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ORIGINAL PAPER

Screen-less expanded bed column: new approach for the recovery and purification of a malaria transmission blocking vaccine candidate from *Pichia pastoris*

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Abstract An experimental malaria transmission blocking vaccine antigen, Pfs25H, expressed and secreted from *Pichia pastoris* was recovered and purified using a screenless expanded bed column equipped with a rotating fluid distribution system. This column was able to accommodate feed stock, containing 30% biomass, at a flow rate of 300–400 cm/h without affecting column stability. This capability is three times higher than the capability of the expanded bed column currently in use, which is equipped with a perforated plate fluid distribution system; this design could accommodate biomass concentrations of only up to 10%. The screen-less design did not affect the binding capacity, purification level or process yield and, therefore, shorten the process. Purified Pfs25H of 6.4 g were recovered from 37 l of *Pichia pastoris* culture in one step.

Keywords Expanded bed adsorption · Malaria vaccine · *Pichia pastoris*

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Introduction

Pichia pastoris is an efficient producer of recombinant proteins. This methylotrophic yeast grows up to 300–400 g/l (biomass) and usually secretes the desired protein into the growth media (Lin Cereghino and Cregg 2000). The biomass is removed by centrifugation and filtration before the secreted protein is recovered and purified. Since the removal of large amounts of biomass is cumbersome and difficult, expanded bed adsorption (EBA) is a viable and attractive alternative (Calado et al. 2004; Charoenrat et al. 2006; Thommes et al. 2001; Trinh et al. 2000).

EBA is an established procedure for the direct adsorption of proteins from crude feed stock, such as fermentation broth or biomass extract, eliminating the need for centrifugation and clarification, thereby greatly simplifying the recovery process (Anspach et al. 1999; Chase 1994; Frej et al. 1994; Johansson et al. 1996; Shiloach and Kennedy 2000). In this operation, the adsorbent bed is expanded by an upward liquid flow which is controlled by a liquid distribution system. A stable, uniformly expanded bed is essential for successful operation. This is achieved through the size and density of the adsorbent particles, the design of the liquid distribution system, and the linear flow rates, usually between 200 cm/h and 400 cm/h.

The expanded bed column currently in use is equipped with a liquid distribution system made of a perforated plate with a 50 μm stainless steel net to contain adsorbent particles. Another net is found at the upper flow adaptor. These nets are susceptible to high concentrations of biomass or debris and, therefore, tend to clog, triggering the collapse of the expanded bed. In addition, this configuration is sensitive to air bubbles that can cause turbulence, adversely affecting column stability as well as protein binding, especially at high biomass concentrations. To prevent collapse of the column, careful evaluation of biomass concentrations and flow rates must be done before the column can be operated (Brixius et al. 2005; Chang and Chase 1996; Fernandez-Lahore et al. 1999; Lin et al. 2004). In addition, careful monitoring of the column's performance and operating parameters must be conducted while it is in use. To eliminate these potential pitfalls and to allow higher biomass concentrations to be loaded, different flow distribution systems have been explored. In one such configuration, the perforated plate distribution was replaced by a tangential flow device. In another, it was replaced with localized mixing. Yet another configuration replaced the perforated plate distribution with a rotating liquid distributor (Hubbuch et al. 2005). In all three of these configurations, the upper flow adaptor was eliminated. These distribution systems have the potential to be less sensitive to air bubbles and more effective in handling biomass concentrations greater than the 100 g/l, which is the limit for the existing distribution system made of perforated plate (Trinh et al. 2000; Thommes et al. 2001; Murasugi et al. 2001).

In this report, the performance of an expanded bed column equipped with a rotating fluid distribution system (Streamline Direct) was evaluated for capturing a secreted protein from *P. pastoris*. The protein was Pfs25H (Zou et al. 2003; Tsai et al. 2006), an experimental malaria transmission blocking vaccine antigen (Kaslow and Shiloach 1994). This *P. pastoris* derived 25 kDa cystine-rich *Plasmodium falciparum* protein was needed in large quantities for a phase I clinical trial.

