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# Recovery of recombinant β-glucosidase by expanded bed adsorption from *Pichia pastoris* high-cell-density culture broth

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**Abstract**

Methanol limited fed-batch cultivation was applied for production of a plant derived β-glucosidase by *Pichia pastoris*. The β-glucosidase was recovered by expanded bed adsorption chromatography applied to the whole culture broth. The new Streamline Direct HST1 adsorbent was compared with Streamline SP. Higher bead density made it possible to operate at two times higher feedstock concentration and at two times higher flow velocity. The higher binding capacity in the conductivity range 0–48 mS $cm^{-1}$ of Streamline Direct HST1 might be caused by the more complex interaction of multi-modal ligand in Streamline Direct HST1 compared to the single sulphonyl group in Streamline SP. Harsher elution condition had to be applied for dissociation of β-glucosidase from Streamline Direct HST1 due to stronger binding interaction. The 5% dynamic binding capacity was 160 times higher for Streamline Direct HST1 compared to Streamline SP. The yield of β-glucosidase on Streamline Direct HST1 (74%) was significantly higher than on Streamline SP (48%). Furthermore, β-glucosidase was purified with a factor of 4.1 and concentrated with a factor of 17 on Streamline Direct HST1 while corresponding parameters were half of these values for Streamline SP. Thus, for all investigated parameters Streamline Direct HST1 was a more suitable adsorbent for recovery of recombinant β-glucosidase from unclarified *P. pastoris* high-cell-density cultivation broth.


*Keywords:* β-Glucosidase; *Pichia pastoris*; Methanol limited fed-batch; Expanded bed adsorption; Multi-modal ligand

*Abbreviations:* AOX, enzyme alcohol oxidase; *aox*, alcohol oxidase gene; EBA, expanded bed adsorption; PI, propidium iodide

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**Nomenclature**

| | |
|---|---|
| $C$ | β-glucosidase activity in effluent (U ml$^{-1}$) |
| $C_{eq}$ | β-glucosidase activity at equilibrium condition (U ml$^{-1}$) |
| $C_0$ | β-glucosidase activity at initial condition (U ml$^{-1}$) |
| DOT | dissolved oxygen tension (%) |
| $Q_B$ | binding capacity (U ml$_{ads}$$^{-1}$) |
| $Q_{eq}$ | equilibrium capacity (U ml$_{ads}$$^{-1}$) |
| $V_{ads}$ | volume of adsorbent (ml) |
| $V_{eff}$ | volume of effluence (ml) |
| $V_l$ | total reaction volume (ml) |
| $V_0$ | system dead volume (ml) |
| $V_x$ | breakthrough volume when $100C/C_0$ is $X$% (ml) |

## 1. Introduction

β-Glucosidase (β-glucoside glucohydrolase; EC 3.2.1.21) catalyzes the hydrolysis of alkyl- and aryl-β-glucosides, as well as diglucosides and oligosacharides, to release glucose and an aglycone (Reese, 1977). These enzymes are found wildly in microorganisms, animals and plants, indicating their general importance to life. β-Glucosidases have been widely studied because of their important roles in medical, agricultural, biotechnological and industrial applications (Gueguen et al., 1997; Ducret et al., 2002). Seeds of Thai rosewood, *Dalbergia cochinchinensis* Pierre, were found to contain high levels of β-fucosidase and β-glucosidase activities (Surarit et al., 1995). The pure enzyme has β-glucosidase as well as β-fucosidase activities, an apparent subunit molecular weight of 66 kDa by SDS-PAGE and an apparent native $M_r$ of approximately 330 kDa (Srisomsap et al., 1996).

*Pichia pastoris* (*P. pastoris*) is frequently applied for production of recombinant proteins, mostly under control of the *aox1* promoter (Cregg et al., 1987). It offers several advantages as production host compared to *Escherichia coli* because it does not carry endotoxin, the product can be secreted to a mineral-salts medium, and it is capable to glycosylate proteins (Cregg et al., 1987; Lin Cereghino and Cregg, 2000). Furthermore, it can be cultured to very high-cell-densities (>130 g l$^{-1}$ cell cell dry weight) (Wegner, 1990; Jahic et al., 2002) which improves the volumetric productivity.

Expanded bed adsorption (EBA) is a technique for single step recovery of bioproducts from crude particulate containing feedstock. Feedstock properties are important parameters to consider during the design and development of adsorbents and EBA process operations, especially the high-cell-density often achieved in *P. pastoris* cultures combined with often rather high conductivity.

Feedstock with high particulate concentration displays high viscosity. It was demonstrated that the variety of feedstock viscosities resulted in different degrees of bed expansion. It was possible to stabilize the bed expansion by adjusting the feedstock viscosity and/or liquid flow velocity, which however resulted in a longer process cycle and lower productivity (Chang and Chase, 1996).

The feedstock conductivity significantly affects the equilibrium binding constant ($Q_{eq}$) on an ion exchanger. In the recovery of human chymotrypsinogen B from *P. pastoris* fermentation broth by the cation exchanger Streamline SP it was shown that $Q_{eq}$ was reduced more than 90% when conductivity was increased from 8.9 to 26.8 mS cm$^{-1}$ (Thömmes et al., 2001). Similar results were also presented in human calcitonin precursor recovery by Streamline SP. The $Q_{eq}$ was reduced from 28 to 16 mg ml$^{-1}$ when conductivity was increased from 7.0 to 11.0 mS cm$^{-1}$ (Sandgathe et al., 2003). The need to dilute high conductivity feedstock will also result in longer process cycles and reduced productivity.

