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# Hydrophobic interaction ligand selection and scale-up of an expanded bed separation of an intracellular enzyme from *Saccharomyces cerevisiae*

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**Abstract**

A prototype Streamline-Phenyl matrix was evaluated in a hydrophobic interaction mode for the direct recovery of alcohol dehydrogenase (ADH) from yeast cell homogenate. At 5% breakthrough of ADH, a yield of 100% was obtained for a dynamic expanded bed capacity of 240 U(ADH)/ml matrix with a purification factor of 9.2. This compared with a dynamic capacity of 3013 U(ADH)/ml matrix for the packed bed equivalent and a purification factor of 18. In both systems the purification factor was found to increase simultaneously with a decrease in yield as the load of homogenate or breakthrough of ADH was increased. The expanded bed mode of operation conferred considerable robustness with respect to process fouling. No loss in yield was seen over five cycles of repeat loading with an unclarified homogenate. By contrast the packed bed media showed a decrease in yield from 86 to 56% over the same period. Successful scale up of the expanded bed protocol for a 20% breakthrough was demonstrated over a fourfold increase in column diameter. The application of hydrophobic interaction chromatography mediated expanded bed adsorption and its scale-up is discussed in the context of large-scale operations.


*Keywords:* Expanded bed chromatography; *Saccharomyces cerevisiae*; Hydrophobic interaction chromatography; Preparative chromatography; Adsorption; Enzymes

## 1. Introduction

Packed bed adsorption chromatography is widely employed for the concentration and purification of proteins [1]. Chromatographic capture is usually achieved early in downstream processing so as to reduce proteolytic degradation [2] and to reduce the overall working volume. Use of packed beds requires the removal of whole cells or colloidal debris. Though often ignored in the development of laboratory isolation schemes, the removal of debris is one of the most difficult downstream operations to achieve with high efficiency [3,4].

An alternative solution, avoiding the need for any

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process stream clarification, is to use fluidized bed and more recently expanded bed adsorption (EBA). Both use the flow of fluid though an initially packed bed structure to achieve a contactor with a high fluid voidage. In the former the adsorbent particles become well-mixed by virtue of the fluid flow. In the latter, which operates at lower velocities, the particles expand to a steady-state condition where they are essentially stationary but separated from one another in the direction of flow. The EBA structure is therefore similar to that of a packed bed but with a very much higher voidage. In operation the ordered packing of EBA provides a much higher degree of chromatographic resolution compared to that of a fluidised bed. Early applications include the isolation of streptomycin [5] and novobiocin [6] from their respective fermentation liquors and the recovery of bovine serum albumin (BSA) spiked into blood plasma and *Saccharomyces cerevisiae* cell suspensions [7] and for protein isolation [8–10]. This research demonstrated the feasibility of using traditional matrices for protein adsorption from crude feed streams. However the low density of the packed bed matrices studied dictated the use of low liquid velocities and the matrices gave significantly lower adsorptive capacities for model proteins from unclarified feed streams.

Matrices for EBA incorporate a dense core for increased particle density and possess a wide particle size distribution [11]. They are available with a wide range of functional groups and have been widely reported upon and reviewed within the literature [12–16]. Though widely used in conventional laboratory isolation techniques ion-exchange matrices are not well suited for the direct capture of proteins due to the high ionic strength of fermentation broth and cell homogenates and the presence of large quantities of contaminating species often possessing a similar charge as the target molecule. To facilitate adsorption under high ionic strength, extensive diafiltration or dilution must first be performed [17].

More selective adsorption techniques have been employed to circumvent this issue. Dye-affinity techniques have showed promise [15] but may be complicated by ligand leakage. Immobilized metal ion affinity chromatography (IMAC) has been reported [16]. Hydrophobic interaction chromatography (HIC) first introduced in 1948 [18] provides a relatively gentle separation technique with elution by decreasing salt concentration. The attractiveness of hydrophobic interaction lies in part with the stabilizing effects of the salt anions used during adsorption. Elution in low salt conditions generally ensures that compounds retain their native conformation and biological activity [19].

The ability for adsorption to occur at high ionic strength in the presence of cellular contaminants and debris offers the potential for process intensification such as the tighter integration of fermentation with purification, where cells are typically washed free of high ionic strength nutrients prior to adsorption. Product elution at low ionic strength is ideal for subsequent high resolution purification steps such as ion-exchange chromatography.

