Original Article

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Process Integration for Purification of Alcohol Dehydrogenase and Invertase from Baker's Yeast (*Saccharomyces Cerevisiae*)

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Abstract

The present work focuses on the integration of aqueous two-phase extraction with membrane processes (ultrafiltration / microfiltration) for the concentration and purification of alcohol dehydrogenase and invertase from crude extract of baker's yeast (*Saccharomyces cerevisiae*). Integration was carried out in three different modes. Integration of microfiltration (using nanofibrous membrane) with aqueous two-phase extraction was found to be best compared to other modes which has resulted in 8.12 fold purification with 647.32 U / mg specific activity and 95% enzyme activity recovery in case of alcohol dehydrogenase and 14.09 fold purification with 54.52 U / mg specific activity and 92.41% enzyme activity recovery in case of invertase.

Keywords: Aqueous Two-Phase Extraction; Bioseparation; Process Integration; Purification; Ultrafiltration; Yeast; Enzymes.

Introduction

Recent advances in biotechnology have focused on downstream processing, which constitutes a major portion of the production costs. However, much research has not been carried out in this area. There is a strong demand for downstream processing methods, which increase the yield, while reducing the process time and capital expenditure [1]. Process integration, wherein two unit operations are combined into one in order to achieve specific goals, which are not effectively met by these unit operations when they employed alone, offers considerable potential benefit for the recovery and purification of biological products [2]. It could be integration of extraction with membrane processes or different membrane processes with each other for achieving desired selectivity and purity of the biomolecule. Several integrated approaches have been developed to optimize productivity and cost effectiveness of different bioprocesses [3-5]. For instance the feasibility of aqueous two-phase extraction and membrane processes was demonstrated for the separation and purification of proteins [6]. Microfiltration and ultrafiltration have been widely used as preferred methods for protein concentration, purification, buffer exchange and has effectively replaced size exclusion chromatography [1, 7-9]. In view of the high commercial potential of the enzymes, several attempts have been made to obtain a stable enzyme preparation suitable for commercial application.

In order to evaluate the efficacy of the process integration, the work was carried out for the downstream processing of alcohol dehydrogenase (ADH) and invertase from baker's yeast (*Saccharomyces cerevisiae*), which are the main commercially valuable enzymes present abundantly in yeast.

Alcohol dehydrogenase (E.C. No. 1.1.1.1) enzyme is widely used in biochemical, forensic science for

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estimating the concentration of primary alcohols, NAD^+, ethylene glycol, numerous aldehydes and enzymatic catalysis of organic solvents and also in biosensors. 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% [10-13].

Invertase (β-D-Fructofuronoside fructohydrolase; E.C. 3.2.1.26) is mainly used in the food industry (confectionery, syrups, condensed milk, infant foods and beverages). It is also used for the manufacture of artificial honey, plasticizing agents used in cosmetics, pharmaceutical and paper industries as well as enzyme electrodes for the detection of sucrose. Various researchers have developed several methods for the purification of invertase [14-18]. 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 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% [19]. Other report on invertase purification using precipitation followed by DEAE-column chromatography resulted in 5.8 fold purification with recovery of 3.2% [20]. However, almost all these methods of purification 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 [21].

ATPE has been employed for the primary recovery and partial purification of a variety of biological products, including proteins, genetic material, low molecular weight products, cells and cell organelles [22-23]. The main advantages of this technique include scaling up feasibility, process integration capability and biocompatibility. The separation involving membrane processing and aqueous two-phase extraction (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) and invertase from baker's yeast to achieve higher purity without losing recovery.

The majority of the commercially available microfiltration / ultrafiltration membranes are inherently non homogeneous (non-uniform in mass and thickness), which 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 the enzymes. Different combinations of ATPE and membrane process were used for downstream processing of these enzymes.