Generally, adsorbents developed for EBA processes are the result of a compromise between the matrix characteristics (particle size, particle density and pore size), which determine the useful range of flow-rates and adsorption kinetics, especially mass transfer limitations. A well performing adsorbent will prevent bed instability and give high breakthrough capacity ($Q_B$) comparable to packed bed adsorbents (Lei et al., 2003; Tong and Sun, 2001; Anspach et al., 1999). By using high density adsorbent, the EBA process can be run at high flow velocity or high particulate containing feedstock without losing of adsorbent in flow-through. The increase of bead density can be done by adding of a densifier such as quartz, steel alloy, $TiO_2$, and more (Lei et al., 2003; Tong and Sun, 2001).

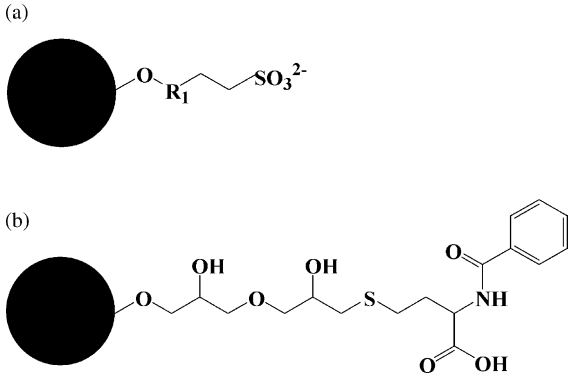


Fig. 1. The structures of ligands in the (a) Streamline SP and (b) Streamline Direct HST1 adsorbent.

Adsorbents developed for use in EBA can be furnished with a wide variety of ligands (Chase, 1998). The Streamline SP bead (GE Healthcare, Uppsala, Sweden) is based on agarose with quartz as densifier (1.2 g $ml^{-1}$) and with a sulphonyl group as the ion exchange ligand (Fig. 1a). This type of adsorbent has also been used for recovery of recombinant protein from *P. pastoris* culture broth. However, about 2–5 times dilution of feedstock was required to reduce both cell concentration and conductivity (Sandgathe et al., 2003; Murasugi et al., 2001; Shepard et al., 2000; Trinh et al., 2000). The new Streamline Direct HST1 bead (GE Healthcare, Uppsala, Sweden) is also based on agarose but with a denser metal alloy as densifier (1.8 g $ml^{-1}$) and carrying a multi-modal ligand composed of a thioether group, a carboxylic group and an aromatic group (Fig. 1b). A similar ligand has been used in packed bed chromatography functional at both high and low ionic strengths (Burton et al., 1997).

The aim of this work was to investigate the feasibility of designing an EBA process for the recovery of recombinant Thai Rosewood β-glucosidase directly from high-cell-density and high conductivity *P. pastoris* culture broth. To do this the possibility to use Streamline Direct HST1 adsorbent for increasing of EBA process efficiency was studied and the performance was compared with Streamline SP adsorbent. The binding and dissociation conditions, and breakthrough capacities were studied to develop the recovery process.

## 2. Materials and methods

### 2.1. Organism

The *P. pastoris* strain Y-11430 (wild-type strain), a kind gift from J. Lin Cereghino (Lin Cereghino and Cregg, 2000), was used in this study. The *β-glucosidase* cDNA gene from Thai Rosewood (*Dalbergia cochinchinensis* Pierre) (Ketudat-Cairns et al., 2000) was cloned into the pPICzαB vector (Invitrogen). The pPICzαB with the *β-glucosidase* gene was then integrated into *P. pastoris* Y-11430 at the *aox1* promoter.

### 2.2. Cultivation

#### 2.2.1. Inoculum preparation

First inoculum was prepared by adding one colony of *P. pastoris* from yeast extract peptone dextrose agar medium (YPD) (yeast extract 10 g $l^{-1}$, peptone 20 g $l^{-1}$ and dextrose 20 g $l^{-1}$) containing 100 μg $ml^{-1}$ zeocin into 20 ml YPD broth. The culture was incubated at 30 °C, 200 rpm for 24 h. A second inoculum was prepared by transferring the entire first inoculum culture into a 1000 ml shake flask containing 80 ml buffered glycerol complex medium (BMGY) (yeast extract 10 g $l^{-1}$, peptone 20 g $l^{-1}$ and glycerol 10 g $l^{-1}$ in 0.1 M potassium phosphate buffer pH 6.0). The culture was incubated under the same condition as the first inoculum culture.

#### 2.2.2. Fed-batch cultivation

The fed-batch cultivation was carried out in a 10 l fermenter (Belach Bioteknik, Stockholm, Sweden). The fermenter was sterilized in situ and the glycerol basal salt medium, GBS (glycerol 40.0 g $l^{-1}$, 85% $H_3PO_4$ 26.7 ml $l^{-1}$, $CaSO_4$ 0.93 g $l^{-1}$, $K_2SO_4$ 18.2 g $l^{-1}$, $MgSO_4{\cdot}7H_2O$ 14.9 g $l^{-1}$, KOH 4.13 g $l^{-1}$, and PTM1 trace salts 4.35 ml $l^{-1}$) was added using 0.2 μm AcroPak™ 20 Filter (Pall Life Sciences, Ann Arbor, MI, USA). The PTM1 trace salts ($CuSO_4{\cdot}5H_2O$ 6.0 g $l^{-1}$, KI 0.08 g $l^{-1}$, $MnSO_4{\cdot}H_2O$ 3.0 g $l^{-1}$, $Na_2MoO_4{\cdot}2H_2O$ 0.2 g $l^{-1}$, $H_3BO_3$ 0.02 g $l^{-1}$, $ZnCl_2$ 20.0 g $l^{-1}$, $FeCl_3$ 13.7 g $l^{-1}$, $CoCl_2{\cdot}6H_2O$ 0.9 g $l^{-1}$, $H_2SO_4$ 5.0 ml $l^{-1}$ and biotin 0.2 g $l^{-1}$) were sterilised using a 0.2 μm syringe filter (Sartorius, Goettingen, Germany) and added separately. The temperature, pH, DOT, aeration rate, pressure, pump speed, agi-

tation rate and antifoam levels were automatically controlled.