This paper reports on the development of an expanded bed adsorption protocol using a prototype HIC matrix for the direct capture of a target molecule, alcohol dehydrogenase, from a crude yeast cell homogenate. Scouting was carried out using 1-ml packed beds, pure protein and clarified feed streams. The optimized adsorption protocol was translated to a laboratory-scale expanded bed system. The fouling characteristics of both packed and expanded bed systems were investigated using a standardized clean-in-place (CIP) protocol.

## 2. Materials and methods

### 2.1. Assays

Total protein concentration was determined by the Bradford assay [20]. Alcohol dehydrogenase (ADH, E.C. 1.1.1.1) activity was determined by the method of Bergmeyer [21]. Assays were performed in duplicate with a reproducibility of $\pm 5\%$.

### 2.2. Cell disruption and clarification

Bakers' yeast (DCL, Crawley, UK) was suspended to 450 g/l (wet mass) in buffer (0.1 *M* $KH_2PO_4$, pH 6.5) and disrupted by high-pressure homogenizers [$<$300 ml, Model Lab 40; $<$10 l, Model Lab 60; $>$10 l, Model K3 (all APV, Crawley, UK)]. Homogenization was for five discrete passes at 500 bar (*g*). Clarification of the homogenate prior to

loading onto packed bed columns was by centrifugation (Model J2-M1, Spinco, Beckman Instruments, Palo Alto, CA, USA) with a fixed angle rotor (type JA-18) at 16 000 rpm (37 800 *g*) for 1 h.

### 2.3. Chromatography columns and equipment

Ligand selection was carried out on 1-ml HiTrap columns and a fast protein liquid chromatography (FPLC) system incorporating a conductivity meter. Voltage output signals from the on-line absorbance at 280 nm and conductivity monitors were collected using two A/D interfaces (Model 970/0, Perkin-Elmer, Nelson Systems, CA, USA) connected to an IBM compatible personal computer before processing using chromatography software (Turbochrom v.4.1, Perkin-Elmer, CA, USA).

Intermediate-scale chromatography trials (300 ml bed volume) were performed with 50 mm diameter XK50/40 packed or Streamline-50 expanded bed columns. Packed bed height was constant. Peristaltic pumps (Model 505DU, Watson Marlow, Falmouth, UK) were used for chromatographic operation and hydraulic positioning of the upper expanded bed adapter. On-line absorbance was monitored at 280 nm using a UV meter fitted with an industrial 10 mm path-length flow cell. Salt concentration was monitored using a combined pH and conductivity meter (Model: pH/ion Multiplexing monitor, Sepracor, MA, USA). A fraction collector was used to collect eluate from the chromatography systems.

An FPLC controller was used to control two pumps and two motorized valves to allow both packed and expanded bed columns to be operated using common ancillary equipment. A bubble trap upstream of the flow reversal valve was used to prevent air-bubbles entering both the packed and expanded bed.

Pilot-scale expanded bed adsorption was performed in a 200 mm diameter Streamline column. Peristaltic pumps (Model 505DI, Watson Marlow) were used with 8 mm I.D. silicone tubing for both sample loading and adapter positioning. Two 300-l mobile buffer tanks equipped with top mounted impellers were connected together through a T-piece upstream of a stainless steel 10 mm I.D. four-port–two-way valve. Also connected to this valve via 10 mm I.D. tubing with sanitary fittings, clamps and gaskets was a mobile 100-l buffer tank for preparation and storage of homogenate during loading onto the column. Upstream of the valve was a pressure gauge and a four-port–four-way valve for flow reversal. Eluate from the column was also monitored for UV absorbance at 280 nm and on-line conductivity.

### 2.4. Hydrophobic ligand selection

Five matrices were examined; Phenyl Sepharose Fast Flow (FF) [(low substitution (~mg/ml) and high substitution (mg/ml)], Phenyl Sepharose High Performance and Butyl Sepharose Fast Flow and Octyl Sepharose Fast Flow. Columns were equilibrated with buffer A [10 column volumes (CVs); 0.78 *M* $(NH_4)_2SO_4$ in 0.02 *M* $KH_2PO_4$, pH 7] and the retention characteristics of a 2 CV load of 2 mg (pure ADH)/ml (buffer A) evaluated for each matrix. Non-bound protein was washed from the columns using 11 CVs of buffer A. Elution was performed using a 10 CV linear gradient from 0.78 to 0 *M* ammonium sulfate.