Materials and methods

Pichia pastoris strain and the recombinant protein

Plasmodium. falciparum protein, Pfs25H, was expressed in *P. pastoris* strain GS115 (Invitrogen Corporation, Carlsabad, CA), that was modified for over-expression of the *P. pastoris* protein disulfide isomerase (PDI) (Tsai et al. 2006). The Pfs25H, a malaria transmission blocking vaccine candidate, is a 25 kDa histidine-tagged protein that was expressed extracellularily from the *P. pastoris* clone Pfs25H-PpPDI (Tsai et al. 2006).

Production of Pfs25H

P. pastoris fermentation was performed based on a previously developed procedure (Trinh et al. 2003). The yeast was grown in BMGY medium which consists of: 20 g Bacto-peptone/l, 10 g Bacto-yeast extract/l, 11.9 g KH_2PO_4/l, 2.14 g K_2HPO_4/l, 13.4 g yeast nitrogen base/l (without amino acids), 1 mg biotin/l, and 40 g glycerol/l. For bench top studies, a 5-l working volume fermentor (New Brunswick Scientific Inc., Edison, NJ) was inoculated with 100 ml of overnight culture. For pilot studies in a 60-l fermentor (Bioflo 5000 New Brunswick Scientific Inc., Edison, NJ), 1 l of overnight culture was used as inoculum. After 16 h growth at 30°C, pH 6.0, and dissolved O_2 controlled at 30% air saturation via adaptive control software, an $OD_{600\ nm}$ of approximately 70 (100 g/l biomass) was reached. At this time, glycerol feeding was initiated by pumping 50% (w/v) glycerol containing 12 ml of trace elements solution (per liter: 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g H_3BO_4, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin, 5 ml H_2SO_4) at a rate of 20 ml/l h^{-1}, the pH was allowed to drop to 3.5 and the temperature was decreased to 22°C. After about 2 h, (when the cell density reached an $OD_{600\ nm}$ of approximately 100 (150 g/l biomass) the glycerol feed-rate was gradually decreased to 2 ml/l h^{-1} while induction commenced by adding methanol containing 12 ml PTMI per liter. The methanol was added via a programmable pump (Scilog,

Middleton, Wis) based on the following equation: $r_{methanol} = 2.54\ e^{0.02t}$.

Determination of total & dynamic binding capacity of Streamline chelating resin for Pfs25H

Total binding capacity was determined by overloading a packed column of streamline chelating resin (Amersham Biosciences) with clear culture supernatant. The column was then washed and eluted, and the amount of Pfs25H in the eluant was determined. Dynamic binding capacity was determined by loading the culture supernatant on a packed column and following the Pfs25H in the run-through. When the concentration of the protein in the run-through reached 10% of the original concentration, the amount of protein loaded was calculated and the binding capacity was determined.

Expanded bed column operation

Two types of expanded bed columns were used: Streamline and Streamline Direct (Amersham Biosciences). The Streamline column is equipped with a perforated plate fluid distribution system and a hydraulic flow adaptor. The Streamline Direct column, on the other hand, is equipped with a screen-free rotating fluid distribution system of 4 oscillating arms with holes in their undersides (Fig. 1).

These two columns were loaded with high biomass suspension culture, applied to the expanded resin from the bottom in an upward direction. The protein Pfs25H was captured on the resin while cells, cells particulates, and other impurities passed through the expanded bed. Protein elution from the Streamline column (equipped with perforated plate) was carried out in downward direction with the resin bed packed by lowering the hydraulic flow adaptor. Protein elution from the Streamline Direct column (equipped with rotating fluid distribution system) could only be carried out in the upward direction.

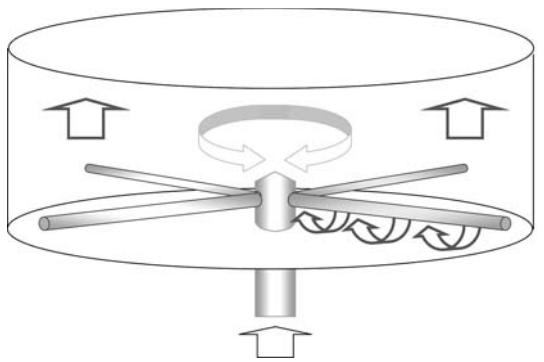

Fig. 1 Schematic drawing of the bottom inlet distribution system for Streamline Direct columns (courtesy of Amersham Biosciences)