The fermentation process was divided into four stages (glycerol batch, glycerol exponential fed-batch, methanol exponential fed-batch and constant methanol fed-batch) as described by Jahic et al. (2002). In the protein production phase, the methanol was fed to keep DOT constant at about 25%. The temperature, aeration, agitation and pH were controlled at 30 °C, 6 l min$^{-1}$, 1000 rpm and 5.0, respectively. Twenty-five percent $NH_4OH$ was used as the alkaline to control the pH. All feed solutions contained 12 ml l$^{-1}$ of PTM1 trace salts.

#### 2.2.3. Harvest

The unclarified culture broth was collected from the bioreactor at the end of the cultivation. A part of the culture broth was used to prepare clarified culture supernatant by centrifuging at 10,000 rpm (16,915 × *g*) for 20 min. Both preparations were stored at 4 °C until use.

### 2.3. Adsorbents

Streamline SP (GE Healthcare, Uppsala, Sweden), is an agarose based cation exchanger with mean particle size 200 μm, density 1.2 kg dm$^{-3}$ and sulphonate groups as ion exchange ligand (Fig. 1a). Streamline Direct HST1 (GE Healthcare, Uppsala, Sweden), is agarose based, with mean particle size 140 μm, density 1.8 kg dm$^{-3}$ and carries multi-modal ligand, as described in Fig. 1b.

### 2.4. Binding conditions

The binding conditions of β-glucosidase, measured as equilibrium capacity ($Q_{eq}$), were tested at different pH and conductivity values. The adsorbents were prepared by placing 0.5 ml of adsorbent in test-tubes and equilibrated in steps with buffer at specified pH and conductivity values for about 2 min by mixing end over end. In the first step 5 ml of 500 mM sodium-acetate buffer was used 10 times followed by a second step with 5 ml of 50 mM sodium-acetate buffer for five times. The samples were prepared from clarified *P. pastoris* culture supernatant by dialysis against 50 mM sodium acetate buffer pH 5.0 and then the pH and conductivity were adjusted with 1 M NaOH or 1 M acetic acid and with 1 M NaCl, respectively. One millilitre of prepared sample was then added to the prepared adsorbent and incubated end-over-end mixing for 1 h. The adsorbent was allowed to settle and then the supernatant was assayed for β-glucosidase activity. The initial β-glucosidase activity ($C_0$) and equilibrium β-glucosidase activity ($C_{eq}$) were determined and the equilibrium capacity ($Q_{eq}$) was calculated as:

$$Q_{eq} = \frac{(C_0 - C_{eq})V_l}{V_{ads}} \quad (1)$$

where $V_l$ andd $V_{ads}$ are the total volume in the test-tube (ml) and the volume of adsorbent (ml), respectively.

### 2.5. Dissociation conditions

Elution buffers (50 mM sodium acetate and 250 mM potassium acetate) with different pH (4–7) and conductivity (1–44 mS cm$^{-1}$) were prepared. Two molars of NaCl solution was add for adjusting conductivity. One millilitre of a certain elution buffer was added into 0.5 ml of adsorbent previously adsorbed with β-glucosidase as described for the binding condition tests. The samples were mixed for 1 h and then the adsorbent was allowed to settle. The supernatant was assayed for β-glucosidase activity and the percentage of β-glucosidase that had dissociated from the adsorbent was calculated.

### 2.6. Breakthrough capacity determination

The expanded bed system used in this work consisted of a Streamline C-25 column (25 mm i.d.) (GE Healthcare, Uppsala, Sweden) packed with 100.7 ml (20.5 cm bed height) of Streamline SP or Streamline Direct HST1, connected to a peristaltic pump and a UV detector.

The adsorbent was equilibrated with 500 mM sodium-acetate buffer, pH 4.0, for 2 column volumes and 50 mM sodium-acetate, pH 4.0, for 4 column volumes in expanded mode at flow velocities of 300 and 600 cm h$^{-1}$ for Streamline SP and Streamline Direct HST1, respectively.