Materials and Methods

Materials

Polyethylene glycol (PEG, mol. wt 6000 and 20,000), peroxidase, sucrose, o-dianisidine, glucose oxidase, glycerol and glucose were procured from Sigma Aldrich, MO, USA. Potassium phosphate salts (KH_2PO_4, K_2HPO_4), trisodium citrate ($C_6H_5O_7Na_3$), hydrochloric acid (HCl), sodium sulphate (Na_2SO_4) were from Merck, Mumbai, India. Nicotinamide adenine dinucleotide (NAD^+) from Himedia (India), Ammonium sulphate ($(NH_4\text{-})_2SO_4$), magnesium sulphate ($MgSO_4$), sodium phosphate (Na_2HPO_4, NaH_2PO_4), were purchased from Ranbaxy Chemicals Gurgaon, India. All the chemicals used were of analytical grade. Baker's yeast was procured from local super market. Ultrafiltration membranes (100 kDa) and PVDF microfiltration membranes (0.45 mm, 47 mm) were procured from Millipore, USA and Microfiltration nanofibrous membranes were obtained from National University of Singapore (NUS).

Methods

Crude Extract Preparation

Crude extract was prepared using Baker's yeast (dry form, 1:10 ratio (w / v)) in 10 mM sodium phosphate buffer for ADH and in Tris buffer (50 mM, pH 7.5) for invertase extraction (8, 24). 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.

Protein Concentration

Bradford method [25] was used to determine the concentration of the protein using coomassie brilliant

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).

Enzyme Activity

ADH assay was carried out using ethanol as substrate in presence of NAD^+ (26). 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 mmole acetaldehyde from ethanol per minute, at pH 8.8 at 25°C [27].

Invertase activity was measured as per the protocol described earlier [24] using sucrose as substrate and glucose was determined by glucose oxidase method One unit of invertase activity is defined as the amount of enzyme at pH 4.9 which hydrolyzes sucrose to produce 1 µmole of glucose / minute at 30^0 C [24].

Aqueous Two-Phase Extraction

Aqueous two-phase extraction experiments were carried out in the following manner. Predetermined quantities of polymers and salts from the phase diagrams [22, 28] 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%.

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 mm 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 N_2 gas. Transmembrane flux was calculated based on the average flux.

Results and Discussion

Purification of alcohol dehydrogenase and invertase was carried out separately by employing three different modes (presented in Figure 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. ATPE experiments were carried out by selecting the standardized phase compositions from literature [8, 24].

Purification of ADH

ATPE Followed by UF (Mode 1)

Aqueous two-phase extraction of ADH was carried out employing PEG-20000 / potassium phosphate system (12 / 7.33 %, w / w). The top and bottom phases were separated after ATPE and the volumes were measured. The results are given in the Table 1. From the table it can be seen that ADH preferentially partitioned to the bottom phase with 96.94 % 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 is shown in Figure 2. Ultrafiltration has 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 from 96.94 to 91.38 %.

UF Followed by ATPE (Mode 2)

The crude extract (50 ml) was subjected to ultrafiltration for 2 hr at 1.5 bar pressure using 100 kDa membrane to obtain 45 ml of permeate. Fig. 3 shows the comparison of flux rates in case of UF in normal mode and UF in diafiltration mode. Diafiltration mode was used to reduce both concentration polarization and membrane fouling in order to maintain the flux. It can be seen from the figure that UF in diafiltration has shown higher flux compared to UF (in normal mode). Around 20% of the contaminant proteins were removed from the crude extract during ultrafiltration (diafiltration mode) resulting in 1.24 fold enrichment of the enzyme. The retentate obtained was subjected to ATPE for further purification.

After ATPE, around 7.53 fold purification of ADH was observed with 97.4 % activity recovery in the bottom phase. Specific activity has enhanced to 600.29 U / mg compared to crude extract of 79.67 U / mg (Table 1).

MF followed by ATPE (Mode 3)

In this mode microfiltration was employed followed by ATPE for the purification of ADH. MF, using membrane with MWCO 0.45 mm, was employed for the clarification of crude extract which has resulted in around 1.3 fold enrichment. However, the recovery was slightly low (90%). MF was also carried out using nanofibrous MF membranes. Figure 4 shows the flux rates of normal PVDF and nanofibrous membranes and it can seen that nanofibrous membranes have resulted in higher flux (19 L / m^2h) with high recovery (96 %) compared to conventional PVDF membranes (6.5 L / m^2h). The permeate obtained was subjected to ATPE for further purification. After ATPE, 8.12 fold purification of ADH was observed in the bottom phase. 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.