Analytical methods

Pfs25H concentration during the binding experiments was determined using HPLC equipped with 0.75 mm i.d. by 7.5 cm TSK-Gel nickel chelating column (TOSOH Bioscience, Montgomeryville, PA). The column, held at 30°C, was equilibrated with 20 mM phosphate buffer pH 7.4 containing 0.75 M NaCl. After loading, the column was washed with 20 mM phosphate buffer pH 6.9 containing 0.75 M NaCl for 20 min. The protein was eluted with 20 mM phosphate buffer pH 3.3 containing 0.75 M NaCl for 30 min at 0.5 ml/min and the eluant was monitored at 210 nm and 280 nm.

The final product purity was evaluated by acidifying the protein solution with 10% (v/v) trifluoroacetic acid (TFA) to give 0.1% TFA and analysis on Vydac C4 reverse phase column (2.1 mm × 250 mm). The initial mobile phase combined 99% mobile phase A (0.1% TFA in water) and 1% mobile phase B (0.1% TFA in acetonitrile). Recombinant Pfs25H was eluted with increasing concentrations of mobile phase B with the flow rate maintained at 0.2 ml/min over 60 min.

Total protein was determined using the BCA protein assay kit (Pierce, Rockford, IL USA). The characterization of protein preparations (before and after purification) was done by SDS PAGE (4–20%).

Results

Growth and protein production

A typical fermentation process for Pfs25H production from *Pichia pastoris* overly produced PDI

is shown in Fig. 2. After initial growth on glycerol, lasting 20 h, the carbon source was changed to methanol which was introduced at a predetermined exponential rate (Trinh et al. 2003) for an additional 40–45 h. At the end of the fermentation; the culture volume increased from 30 l to 37 l, the biomass concentration was 205 g/l, total biomass was 7.6 kg, the Pfs25H concentration was 173 mg/l culture, and the total Pfs25H was 6.4 g.

Total and dynamic binding capacity of Pfs25H to Streamline chelating resin

Expressed Pfs25H has a 6× histidine tag at the *C*-terminal and, therefore, adsorption to the chelating resin in an expanded column was considered the first recovery step. EBA was chosen because it can potentially eliminate the tedious biomass removal steps that precede adsorption to a packed column. Total and dynamic binding capacities of Pfs25H had to be determined before evaluating the direct adsorption of the protein from the culture supernatant. The total binding capacity of Pfs25H to Streamline chelating resin was determined by loading 1.4 l of supernatant at a pH 7.4, containing 9.0 g total protein (of which 410 mg were Pfs25H) on a 33.5 ml column (1.6 cm × 16.7 cm) at a flow rate of 300 cm/h. After washing, the column was eluted with 0.25 M sodium acetate buffer (pH 4.5) at a flow rate of 100 cm/h. The elution fractions contained 183 mg Pfs25H, representing maximum binding of 5.5 mg/ml resin.

The dynamic binding of Pfs25H from *P. pastoris* culture supernatant was determined by loading the supernatant on a 33.5 ml packed column (1.6 cm × 16.7 cm) of Streamline chelating resin at a flow rate of 300 cm/h. When 10% break through of Pfs25H was observed, 185 mg of Pfs25H were loaded, representing a dynamic binding capacity of 5.5 mg/ml resin.

Recovery of Pfs25H from P. pastoris culture by adsorption onto Streamline chelating resin

To evaluate the recovery efficiency of Pfs25H from the culture broth, the following three conditions were compared: (i) clear supernatant on packed column, (ii) diluted fermentation broth on traditional expanded bed column (equipped with perforated fluid distribution system and upper flow adaptor), and (iii) undiluted fermentation broth on the newly designed expanded bed column (Streamline Direct), in which the perforated fluid distribution system was replaced with a rotating fluid distribution system and the upper flow adaptor was eliminated. In all three cases, the columns were loaded with approximately 80% of the maximum capacity determined earlier. The pH of each sample was adjusted to 7.4, the loading flow rate was 300 cm/h, and the