The unclarified *P. pastoris* culture broth was adjusted to pH 4.0 and to conductivity 5.0 mS cm$^{-1}$ (920 ml l$^{-1}$ supernatant volume, 105 g l$^{-1}$ cell wet

weight and 24 g $l^{-1}$ cell dry weight), and to pH 4.0 and conductivity 15.0 mS $cm^{-1}$ (783 ml $l^{-1}$ supernatant volume, 248 g $l^{-1}$ cell wet weight and 60 g $l^{-1}$ cell dry weight) for Streamline SP and Streamline Direct HST1, respectively. Acetic acid, 0.5 M, was used as acid for adjusting pH. In the case of conductivity adjustment, 2 M NaCl solution and 50 mM sodium-acetate buffer were used. Then the samples were applied to the column at the same flow velocity as used at equilibration. The breakthrough was monitored by taking out fractions from the effluent and assay for the β-glucosidase activity. In order to determine the breakthrough capacity ($Q_B$) the normalized effluent concentration ($C/C_0$) was plotted versus the amount of β-glucosidase loaded per volume of adsorbent [$(V_{eff}C_0)/V_{ads}$]. The breakthrough capacity ($Q_B$) of the adsorbent was determined as follows:

$$Q_{BX\%} = \frac{C_0(V_x - V_0)}{V_{ads}} \quad (2)$$

where $V_{eff}$ is the volume of effluent (ml), $V_0$ is the dead volume of the system (ml) and $V_x$ is the effluent volume at which $100C/C_0$ is $X$% (ml).

### 2.7. The EBA recovery process

The equilibration, washing and elution buffers used for the recovery of β-glucosidase from unclarified *P. pastoris* culture broth were selected from the above described screening experiments. The equilibration and sample application steps were performed as described for the $Q_B$ experiment above. Then, washing was performed with 50 mM NaCl in 50 mM sodium-acetate buffer, pH 4.0, for Streamline SP and 50 mM sodium-acetate buffer, pH 5.0, for Streamline Direct HST1 until all residual cell and unbound proteins were removed ($A_{280}$ back to base-line). Elution was performed using 250 mM NaCl in 50 mM sodium-acetate buffer, pH 5.0 (conductivity 24.7 mS $cm^{-1}$) for Streamline SP, and 250 mM potassium-phosphate buffer, pH 7.0, (conductivity 30.5 mS $cm^{-1}$) for Streamline Direct HST1 at flow velocity of 100 cm $h^{-1}$ in expanded mode. Cleaning-in-place (CIP) of the adsorbents was performed using 1 M NaCl in 0.5 M NaOH, distilled water, 25% acetic acid in 20% ethanol and distilled water again as recommended by the manufacturer.

### 2.8. Analyses

#### 2.8.1. Cell concentration

Cell concentration was monitored by measuring the optical density at 600 nm ($OD_{600}$) and cell dry weight. Cell dry weight was determined by centrifugation of 5 ml of culture broth at 4500 rpm (1400 × $g$) for 10 min, and the supernatant was collected for analyzes of β-glucosidase activity, total protein and SDS-PAGE. The pellet was washed with distilled water once and dried at 105 °C, till constant weight.

#### 2.8.2. Total protein concentration

The total protein concentration in the supernatant was analyzed according to Bradford (Bradford, 1976). Bovine serum albumin was used as standard protein.

#### 2.8.3. β-Glucosidase activity

β-Glucosidase activity was assayed by the method of Evans (1985). This method is a spectrophotometric assay that measures the release of *p*-nitrophenol from *p*-nitrophenol-β-D glucopyranoside (3.3 mM) catalysed by β-glucosidase. One unit of enzyme was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute at 30 °C in 0.1 M sodium acetate buffer at pH 5.0.

#### 2.8.4. SDS-PAGE analysis

The sample, containing 60 μl of supernatant, 25 μl of sample buffer (NuPAGE LDS 4× sample buffer, Invitrogen, CA, USA), 10 μl of 0.5 M dithiothreitol and 5 μl of 3.5% PMSF in ethanol was incubated for 10 min at 95 °C. SDS-PAGE was performed on NuPAGE® Novex 4–2% Bis–Tris Gel (1.0 mm × 10 well) (Invitrogen, CA, USA) using MOPS running buffer. Ten microliters of prepared sample was loaded to the well and run at 200 V for 60 min. The gel was stained with Coomassie Blue R-250 (Merck, Darmstadt, Germany) for 30 min and destained (destain solution: methanol 100 ml $l^{-1}$ and glacial acetic acid 100 ml $l^{-1}$) for 1–2 h.

#### 2.8.5. Cell viability

A Partec PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with a 488 nm argon laser was used for analysis of the total number of cells and the number of cells stained by propidium iodide (PI) (Sigma-Aldrich, Stockholm, Sweden). Samples taken from the fermenter were diluted with PBS (0.16 M

NaCl, 0.003 M KCl, 0.008 M $Na_2HPO_4$ and 0.001 M $KH_2PO_4$, pH 7.3). For staining, 25 μl of stock solution containing 200 μg $ml^{-1}$ of PI dissolved in water was added to 975 μl of diluted sample at room temperature. Samples were analys, without further incubation, at a data rate of about 1500 counts $s^{-1}$. A total count of 50,000 was collected in each measurement. Measurements were calibrated using 3 μm diameter fluorescent beads (Standard 05-4008, Partec GmbH, Münster, Germany). The viability was expressed as percentage of PI negative cells in the population.

*2.8.6. Alcohol oxidase activity*

The intracellular alcohol oxidase activity was assayed with the method described recently (Jahic et al., 2002). Alcohol oxidase units were expressed as a μmol of methanol oxidized per minute.

*2.8.7. Methanol concentration*

The concentrations of methanol was continuously analyzed using Industrial Emissions Monitor Type 1311 (Brüel & Kjær, Innova, Denmark) as described recently (Jahic et al., 2002).