### 2.5. Bed expansion characteristics

The expansion characteristics of the prototype matrix Streamline-Phenyl (low substitution) were investigated in a Streamline-50 column at 20 °C. The settled bed (350 ml) was expanded using deionized water and subsequently equilibration buffer A increasing the velocity in 0.5 m/h steps, every 0.5 h, to a final liquid velocity of 4 m/h. Data were fitted to the Richardson–Zaki correlation [22] yielding the expansion coefficient ''*n*'', and the experimental terminal velocity of the mean particle size.

### 2.6. Characterization of the dynamic and total binding capacity for ADH in expanded bed systems

For the expanded bed system, a Streamline-50 column containing the prototype Streamline-Phenyl matrix (250 ml) was equilibrated and expanded at 2 m/h using buffer A. The upper adapter was set to a constant height of 0.7 m. Unclarified yeast homogenate was adjusted to 0.78 *M* ammonium sulfate with saturated salt and diluted to 10 mg (total protein)/ml buffer. Increasing volumes of homogenate were

loaded. Cell debris and non-bound protein were removed from the expanded bed by washing with buffer A until the eluate absorbance had returned to baseline. The bed was then allowed to gravity settle. Elution of the now packed matrix was performed in the reverse flow direction at 0.75 m/h using a step decrease in salt concentration from 0.78 to 0 *M* ammonium sulfate.

### *2.7. Characterization of fouling*

Fouling of the EBA system during repeated adsorption cycles was examined in an XK50/40 column packed with Phenyl Sepharose Fast Flow (low substitution) to a final bed height of 0.17 m by repeated loading of 1 CV of clarified yeast supernatant. Non-bound protein was washed from the column using 6 CVs of buffer A. Elution was performed using a step decrease in salt concentration as before. Column breakthrough, wash and elution fractions were collected and assayed for ADH and total protein. A CIP protocol described below was performed between each cycle to regenerate the matrix. In a similar fashion, one settled bed volume (350 ml) of unclarified homogenate was repeatedly loaded onto a Streamline-50 column containing Streamline-Phenyl.

### *2.8. Cleaning-in-place protocols*

The CIP protocol used 1 *M* NaOH at a velocity of 3 m/h pumped onto the column to move the adapter to its uppermost position. This procedure was employed to rinse the column wall and space above the adapter. The flow direction was reversed and the plate and net in the upper adapter were rinsed at 1 m/h for 0.1 h. Flow was returned to the upward direction, the adapter height lowered to twice the settled bed height and the automated CIP protocol as follows was used [1 *M* NaOH, 8 CVs; deionized water, 3 CVs; 30% (v/v) isopropanol, 3 CVs; acetic acid, 3 CVs; deionized water, 5 CVs; 20% (v/v) ethanol, 5 CVs]. Ethanol was included in the CIP protocol as a preservative for long term storage.

### *2.9. Scale-up of expanded bed adsorption using Streamline-Phenyl*

For the verification of large-scale expanded bed (Streamline-200 column) performance, a partial breakthrough curve was generated by loading of homogenate onto a Streamline-50 column containing 308 ml of the same batch of matrix. Unclarified homogenate was loaded onto the column to an ADH breakthrough of 20% of the total inlet concentration. Column washing elution and regeneration were performed as previously described. Scale-up trials were conducted using 5 l of Streamline-Phenyl (low substitution) matrix equivalent to a settled bed height of 0.157 m. Homogenate was loaded onto the Streamline-200 column, expanded to approximately three times the settled bed height with equilibration buffer at 2 m/h. Wash, elution and column regeneration were performed as previously described, scaled for the increased volume requirements of the larger diameter column.

## 3. Results and discussion

The use of ammonium sulfate for the salting out of alcohol dehydrogenase from clarified yeast homogenate has been reported [23,24]. Concentrations of up to 40% saturation (1.56 *M*) do not precipitate ADH. To avoid conditions under which precipitation may occur, a salt concentration of 20% saturation (0.78 *M*) was selected as a starting point for further method development. The hydrophobic matrices were evaluated under identical chromatographic conditions for the retention of 800 U of ADH. The full chromatograms for the four matrices are presented in Fig. 1. The interaction with Phenyl Sepharose Fast Flow (high substitution) was too strong and resulted in only 28% of the mass and 34% of initial activity of ADH loaded being recovered. Butyl, Octyl and Phenyl Sepharose all allowed for the recovery of 100% of the enzyme activity, but both Octyl and Phenyl Sepharose Fast Flow (low substitution) gave slightly lower yields of ADH mass due to broadening of the eluted peak.