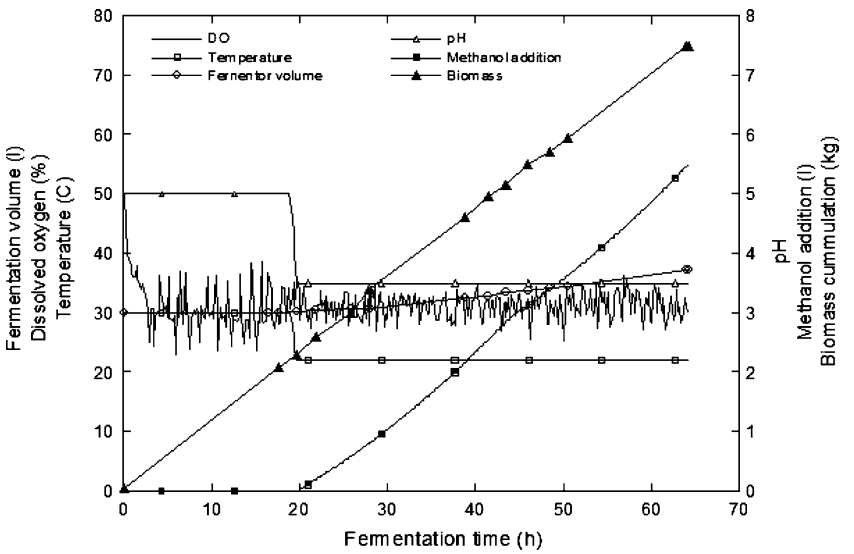

Fig. 2 Growth of *Pichia pastoris* for Pfs25H production. During the first 20 h the yeast grew on glycerol at 30°C and pH 5.0. After 20 h, the temperature was lowered to 22°C, the pH to 3.5 and methanol was fed into the culture to keep the growth rate at 0.02 h^{-1}, after 45 h of growth on methanol the culture was processed

elution flow rate was 100 cm/h. The results are summarized in Table 1 and Fig. 3. The degree of purification and recovery were similar for the three methods.

Pilot scale production and recovery of Pfs25H

Pilot-scale production of Pfs25H was performed at an initial volume of 30 l. After 65 h of fermentation, including 45 h in which the culture was under limited methanol induction, the culture volume increased to 37 l, and the biomass had accumulated to 7.6 kg, expressing 6.4 g Pfs25H. The 37 l fermentation broth was loaded directly on a 10 cm Streamline Direct column containing one packed Streamline chelating resin at a flow rate of 300 cm/h. After washing, the protein was eluted in an upward direction at a flow rate of 100 cm/h. 6.4 g of purified Pfs25H were collected in a total volume of 16 l. The recovery parameters are summarized in Table 2. A comparison of the process using the Streamline Direct column and the Streamline column (extrapolated values) is outlined in Fig. 4.

Final purification and characterization

The 6.4 g Pfs25H solution eluted from the 10 cm Streamline Direct column was ultrafiltered and diafiltered using 5000 MWCO membranes to a final volume of 600 ml in PBS buffer. Ammonium sulfate was added to a final concentration of 1.8 M and the protein was loaded on a 6 cm × 20 cm phenyl Spharose column at 100 cm/h. The Pfs25H was eluted using step elution, concentrated, and polished by loading on 6 cm × 60 cm S 75 gel filtration column (6% v/v) at a linear flow rate of 25 cm/h. The eluted peak was analyzed by SDS PAGE (Fig. 5) and HPLC and was compared to the material recovered and purified using packed bed column (Table 3).

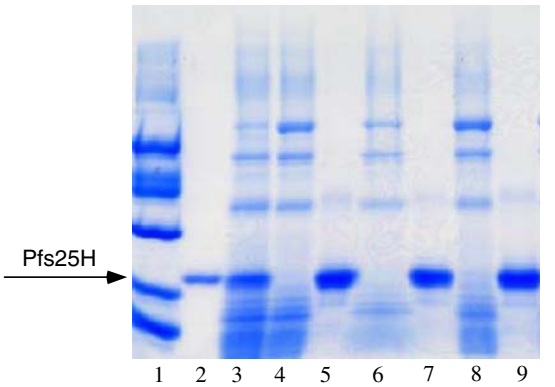

Fig. 3 SDS PAGE of samples from different column configurations. Lane 1: Molecular weight marker, Lane 2: Pfs25H standard, Lane 3: *Pichia Pastoris* culture supernatant before purification, Lane 4: Run-through from Streamline Direct column, Lane 5: Elution from Streamline Direct, Lane 6: Run-through from conventional EBA column, Lane 7: Elution from conventional EBA column, Lane 8: Run through from packed column, Lane 9: Elution from packed column