## 3. Results and discussion

### *3.1. Fed-batch production of β-glucosidase*

A rapid increase of AOX activity was observed after start of the methanol feed (Fig. 2a). From this time the β-glucosidase activity increased steadily in the medium, and reached about 5100 U $l^{-1}$ after 125 h induction time (Fig. 2c). Initially also the biomass concentration increased rapidly, but it levelled off and ended at about 135 g $l^{-1}$. The high-cell-density in *P. pastoris* processes is important since it compensates for the relatively low specific productivity per cell unit, resulting in production of several grams of product per litre culture broth (Lin Cereghino and Cregg, 2000; Jahic et al., 2002).

An additional advantage of the *P. pastoris* expression system is that the product often can be secreted to the medium which in many cases can be a mineral salt medium without contaminating proteins. In the present investigation the mass of β-glucosidase could not be measured due to problems to obtain the specific activity of pure recombinant enzyme. However, SDS-PAGE analyses (Fig. 2d) indicate that the β-glucosidase was the dominating protein in the cultivation broth in which the total protein concentration was about 700 mg $l^{-1}$. The estimated molecular weight of this recombinant β-glucosidase under denaturing condition was approximately 88 kDa, which is larger than the natural enzyme purified from Thai Rosewood (66 kDa) (Ketudat-Cairns et al., 2000). There is a band with increasing density at about 28 kDa (Fig. 2d), which may be either a proteolysis product or a contamination of host cell proteins. Studies on the stability of the product when incubated in broth with and without cells indicated that the product was proteolytically stable (data not shown).

The start of the methanol feed resulted in a short rapid transient accumulation of methanol in the medium. It reached 600 mg $l^{-1}$ within 50 min, but declined below the measurement limit (25 mg $l^{-1}$) within 5 h (Fig. 2b). The viability dropped from about 99% to about 96%, within 25 h, where it remained throughout the process. The conductivity of the broth declined with increasing biomass from about 45 mS $cm^{-1}$ at the beginning of the process to about 18 mS $cm^{-1}$ at the end (Fig. 2b).

β-Glucosidase was harvested in a culture broth at very high-cell-density (135 g $l^{-1}$ dry cell weight, 450 g $l^{-1}$ wet cell weight, 625 ml $l^{-1}$ supernatant volume) and rather high conductivity (18 mS $cm^{-1}$) (Fig. 2). Without pre-treatment of the broth these typical properties of a *P. pastoris* process liquid will limit the choice of initial operations for cell removal and product recovery.

### *3.2. Binding conditions of β-glucosidase on the adsorbents*

The study of binding conditions of β-glucosidase on the two adsorbents was focused on the effect of pH and conductivity by using cell free supernatant. At pH 4.0 Streamline Direct HST1 had about twice as high $Q_{eq}$ as Streamline SP, but for both adsorbents $Q_{eq}$ decreased when pH was increased (Fig. 3a). The conductivity significantly affected the $Q_{eq}$ in Streamline SP. At constant pH 4.0, when the conductivity was changed from 6.4 to 15.3 mS $cm^{-1}$ the $Q_{eq}$ decreased with 40%. However, in the conductivity range of 6.4–47.0 mS $cm^{-1}$ at pH 4.0 the $Q_{eq}$ for Streamline Direct HST1 declined only by about 15% (Fig. 3b).