Gagnon et al. [25] discussed hydrophobic ligand selection in the context of large scale process development and suggested that the best matrix for hydrophobic interaction chromatography provided for the highest mass and activity recovery without producing aggregation or on-column conformational modifications. For expanded bed adsorption from crude cell homogenates other hydrophobic species

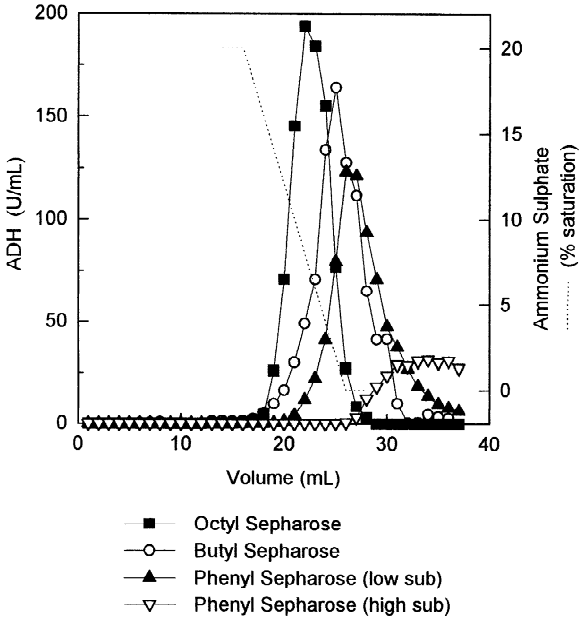


Fig. 1. Rapid ligand selection using a HiTrap HIC test kit.

would be expected to reduce the effective capacity of the matrix, a ligand demonstrating very high interaction strength whilst still meeting the above criteria was needed. Phenyl Sepharose Fast Flow (low substitution) was selected for further study.

Experimentally-derived values for the Richardson–Zaki coefficient [22] were consistent with those for Streamline-DEAE indicating that the prototype matrix possessed similar fluidization properties to commercially available matrices.

Studies of the dynamic and total binding capacity for the packed bed matrix (data not shown) gave a dynamic binding capacity at 5% breakthrough of ADH of was 1021 U/ml with a purification factor of 6.8 and yield of 92%. At 100% breakthrough the dynamic capacity was 3013 U/ml with a purification factor of 18 but a yield of only 26%. The trade offs in yield purity and purification factor for this system are examined in Fig. 2 for increasing loads of clarified homogenate passed to the packed bed system and show the expected fall off in yield as capacity and purification factor each rise.

The results of mass balances conducted over five repeat cycles of loading partially clarified homogenates to the 5 cm expanded bed system are summarized in Table 1. A typical chromatogram is illus-

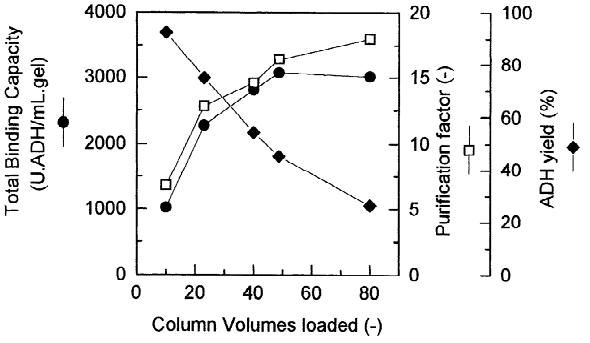


Fig. 2. Yield, purity and binding capacity operating curves for the adsorption of ADH from clarified cell homogenate using the packed bed matrix Phenyl Sepharose FF (low substitution).

trated in Fig. 3, resulting from a 2-CV load of homogenate, equivalent to a 5% breakthrough of ADH. Under this load a yield of 93% and a purification factor of 9.2 were obtained. These values are similar in magnitude to those obtained with the equivalent 10-CV packed bed loading illustrated in Fig. 2, however as expected for the expanded bed the dynamic capacity of the matrix was much lower at 240 U/ml (settled matrix). This represents an approximate fourfold reduction in available capacity of the expanded bed over that of the packed bed of comparable volume but packed with a smaller diameter adsorbent particles.

