH\textsubscript{2}PO\textsubscript{4}) were purchased from Ranbaxy Chemicals Gurgaon, India. Potassium phosphate (K\textsubscript{2}HPO\textsubscript{4}, KH\textsubscript{2}PO\textsubscript{4}) were purchased from Merck, Mumbai, India. Baker’s yeast was procured from AB Mauri, India Pvt Ltd. All the chemicals used were of analytical grade. Ultrafiltration membranes (100 kDa) and PVDF microfiltration membranes (0.45 \textmu m, 47 mm) were procured from Millipore, USA and Microfiltration nanofibrous membranes were obtained from National University of Singapore (NUS).

3A.2.2. Methods

3A.2.2.1. Crude extract preparation and activity measurements

Crude extract was prepared as mentioned in section 1A.2.2 using Baker’s yeast (dry form) in 10 mM Sodium phosphate buffer with 15% PEG-6000 for ADH extraction. The clear supernatant obtained (crude extract) was used for the experiments. Measurements of enzyme activity, protein concentration and specific activity were carried out as per the protocol described in section 1A.2

3A.2.2.2. Aqueous two-phase extraction

ATPE experiments were carried out employing PEG-20000/potassium phosphate system for downstream processing of ADH (selected from chapter 1B).
3A.2.2.3. Membrane processes

Microfiltration and ultrafiltration were carried out using stirred cell module (Amicon solvent resistant stirred cell module, Millipore, USA; capacity of 50 ml). 100 kDa membrane disc of 47 mm diameter (Millipore, USA) was used for ultrafiltration. The nanofibrous membrane sheet (obtained from NUS, Singapore) was cut into discs of 47 mm diameter and PVDF membrane of 47 μm diameter (Millipore, USA) were used for microfiltration. The filtration experiments were carried out for 2h by maintaining the pressure, stirring speed and temperature constant throughout the experiment at 1.5 bar, 250 rpm and 25 ± 2 ºC, respectively. The pressure was applied using N₂ gas. Transmembrane flux was calculated based on the average flux.

3A.3. Results

Purification of alcohol dehydrogenase was done by employing three different modes (presented in Figure 3A.1) from the crude extract of baker’s yeast namely, Mode 1: ATPE followed by UF, Mode 2: UF (in diafiltration mode) followed by ATPE and Mode 3: MF followed by ATPE. Obtained results were tabulated in the Table 3A.1.

Mode 1: ATPE followed by UF

Aqueous two phase extraction of ADH was carried out employing PEG-20000/potassium phosphate system (12/7.33 phase composition selected from chapter 1B). After ATPE, the top and bottom phases were separated and the volumes were measured. The results obtained are given in the Table 3A.1.
the table it can be seen that ADH preferentially partitioned to the bottom phase and has resulted in 97% enzyme activity recovery. From ATPE alone 6.6 fold purification was obtained with specific activity of 522.62 U/mg. Further, the bottom phase was subjected to UF. Equipment used for membrane processing was shown in Figure 3A.2. Ultrafiltration was shown some improvement in the purification factor (6.6 to 7.32) along with the removal of phase components and at the same time recovery was slightly reduced (97 to 91%).

**Mode 2: UF followed by ATPE**

The crude extract was directly subjected to the ultrafiltration using 100 kDa membrane. UF was taken around 2 hr of time at 1.5 bar pressure to obtain 45 ml of permeate. One of the critical issues in the performance of ultrafiltration is the decline in transmembrane flux that occurs as a result of both concentration polarization and membrane fouling. Hence, diafiltration mode was used to reduce these and to maintain the flux. Figure 3A.3 shows the comparison of flux rates in case of UF alone and UF in diafiltration mode. Diafiltration has shown higher flux compared to UF alone hence, diafiltration was used for the study. Ultrafiltration has shown removal of around 20% of the contaminant proteins from the crude extract resulting in 1.24 fold enrichment of the enzyme. Obtained retentate was subjected to ATPE for further purification.

After ATPE, bottom phase has resulted in 7.53 fold purification of ADH with 97.4% activity recovery. Specific activity has enhanced to 600.3 U/mg compared to crude extract of 80 U/mg.
**Mode 3: MF followed by ATPE**

In this mode microfiltration was employed followed by ATPE for the purification of ADH. Membrane with MMWCO 0.45 \( \mu \text{m} \) was used. MF was used for the clarification of crude extract which has resulted in around 1.3 fold enrichment. However, the recovery was slightly low (90%). Alternately, MF was also carried out using nanofibrous MF membranes. The Figure 3A.4 shows the flux rates from normal PVDF and nanofibrous membranes. Nanofibrous membranes have shown higher flux compared to conventional membranes (PVDF) with high recovery (96 %). Obtained permeate was subjected to ATPE for further purification. After ATPE, highest degree of purification of ADH (8.12 fold) was observed in the bottom phase compared to crude extract. In this mode, highest specific activity of 647.32 U/mg was obtained with 95 % activity recovery which is the best among the studied combinations.