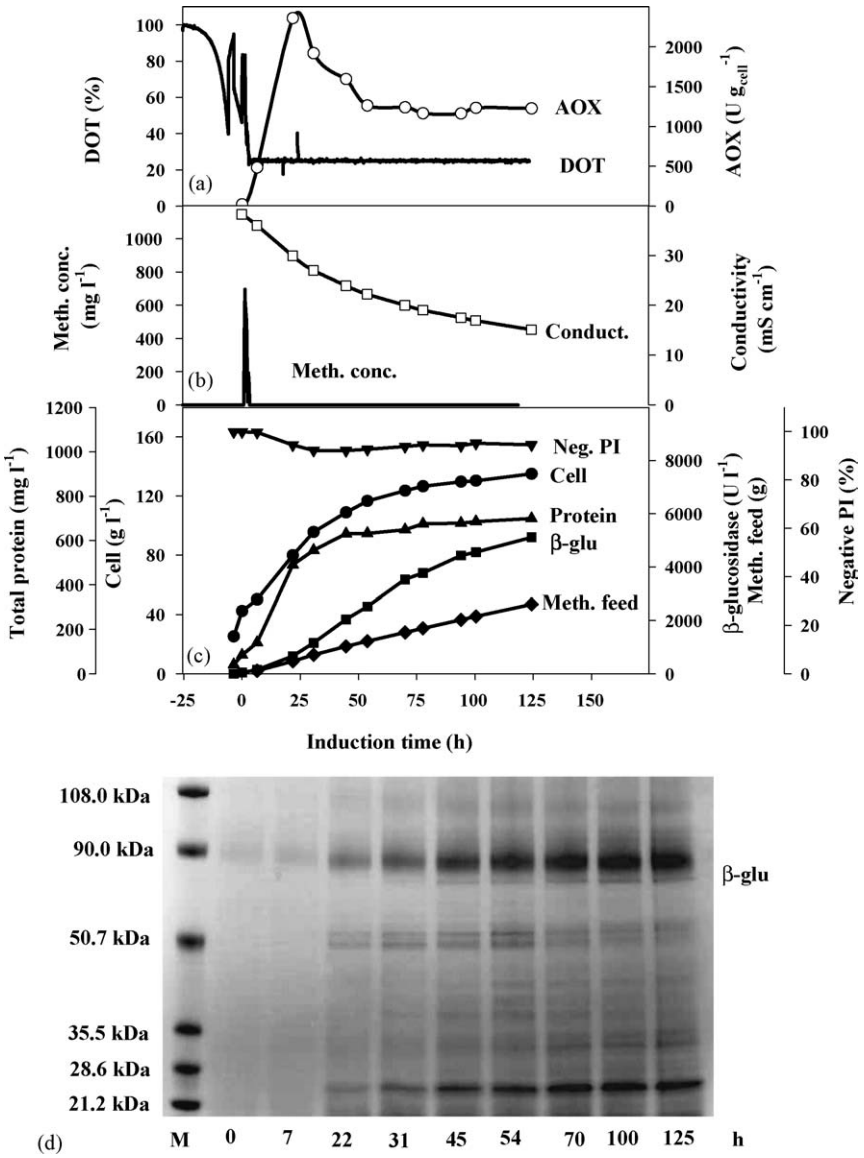


Fig. 2. Cultivation profile of recombinant β-glucosidase production with *P. pastoris*. (a) DOT (continuous line), intracellular AOX activity (–○–); (b) the methanol concentration (continuous line) and conductivity (–□–); (c) PI negative (viable) cells (–▼–), biomass (–●–), total protein (–▲–), β-glucosidase (–■–), total methanol feed (–♦–). (d) SDS-PAGE of the culture supernatants withdrawn at different times.

The decrease of $Q_{eq}$ for both adsorbents when pH was increased indicated that an ion exchange mechanism was involved in the interaction between β-glucosidase and adsorbent (Fig. 3a). However, the Streamline Direct HST1 adsorbent was shown to be much more salt tolerant with respect to binding of β-glucosidase compared to Streamline SP at pH 4 (Fig. 3b). In fact, the binding was achieved well above the conductivity of the *P. pastoris* culture broth (18 $mS^{-1}$). Furthermore, the higher $Q_{eq}$ of Streamline Direct HST1 than of Streamline SP at all measured pH and conductivity values is probably due to other interactions, e.g. like hydrophobic interaction from the aromatic ring and thiophilic interaction from the

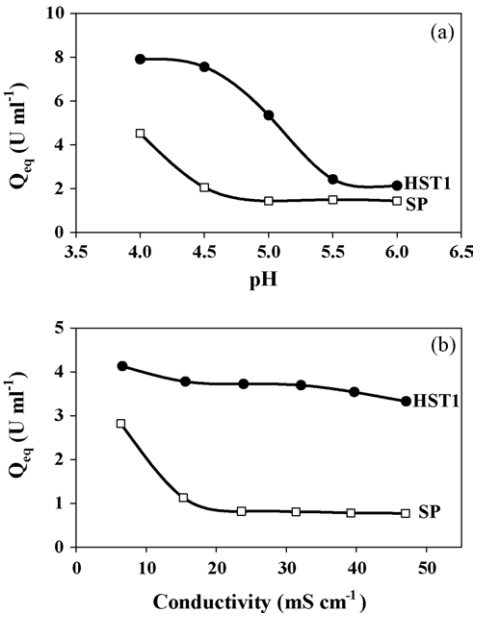


Fig. 3. Equilibrium capacity ($Q_{eq}$) for adsorption of β-glucosidase to Streamline SP (–□–) and Streamline Direct HST1 (–●–), (a) at various pH with constant $C_0 = 2.687$ U ml$^{-1}$ and conductivity 15.0 mS cm$^{-1}$ and (b) at various conductivity values with constant $C_0 = 1.219$ U ml$^{-1}$ and pH 4.0.

thioether spacer arm. However, a high salt concentration is required for promoting protein adsorption in both traditional hydrophobic interaction chromatography and traditional thiophilic interaction chromatography (Porath, 1990) but for Streamline Direct HST1 the β-glucosidase was bound even at low conductivity, thus indicating a more complex interaction. Interestingly, a modified thiophilic gel containing a thioether spacer arm has been used as salt-independent adsorbent, i.e. adsorbing protein at both high and low salt concentrations (Scholz et al., 1998).

The interaction between β-glucosidase and Streamline Direct HST1 very much resemble the interaction described to take place in hydrophobic charge induction chromatography (Burton and Harding, 1998) and mixed mode chromatography (Burton et al., 1997). When a ligand similar to Streamline Direct HST1 was studied that contained a thiophilic spacer arm with a heterocyclic function the suggested binding mechanism was described to involve ionic, hydrophobic and thiophilic interactions. One advantage was that the adsorption could take place at both high and low conductivities; a pre-treatment step to adjust the salt concentration of feedstock was not required (Burton et al., 1997).

### 3.3. Dissociation of β-glucosidase from the adsorbents

The eluting conditions were determined in test tube experiments by varying pH and conductivity. β-Glucosidase was dissociated from Streamline SP at pH values above pH 4.5 at a conductivity of 0.8 mS cm$^{-1}$ (Fig. 4a). At a constant pH of 4.0, the dissociation started when the conductivity exceeded about 10 mS cm$^{-1}$. Based on this, 250 mM NaCl in 50 mM sodium acetate buffer of pH 5.0 (corresponding to a conductivity of 24.7 mS cm$^{-1}$) was selected for dissociation of β-glucosidase from Streamline SP.