In the EBA runs the majority of contaminating proteins were immediately eluted in a narrow peak. The more strongly adsorbed ADH eluted as a broader peak possibly caused by the interaction of desorbed enzyme with hydrophobic regions further along the length of the column, not saturated with ADH, at salt concentrations between 0.78 and 0 *M*. Successive loads of clarified homogenate resulted in broadening of the ADH peak due to rapid irreversible fouling of the matrix. The elution volume over which ADH was collected during the first loading to virgin matrix was set constant and ADH eluting outside of range volume during successive chromatography cycles was discarded. As shown in Table 2 the fouling of the column resulted in a decrease in yield for the packed bed from 86 to 56% over five cycles.

Results of the comparative EBA loading study are shown in Fig. 4. A considerable volume of wash buffer (approximately 10 CVs) was required to remove colloidal material and unbound protein from

Table 1
Mass balances for the repeated load of one column volume (1 CV) of clarified supernatant onto a 5 cm diameter packed bed of Phenyl Sepharose FF (low substitution)

| Cycle number | ADH load (U) | Protein load (mg) | ADH wash (U) | Protein wash (mg) | ADH eluted* (U) | Protein eluted (mg) | Yield ADH (%) | Mass balance ADH (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 31 774 | 3374 | 11 | 1452 | 27 356 | 794 | 86 | 86 |
| 3 | 39 622 | 3388 | 3 | 1498 | 28 588 | 860 | 72 | 72 |
| 5 | 37 953 | 3468 | 4 | 1405 | 21 817 | 740 | 58 | 58 |

*Elution volume set equal to the elution volume range of ADH collected over cycle 1

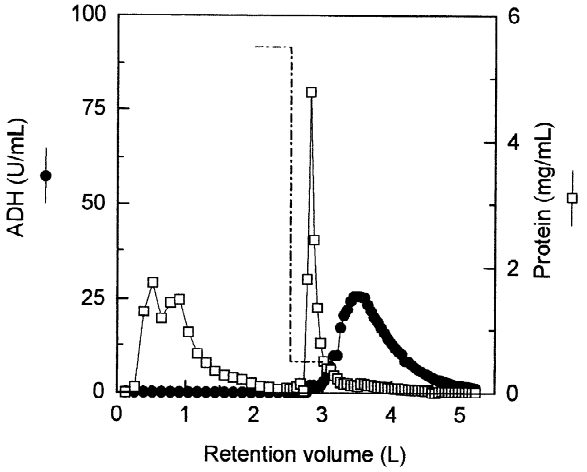


Fig. 3. Expanded bed recovery (5% breakthrough) of ADH directly from crude cell homogenate. Ammonium sulfate salt gradient (.).

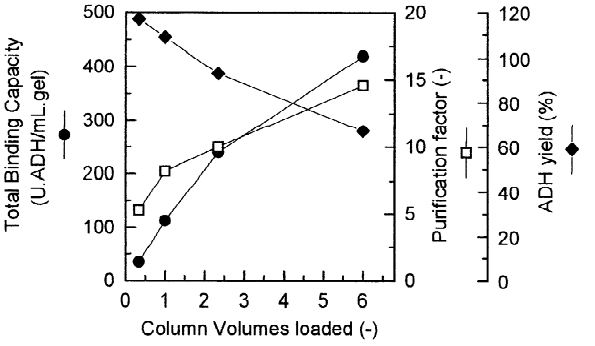


Fig. 4. Yield, purity and binding capacity operating curves for the adsorption of ADH from unclarified cell homogenate using the expanded bed matrix Streamline-Phenyl (low substitution).

the expanded bed. Others [38] addressed this issue by including 25% (v/v) glycerol in the wash buffer to reduce the overall volume of buffer and shorten cycle times. For extensive loads, it was necessary to reverse momentarily the direction of flow to the column several times to dislodge cellular debris which had accumulated at the top adapter. Elution of both protein and ADH was in relatively narrow peak widths unlike those observed with the packed bed systems. This was thought to be a consequence of reversing the elution direction in the EBA system such that desorbed ADH was not able to re-bind further down the column.