**3A.4 Conclusions**

The efficacy of integration of membrane processes with ATPE was demonstrated for the downstream processing of ADH from baker’s yeast for achieving the higher degree of purification of ADH without losing the yield. Integration of nanofibrous microfiltration with aqueous two phase extraction has resulted in 8.12 fold purification with 95% activity recovery. MF followed by ATPE was found to be the best among the modes studied.
Table 3A.1 Integrated approach for the concentration and purification of alcohol dehydrogenase

<table>
<thead>
<tr>
<th>Operation</th>
<th>Phase</th>
<th>Protein Concentration (mg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Enzyme Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Enzyme activity Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td></td>
<td>85.67</td>
<td>6.8</td>
<td>79.67</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>ATPE</td>
<td>Top</td>
<td>17.27</td>
<td>0.5</td>
<td>27.93</td>
<td>0.35</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>45.87</td>
<td>24.0</td>
<td>522.62</td>
<td>6.56</td>
<td>96.94</td>
</tr>
<tr>
<td>ATPE-UF (100 kDa)</td>
<td>Bottom</td>
<td>41.00</td>
<td>23.9</td>
<td>582.90</td>
<td>7.32</td>
<td>91.38</td>
</tr>
<tr>
<td>UF- (100 kDa)</td>
<td>Retentate</td>
<td>67.93</td>
<td>6.7</td>
<td>98.69</td>
<td>1.24</td>
<td>98.59</td>
</tr>
<tr>
<td></td>
<td>Top</td>
<td>16.67</td>
<td>0.5</td>
<td>30.39</td>
<td>0.38</td>
<td>5.96</td>
</tr>
<tr>
<td>UF-ATPE</td>
<td>Bottom</td>
<td>40.13</td>
<td>24.1</td>
<td>600.29</td>
<td>7.53</td>
<td>97.43</td>
</tr>
<tr>
<td>MF</td>
<td>Permeate</td>
<td>65.73</td>
<td>6.9</td>
<td>104.56</td>
<td>1.31</td>
<td>96.02</td>
</tr>
<tr>
<td></td>
<td>Top</td>
<td>15.73</td>
<td>0.4</td>
<td>27.59</td>
<td>0.35</td>
<td>5.11</td>
</tr>
<tr>
<td>MF- ATPE</td>
<td>Bottom</td>
<td>39.27</td>
<td>25.4</td>
<td>647.32</td>
<td>8.12</td>
<td>95.32</td>
</tr>
</tbody>
</table>
Figure 3A.1 Different combinations of ATPE and membrane processes for downstream processing
Figure 3A.2 Membrane processing set up used for the study. A) Ultrafiltration unit; B) Ultrafiltration with diafiltration mode
Figure 3A.3 Transmembrane flux during ultrafiltration of ADH from crude yeast extract.
Figure 3A.4 Transmembrane flux during microfiltration of ADH from crude yeast extract.
Chapter 3B

Process Integration for Downstream Processing of Invertase from Baker’s Yeast.
3B. 1 Introduction

Invertase (β-D-fructofuranoside fructohydrolase) which catalyses the hydrolysis of sucrose and related glycosides is one of the simplest commercial carbohydrases. Though they are widespread in distribution (Aleksanyan and Markosyan, 1986), the enzymes of commercial interest originate from strains of Saccharomyces sp. Invertase is used for the inversion of sucrose in the preparation of invert sugar and high fructose syrup (HFS). It is one of the most widely used enzymes in food industry where fructose is preferred than sucrose especially in the preparation of jams and candies, because it is sweeter and does not crystallize easily. Invertase is also used whenever sucrose containing substrates are subjected to fermentation viz. production of alcoholic beverages, lactic acid, glycerol etc. Other uses of the enzyme include, manufacture of artificial honey, plasticizing agents used in cosmetics, drug and paper industries and as enzyme electrodes for the detection of sucrose (as biosensors). Enzymatic hydrolysis of sucrose is preferable to acid hydrolysis as it does not result in the formation of undesirable flavoring agents as well as coloured impurities. Additionally, the use of immobilized invertase for the continuous hydrolysis of sucrose can be advantageous because the shifts in the pH brought about as a result of immobilization can be exploited to prevent the formation of oligosaccharides by the transferase activity associated with the soluble enzyme (Wiseman, 1978).