For Streamline Direct HST1, the complex interaction with the ligand also affected the desorption. The β-glucosidase could not be dissociated efficiently at low pH even at very high conductivity. In addition, decrease of salt concentration could not be applied to dissociate, thus indicating that the β-glucosidase did not bind to Streamline Direct HST1 through pure hydrophobic interaction. The pH change resulted in a change of interaction between β-glucosidase and the adsorbent and hence β-glucosidase was dissociated rapidly. At pH 7 conductivity values up to as high as 100 mS cm$^{-1}$ were found to be suitable conditions for the dissociation of β-glucosidase from Streamline Direct HST1. The dissociation pattern observed as a function of ionic strength indicated the existence of a dissociation optimum. At low salt concentration the charge interaction dominates and the electrostatic change can be applied to dissociation. However, the hydrophobic interaction was increased at higher salt concentration (>100 mS cm$^{-1}$) while the dissociation ratio decreased and β-glucosidase was bound to the adsorbent again (Fig. 4c). This again indicated the similarity of the behaviour of Streamline Direct HST1 ligand with hydrophobic charge induction chromatography (Burton and Harding, 1998) as discussed above.

### 3.4. Breakthrough capacity in EBA

The breakthrough capacities ($Q_B$) of Streamline SP and Streamline Direct HST1 were compared in

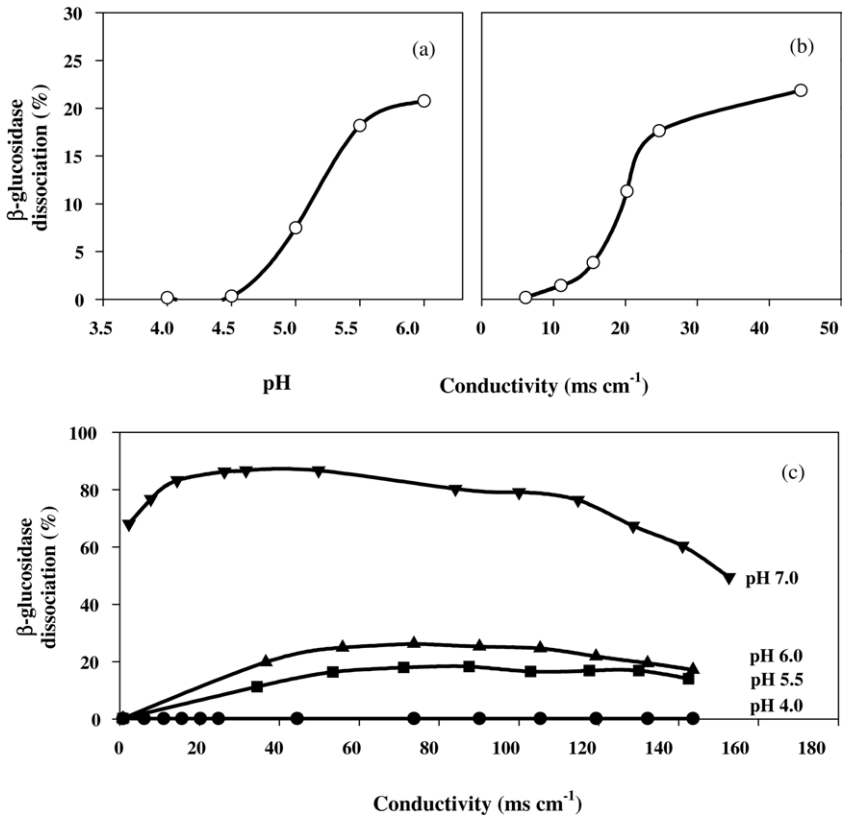


Fig. 4. Dissociation of β-glucosidase from (a) Streamline SP at various pH with constant conductivity 0.8 mS $cm^{-1}$, (b) Streamline SP at various conductivity with constant pH 4.0, (c) Streamline Direct HST1 at various pH (4.0 (–●–), 5.5 (–■–), 6.0 (–▲–), and 7.0 (–▼–)) and conductivity in range of 0.8–152.6 mS $cm^{-1}$.

expanded bed using cultivation broth. The experimental conditions are summarised in Table 1. The normalized effluent concentration ($C/C_0$) was plotted versus the β-glucosidase loading per volume of adsorbent [$(V_{eff}C_0)/V_{ads}$] in order to determine the $Q_B$ (Fig. 5). The 5% breakthrough capacity ($Q_{B5\%}$) for β-glucosidase was about 160 times higher on the Streamline Direct HST1 (210 U $ml_{ads}^{-1}$) compared to Streamline SP (1.3 U $ml_{ads}^{-1}$).

### 3.5. Recovery of β-glucosidase from unclarified culture broth by EBA

According to the binding and dissociation conditions, and the breakthrough capacity experiments the conditions for EBA process operations have been designed (Table 1). By using the denser Streamline

Table 1
Comparison of binding capacity at 5% breakthrough between Streamline SP and Streamline Direct HST1 for recombinant β-glucosidase recovery from *P. pastoris* unclarified cultivation broth

| Conditions | Streamline SP | Streamline Direct HST1 |
|---|---|---|
| Feed cell dry weight (g $l^{-1}$) | 24 | 60 |
| Feed cell wet weight (g $l^{-1}$) | 105 | 248 |
| Feed wet cell volume (ml $l^{-1}$) | 80 | 217 |
| Feed cell count (cell) | $6 \times 10^{12}$ | $4 \times 10^{13}$ |
| β-Glucosidase ($C_0$) (U $ml^{-1}$) | 1.6 | 4.3 |
| Conductivity (mS $cm^{-1}$) | 5 | 15 |
| pH | 4.0 | 4.0 |
| Sedimented bed height (cm) | 20.5 | 20.5 |
| Adsorbent volume ($V_{ads}$) (ml) | 100.7 | 100.7 |
| Liquid velocity (cm $h^{-1}$) | 300 | 600 |
| Bed expansion | 3.9 | 3.3 |
| $Q_{B5\%}$ (U $ml_{ads}^{-1}$) | 1.3 | 210.0 |