Table 2 Recovery of malaria transmission blocking vaccine, Pfs25H, from pilot scale *Pichia pastoris* culture using Streamline Direct expanded bed

Bed volume (l)	Volume loaded (l)	Biomass total (kg)	Volume eluted (l)	Protein eluted(g)
1	37	7.6	16	6.4

Discussion

EBA is a widely used method for capturing proteins from crude feed stock. It replaces centrifugation and filtration and is especially

Table 1 Comparison of capturing malaria transmission blocking vaccine, Pfs25H, from *Pichia pastoris* culture using three different approaches: (1) packed bed (2) Streamline expanded bed and (3) Streamline Direct

Capturing method	Streamline direct with undiluted culture broth	Traditional Streamline EBA with diluted culture broth	Packed-bed with clear culture supernatant
Bed volume (ml)	126	314	10
Volume loaded (l)	4.1	24	0.4
Biomass (g/l)	261	100	0
Loading ratio (%)	85	79	87
Volume eluted (ml)	600	958	40
Protein eluted (g)	0.52	1.5	0.038
Recovery (%)	91	109	83

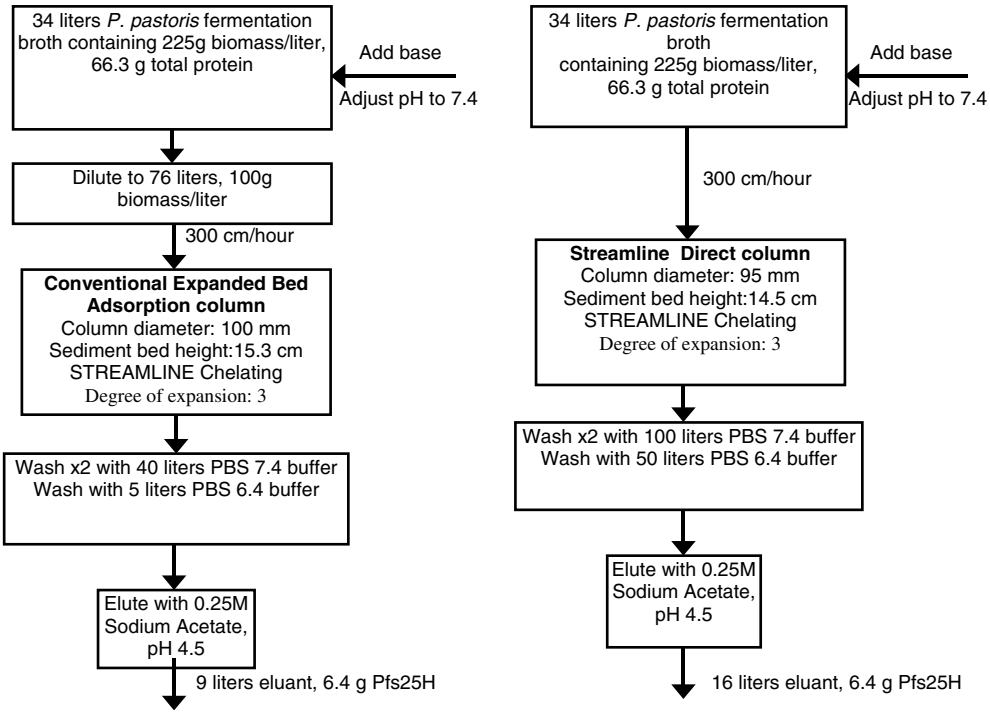

Fig. 4 Comparison of the recovery process of Pfs25H from *Pichia pastoris* culture. (**A)** Recovery process using expanded bed column equipped with perforated fluid distributor system (Streamline), (**B)** Recovery process using expanded bed column equipped with rotating fluid distribution system (Streamline Direct)