Purification of Invertase

ATPE followed by UF (Mode 1)

ATPE was carried out at standardized conditions employing PEG-3350 / magnesium sulphate system (14 / 15, % w / w) for downstream processing of invertase [24]. The top and bottom phases were separated after ATPE and measured the phase volumes. The results obtained are given in the Table 1. From the table it can be seen that invertase preferentially partitioned to the bottom phase and resulted in 8.5 fold purification with 86.75 % enzyme activity recovery. The bottom phase was subjected to UF which has enhanced the purification factor from 8.5 to 10.89 fold, at the same time with a slight reduction in enzyme activity recovery (86.7 to 80.3%).

UF followed by ATPE (Mode 2)

Ultrafiltration of crude extract was carried out employing 100 kDa membrane. The system used for membrane process is same as shown in Figure 2. UF in diafiltration mode was used to for the processes because, UF in normal mode has shown lower permeate flux (4 L / m^2h at 120 min) compared to UF in diafiltration (7 L / m^2h at 120 min) (Figure 5). UF has shown removal of around 30% other low molecular weight contaminant proteins from the crude extract resulting in increased purification of 1.96 fold (Table 1). The retentate obtained was subjected to ATPE for further purification.

Table 1: Integrated approach for the purification of alcohol dehydrogenase

Mode of	operation	Phase	Protein Concentration (μg/ml)	Enzyme activity (U/ml)	Enzyme Specific activity (U/mg)	Degree of Purification (fold)	Enzyme activity Recovery (%)
	-	Crude	85.67	6.8	79.67	-	-
Mode 1	ATPE	Top	17.27	0.5	27.93	0.35	5.67
		Bottom	45.87	24.0	522.62	6.56	96.94
	ATPE-UF	Bottom	41.00	23.9	582.90	7.32	91.38
Mode 2	UF	Retentate	67.93	6.7	98.69	1.24	98.59
	UF-ATPE	Top	16.67	0.5	30.39	0.38	5.96
		Bottom	40.13	24.1	600.29	7.53	97.43
Mode 3	MF	Permeate	65.73	6.9	104.56	1.31	96.02
	MF- ATPE	Top	15.73	0.4	27.59	0.35	5.11
		Bottom	**39.27**	**25.4**	**647.32**	**8.12**	**95.32**

System: PEG-20000 / potassium phosphate (12 / 7.33 %, w / w)

Table 2: Integrated approaches for the purification of invertase.

operation	Phase	Protein Concentration (mg/ml)	Enzyme activity (U/ml)	Enzyme Specific activity (U/mg)	Degree of Purification (fold)	Enzyme activity Recovery (%)
-	Crude	2.87	11.1	3.87	-	-
ATPE	Top	2.55	5.0	1.94	0.50	17.86
	Bottom	0.88	28.9	32.94	8.51	86.75
UF	Bottom	0.63	26.8	42.16	10.89	80.34
UF	Retentate	1.60	12.1	7.59	1.96	94.76
UF-ATPE	Top	2.90	4.4	1.53	0.40	16.02
	Bottom	0.69	34.4	50.09	12.94	88.96
MF	Permeate	1.55	12.7	8.24	2.13	99.43
MF-ATPE	Top	2.86	5.0	1.76	0.46	18.17
	Bottom	**0.59**	**32.4**	**54.52**	**14.09**	**92.41**

System: PEG 3350 / Magnesium sulphate (14 / 15 % w / w)

The top and bottom phases were separated after ATPE and measured the phase volumes. Around 12.94 fold purification of invertase was observed with 88.96 % enzyme activity recovery in the bottom phase. Specific activity has enhanced to 50.09 U / mg compared to crude extract of 3.87 U / mg (Table 1).