In view of the high commercial potential of the enzyme, several attempts have been made to obtain a stable enzyme preparation suitable for commercial
application. Various researchers have developed several methods for the purification of invertase (Gascon et al., 1968; Mislovicova et al., 1995; Chen et al., 1996; de-Almeida et al., 2005; Basu et al., 2008). However, almost all these methods of purification of invertase involve a number of steps, namely, precipitation, ion-exchange chromatography, gel filtration chromatography etc., and it is known that higher the number of steps higher is the loss of product yield (Kula et al., 1982). There are many reports available for the purification of invertase resulted with good degree of purification however, the recovery was low. For example, purification of soluble acid invertase (SAI) of sugarcane (Saccharum officinarum L.) was reported using combinations of precipitation and different kinds of chromatographic methods obtained about 13 fold purification with a recovery of 35% (Hussain et al., 2009). Other report on invertase purification using precipitation followed by DEAE-column chromatography resulted in 5.8 fold purification with recovery of 3.2% (Uma et al., 2010). The productivity of a given bioprocess can be considerably improved by relatively new strategy of process integration. It could be by integrating membrane process and ATPE to achieve higher degree of purification with good recovery. In this regard attempts in similar lines described in section 3A (for ADH) were made to develop an integrated process for the downstream processing of invertase from crude extract of baker’s yeast.
3B.2. Materials and Methods

3B.2.1. Materials

Polyethylene glycol (PEG, mol. wt. 3350), glucose oxidase, peroxidase, sucrose, o-dianisidine, glycerol, glucose were procured from Sigma Aldrich, MO, USA. magnesium sulphate (MgSO$_4$), sodium phosphate (Na$_2$HPO$_4$, NaH$_2$PO$_4$), were purchased from Ranbaxy Chemicals, Gurgaon, India. Potassium phosphate salts (KH$_2$PO$_4$, K$_2$HPO$_4$), hydrochloric acid (HCl) were from Merck, Mumbai, India. Baker’s yeast (dry form) was procured from local super market. Ultrafiltration membranes (100 kDa) and Microfiltration PVDF membranes (MWCO 0.45 µm, 47mm dia) were procured from Millipore, USA and Microfiltration nanofibrous membranes were obtained from National University of Singapore (NUS). All the chemicals used were of analytical grade.

3B.2.2. Methods

3B.2.2.1. Preparation of crude extract

The Baker’s yeast (dry form) was suspended in Tris buffer (50 mM, pH 7.5, 1:10 ratio (w/v)) and stirred for 1 hour. Disruption of yeast suspension was carried out using homogenizer (Ika, labortechnik, India) for 10 minutes at 10,000 rpm. The homogenate was centrifuged (CPR-24, Remi, India) for about 10 minutes at 10,000 rpm and the clear supernatant obtained (crude extract) was used for the experiments.
3B.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 enzyme extract making the total weight of the system 100% on w/w basis. The contents were mixed thoroughly for 1 hour using a magnetic stirrer and were allowed to separate for about 8 hours in a separating funnel to obtain clear phase separation. The top and bottom phases were collected, volumes were noted and analyzed for protein and enzyme activity. An average of three replicates was considered. The error in the analysis was within ±2%.

3B.2.2.3 Membrane processes

Microfiltration and ultrafiltration were carried out using stirred cell module (Amicon solvent resistant stirred cell module, Millipore, USA; capacity of 50 ml). 100 kDa membrane disc (Millipore, USA) of 47 mm diameter was used for ultrafiltration. PVDF microfiltration membrane (MWCO 0.45μm) of 47mm diameter was used for microfiltration. The nanofibrous membrane sheet (NUS, Singapore) was cut into discs of 47 mm diameter and used for microfiltration. The filtration experiments were carried out for 2h while maintaining the pressure (1.5 bar), stirring speed (250 rpm) and temperature (25 ± 2 °C) constant throughout the experiment. The pressure was applied using N₂ gas. Transmembrane flux was calculated based on the average flux.
3B.3. Results and Discussion

Purification of invertase was done by employing three different modes (Figure 2B.1) from the crude extract of baker’s yeast namely, Mode 1: ATPE followed by UF, Mode 2: UF (as diafiltration mode) followed by ATPE and Mode 3: MF followed by ATPE. Obtained results were tabulated in the Table 3B.1