advantageous when the concentration of biomass or other insoluble particles in the feed stock is high. Such is the case for the production of malaria transmission blocking vaccine (Pfs25H) from *P. pastori*s, which grows to biomass concentrations of 200–400 g/l. As was described in earlier studies (Trinh et al. 2000; Thommes et al. 2001; Murasugi et al. 2001), the existing expanded bed design cannot handle biomass concentrations above 100 g/l. This limitation is mainly due to the presence of a perforated liquid flow distributor, which is usually equipped with a 50 μm stainless steel net to contain the adsorbent particles. The liquid flow distributor can get clogged, adversely affecting flow, column stability, and binding. To overcome these difficulties, the bottom flow adaptor was replaced with a rotating fluid distribution system that eliminates the net and allows free flow of the feed stock over the expanded resin. This design was evaluated for its ability to support higher biomass concentrations and for its effect on the dynamic capacity and recovery capability of the malaria transmission blocking vaccine from the *P. pastoris* culture. The modified expanded bed column was compared to an expanded bed with a perforated liquid flow distributor (existing design) and to a common packed bed configuration.

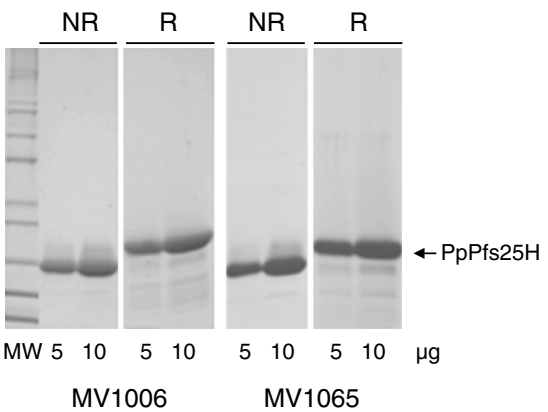

Fig. 5 SDS PAGE of purified Pfs25H captured by packed column (lot MV1006) and Streamline Direct expanded bed (lot MV 1065)

The maximum binding capacity of the Streamline chelating resin for Pfs25H in the

Table 3 Comparison of biochemical and biophysical properties of Pfs25H captured by packed bed and Streamline direct expanded bed

Analytical method	PpPfs25H	
	Packed bed capture Lot MV1006[a]	Streamline Direct Lot MV1065
Reverse-phase HPLC		
Retention time (min)	19.847	19.845
Area (%)	97.39	97.18
SEC-HPLC		
Retention time (min)	18.851	18.698
Area (%)	100	100
MS (Daltons)	20,441.0 + 162[b]	20,439.0 + 162[b]
Amino-terminus	EAEAYVKVTVDT	EAEAYVKVTVDT
Immunoblot		
mAb 4B7	+	+
mAb 1D2	+	+

[a]Analysis of purified recombinant Pfs25H protein was previously reported (Tsai et al. 2006)

[b]Minor presence of 162 Da adducts due to the presence of *O*-linked mannosylation (Tsai et al. 2006)

culture supernatant was found to be 5.5 mg resin/ml, and the dynamic binding at 10% breakthrough at a flow rate of 300 cm/h was the same when the culture supernatant was applied to a packed column. This binding capacity value was then used to compare the three different adsorption methods highlighted above. A biomass concentration of 261 g/l was applied to the Streamline Direct column, and only 100 g/l were applied to the conventional type expanded bed. The level of protein recovery, along with the degree of purity, was similar for all three configurations. Therefore, the high flow rate and high biomass concentration in the feed did not affect the binding or the degree of purification when the modified expanded bed was used. A comparison of the processes used to capture Pfs25H from 37 l *P. pastoris* culture, using both the traditional expanded bed and the modified column is shown in Fig. 4. Since dilution was not required, the loading volume was smaller and the process using the modified column was shorter. However, this system can operate only at an expanded mode and, therefore, larger volumes of buffers were used for washing and elution. Ten times more buffer was needed for washing and the protein was eluted with twice as much elution buffer.

It is likely that increasing the density of the elution and wash buffers or inserting a movable inner adaptor (such and adaptor is found in the 2.5 cm column) can lower the volumes and improve the operation of the column. Further, the rotating fluid distributor system made the expanded bed operation less sensitive to biomass concentrations and air bubbles, and affected neither the recovery nor the purification when compared to an expanded bed column equipped with a perforated liquid flow distribution system, or the traditional packed bed column.

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