As shown in Table 3 unlike for the packed bed system, there was no reduction in the recovery of ADH and up to five cycles were possible with no apparent fouling of the matrix or accumulation of cellular debris anywhere in the system. The profiles

Table 2
Mass balances for the repeated load of one column volume (1 CV) of crude cell homogenate onto a 5 cm diameter expanded bed of Streamline-Phenyl (low substitution)

| Cycle number | ADH load (U) | Protein load (mg) | ADH wash (U) | Protein wash (mg) | ADH eluted* (U) | Protein eluted (mg) | Yield ADH (%) | Mass balance ADH (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | 32 468 | 3537 | 2364 | 2394 | 30 379 | 348 | 93 | 96.3 |
| 3 | 28 113 | 3501 | 2385 | 2623 | 36 781 | 324 | >100 | >100 |
| 5 | 33 656 | 3722 | 2180 | 2460 | 34 244 | 289 | >100 | >100 |

*Elution volume set equal to the elution volume range of ADH collected over cycle 1

Table 3
Scale-up of expanded bed adsorption of ADH from crude yeast cell homogenate

| | Streamline-50 | | | | | | Streamline-200 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Purification stage | Volume (l) | Total ADH (U, $\cdot 10^3$) | Total protein (mg, $\cdot 10^3$) | Specific activity (U/mg) | PF (–) | Yield (%) | Volume (l) | Total ADH (U, $\cdot 10^3$) | Total protein (mg, $\cdot 10^3$) | Specific activity (U/mg) | PF (–) | Yield (%) |
| [A] Homogenate | 20 | 4454 | 613 | 7.25 | 1 | 100 | 20 | 4141 | 542.2 | 7.63 | 1 | 100 |
| [B] To 0.78 *M* $AmSO_4$ | 25 | 4450 | 623 | 7.13 | 0.98 | 99.9 | 25 | 3974 | 536 | 7.41 | 0.97 | 96 |
| [C] Dilute | 58.5 | 4433 | 624 | 7.09 | 0.98 | 97.5 | 53.6 | 4020 | 553.1 | 7.26 | 0.95 | 97 |
| [D] Load | 1.24 | 93.4 | 13.15 | 7.1 | 1 | 100 | 21.28 | 1,59 | 219.6 | 7.26 | 1 | 100 |
| [E] Breakthrough | 1.24 | 2.01 | 0.92 | – | – | – | 21.28 | 32.1 | 13.1 | – | – | – |
| [F] Wash | 3.27 | 21.5 | 8.79 | – | – | 23 | 48.72 | 344.4 | 171.5 | – | – | 21.5 |
| [G] Elution | 1.32 | 81.1 | 1.14 | 71.2 | 10.0 | 86.8 | 20.43 | 1272 | 21.8 | 58.3 | 8.02 | 79.7 |

for the eluted ADH peak overlapped precisely in each instance (not shown).

In order to validate the scale-up of the hydrophobic expanded bed process, unclarified homogenate was loaded onto a 5 and 20 cm diameter Streamline-50 columns to 20% breakthrough of ADH. The large-scale chromatography cycle was scaled by maintaining the ratio of buffer volume to matrix volume based on the 5 cm diameter process. The data from both the 5 cm and 20 cm expanded bed adsorption cycles are presented in a purification table describing both scales of operation (Table 3).

The chromatograms were essentially identical in profile and the purification tables demonstrate that the scale-up of the hydrophobic expanded bed process was successful. The dynamic binding capacity at 20% breakthrough of ADH for the small scale was 263 U/ml while the large scale capacity was 259 U/ml. Purification factors were similar and the yields attained at both scale were within 10% of each other. Variations in both purity and yield are most likely due to small differences in the specific activity of the feed to both columns and are both bounded by the error of the protein assay used in the study.

## 4. Conclusions

Hydrophobic interaction chromatography in an expanded bed adsorption protocol has been successfully employed for the direct capture of an intracellular enzyme from a realistic bioprocess stream. Using small-scale packed bed method scouting, appropriate adsorption conditions and a suitable hydrophobic ligand were rapidly identified and a prototype Streamline-Phenyl matrix was subsequently investigated for the direct capture of alcohol dehydrogenase. One step purification factors of 8–14 at high yields were possible but the capacity of the prototype matrix was fourfold lower than a high quality packed bed matrix.

This work highlighted several considerations in the implementation of expanded bed adsorption in traditional biochemical recovery processes. The fourfold reduction in capacity of the EBA matrix compared to the packed bed matrix must be considered alongside the process benefits of EBA. Eliminating several clarification stages prior to EBA has the benefit of increasing overall process yields and reducing capital and operating costs associated with key equipment such as centrifuges and membranes. The potential re-use of the expanded bed matrix, due to its low fouling characteristics, is also an important consideration. In this instance, the need to operate a much larger expanded bed column to achieve the same mass recovery of a protein may potentially be offset by the cost savings described above. However the use of high salt concentrations for adsorption may represent a significant challenge for subsequent disposal of effluent though the convenience of product elution in low salt may again offset this apparent disadvantage since it enables more effective process integration.

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