**Mode 1: ATPE followed by UF**

Aqueous two phase extraction was carried out employing PEG-3350/magnesium sulphate system (14/15) for downstream processing of invertase (selected from chapter 2A). After ATPE, the top and bottom phases were separated and measured the volumes. The results obtained were given in the Table 3B.1. From the table it can be seen that invertase preferentially partitioned to the bottom phase and resulted in 86.7% enzyme activity recovery. ATPE has resulted in 8.5 fold purification with 33 U/mg specific activity. Further, the separated bottom phase was subjected to UF. Ultrafiltration was shown some improvement in the purification factor (8.5 to 10.89) due to removal of some contaminants along with phase components, at the same time with slightly reduced recovery (86.7 to 80.3%).

**Mode 2: UF followed by ATPE**

Ultrafiltration of crude extract was carried out employing 100 kDa membrane. The system used for membrane process is shown in Figure 3A.2. Diafiltration mode was used to for the processes because, UF normal mode has shown lower permeate flux than diafiltration (Figure 3B.1). Ultrafiltration has
shown removal of around 30\% other low molecular weight contaminant proteins from the crude extract. UF in diafiltration mode resulted in increased purification of 1.96 fold compared to crude. Obtained retentate was subjected to ATPE for further purification.

ATPE was carried out by employing predetermined concentrations of PEG 3350, magnesium sulphate system. Top and bottom phases were separated, volumes measured and assayed for enzyme activity. From the obtained results, bottom phase was showed around 12.94 fold purification of invertase with 88.96\% activity recovery. Specific activity was enriched to 50.09 U/mg compared to crude extract of 3.87 U/mg.

**Mode 3: MF followed by ATPE**

Microfiltration (MF) was employed for the primary purification of invertase before carrying out ATPE. MF with nanofibrous membrane has resulted in higher transmembrane flux compared to that with conventional PVDF membranes (Figure 3B.2). Microfiltration has clarified the crude extract and removed some of the contaminants (solute/proteins) from the crude extract helping in improving the purification of invertase. Permeate from MF of crude extract has resulted in 2.13 fold purification and recovery of 99.43\%. Obtained permeate was subjected to ATPE for further purification. After ATPE, highest invertase purification of 14.09 fold compared to crude extract was observed in bottom phase. From this mode highest specific activity of 54.52 U/mg was obtained with 92.41\% activity recovery and found to be the best among the studied combinations.
3B.4 Conclusions:

Integration of membrane process (ultrafiltration in diafiltration mode and microfiltration using nanofibrous membranes) with ATPE facilitated increased purification of invertase without losing much yield from crude extract. Mode 3 (MF followed by ATPE) has shown highest purification and found to be the best among the studied combinations. Process integration of MF with ATPE has resulted with over all increased purification of invertase 14.09 fold with 92.41% activity recovery.
Table 3B.1 Integrated approach for the concentration and purification of invertase.

<table>
<thead>
<tr>
<th>Operation</th>
<th>Phase</th>
<th>Protein Concentration (mg/ml)</th>
<th>Enzyme activity (U/ml)</th>
<th>Enzyme Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Enzyme activity Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td></td>
<td>2.87</td>
<td>11.1</td>
<td>3.87</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ATPE</td>
<td>Top</td>
<td>2.55</td>
<td>5.0</td>
<td>1.94</td>
<td>0.50</td>
<td>17.86</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>0.88</td>
<td>28.9</td>
<td>32.94</td>
<td>8.51</td>
<td>86.75</td>
</tr>
<tr>
<td>UF (100 kDa)</td>
<td>Bottom</td>
<td>0.63</td>
<td>26.8</td>
<td>42.16</td>
<td>10.89</td>
<td>80.34</td>
</tr>
<tr>
<td>UF (100 kDa)</td>
<td>Retentate</td>
<td>1.60</td>
<td>12.1</td>
<td>7.59</td>
<td>1.96</td>
<td>94.76</td>
</tr>
<tr>
<td></td>
<td>Top</td>
<td>2.90</td>
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<td>16.02</td>
</tr>
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<td>12.94</td>
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<td>32.4</td>
<td>54.52</td>
<td>14.09</td>
<td>92.41</td>
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Figure 3B.1 Transmembrane flux during ultrafiltration of invertase from crude yeast extract.
Figure 3B.2 Transmembrane flux during microfiltration of invertase from crude yeast extract.