MF Followed by ATPE (Mode 3)

MF with nanofibrous membrane has resulted in higher transmembrane flux (21.1 L / m^{2}h at 60 min) compared to that of MF with conventional PVDF membranes (6.5 L / m^{2}h at 60 min) (Figure 6). Microfiltration has clarified the crude extract and removed some of the contaminants (solutes / proteins) resulting in 2.13 fold purification and enzyme activity recovery of 99.43 %. The permeate obtained was subjected to ATPE for further purification. After ATPE, highest invertase purification of 14.09 fold with 92.41% activity recovery was observed in bottom phase.

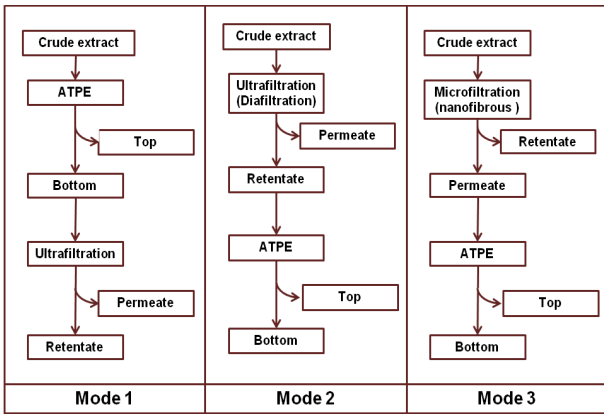


Fig. 1: Different combination of ATPE and membrane processes for downstream processing

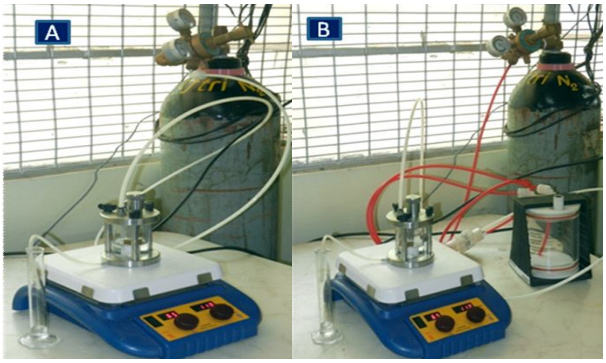
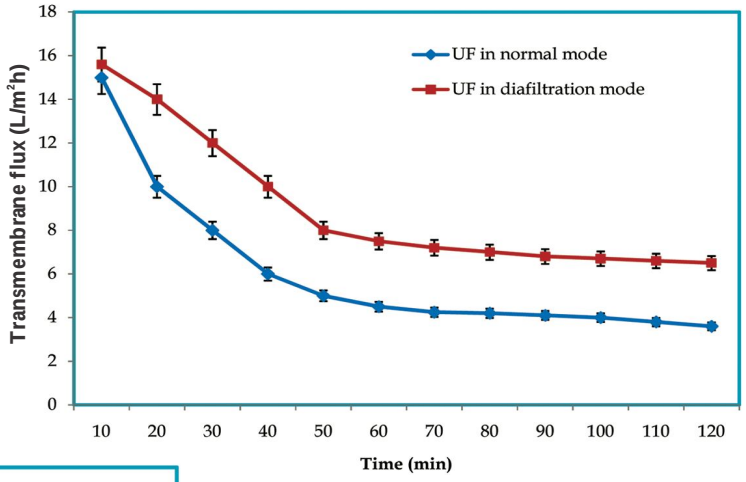


Fig. 2: Membrane processing set up used for the study. A) Ultrafiltration in normal mode; B) Ultrafiltration in diafiltration mode

Fig. 3: Transmembrane flux during ultrafiltration of ADH from crude yeast extract



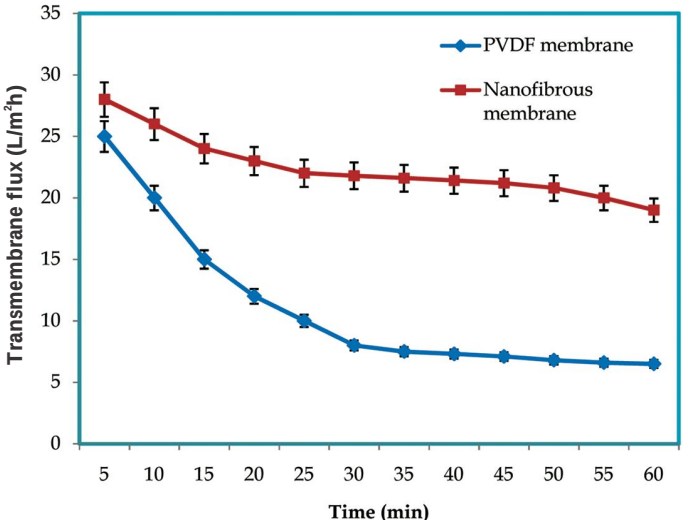


Fig. 4: Transmembrane flux during microfiltration of ADH from crude yeast extract

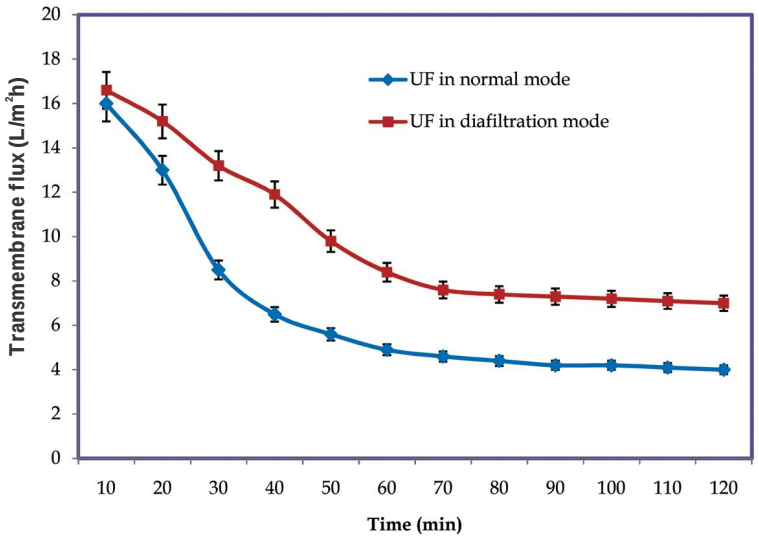


Fig. 5: Transmembrane flux during ultrafiltration of invertase from crude yeast extract

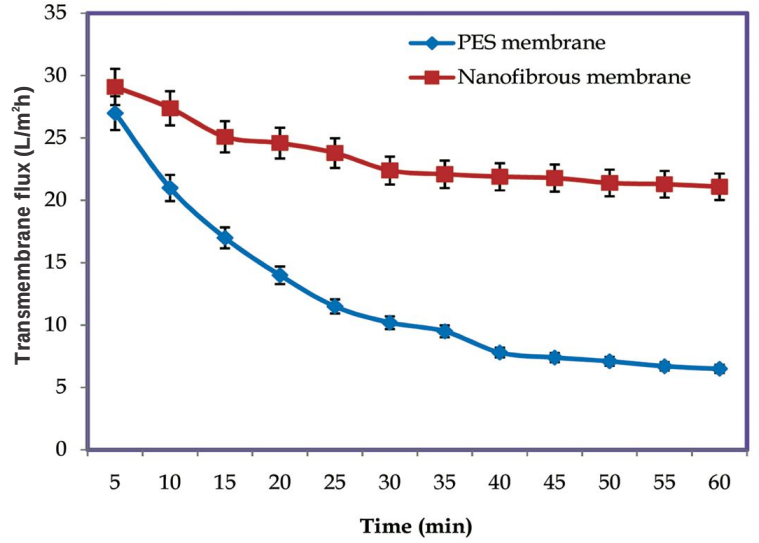


Fig. 6: Transmembrane flux dung microfiltration of invertase from crude yeast extract

Conclusions

The efficacy of integration of membrane processes with ATPE was demonstrated for the downstream processing of ADH and invertase from baker's yeast for achieving the higher degree of purification without losing of much yield. MF followed by ATPE was found to be the best among the combinations studied (3 modes) in case of both the enzymes. Integration of nanofibrous microfiltration with aqueous two-phase extraction has resulted in 8.12 fold purification with 95% activity recovery in case of ADH and 14.09 fold with 92.41% activity recovery in case of invertase.

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