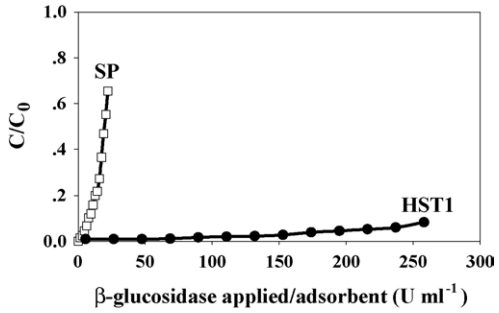


Fig. 5. Breakthrough curve of β-glucosidase from unclarified *P. pastoris* culture broth on expanded bed of Streamline SP (–□–) and Streamline Direct HST1 (–●–). Experimental conditions according to Table 1.

Direct HST1 matrix compared to the Streamline SP, it was possible to design an EBA process where one could use a two times higher flow velocity and a two times more concentrated *P. pastoris* culture broth (246.4 g $l^{-1}$ cell wet weight). Despite the higher concentration and flow velocity still the bed expansion was smaller. The Streamline Direct HST1 also showed higher capacity and the β-glucosidase could be adsorbed at both high and low salt concentrations. The reduced need of diluting the feedstock with Streamline Direct HST1 makes the EBA process a more interesting choice for direct recovery of β-glucosidase from the high-cell-density and high conductivity broth. In contrast, when Streamline SP was applied, a larger dilution of the feedstock was required to decrease both cell density and conductivity, in our case to 105 g $l^{-1}$ and 5 mS $cm^{-1}$, respectively (Table 1). A *P. pastoris* culture broth containing 100 g $l^{-1}$ wet cell weight and 10 mS $cm^{-1}$ conductivity was suggested for use on a Streamline SP matrix by Thömmes et al. (2001). Similar feedstock properties have also been used by others for *P. pastoris* recovery processes (Trinh et al., 2000; Murasugi et al., 2001). Too high a density and viscosity will lead to reduction of the terminal settling velocity of the adsorbent. Furthermore, a consequence of this will be an unreasonably high bed expansion during sample load (Thömmes et al., 2001).

The results of EBA chromatography with Streamline SP adsorbents is shown in Table 2. About 28% of the loaded β-glucosidase was found in the flow through. About 48% of the total β-glucosidase loaded on to the column was recovered in the eluted fraction. SDS-PAGE analysis under denaturing condition of the

Table 2
Recovery of β-glucosidase from *P. pastoris* unclarified culture broth using Streamline SP

| Purification step | Volume (ml) | Flow velocity (cm $h^{-1}$) | Total activity (U) | Total protein (mg) | Specific activity (U $mg^{-1}$) | Purification factor | Recovery of β-glu (%) | β-Glu concentration (U $ml^{-1}$) | Concentration factor |
|---|---|---|---|---|---|---|---|---|---|
| Feed prepared | 1500 | – | 2212 | 145 | 15.3 | (1) | (100) | 1.5 | (1) |
| Flow-through | 1500 | 300 | 608 | 65 | – | – | 27.5 | – | – |
| Wash | 1200 | 300 | 388 | 25 | – | – | 17.5 | – | – |
| Elute | 80 | 100 | 1052 | 33 | 31.8 | 2.1 | 47.6 | 13.2 | 8.8 |
| Total recovery (%) | – | – | 92.6 | 84.8 | – | – | – | – | – |

Experimental conditions as in Table 1.

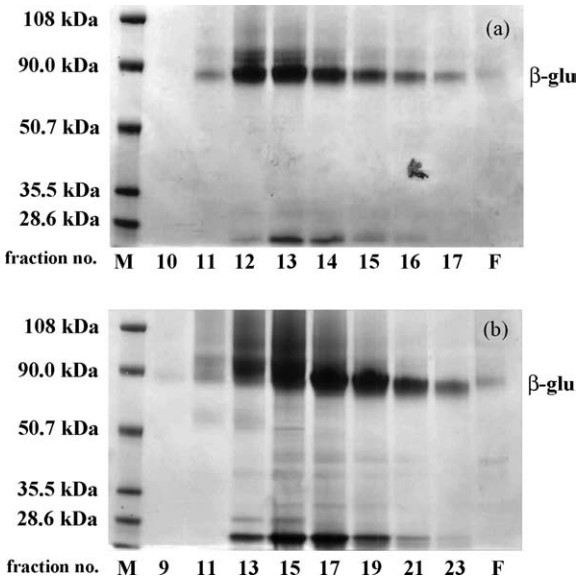


Fig. 6. SDS-PAGE analysis of peak fractions from the elution (a) Streamline SP (b) Streamline Direct HST1 (M: molecular weight marker proteins, F: feed applied to the column).

eluted fraction of β-glucosidase showed one main band of high intensity at the size (Fig. 6a) corresponding to the main band observed during cultivation (Fig. 2d).

For the EBA on Streamline Direct HST1 media (Table 3), a small amount of the loaded β-glucosidase was lost in the flow through (4.5%). About 74% of β-glucosidase was recovered in the eluted fraction. SDS-PAGE analysis under denaturing condition of the eluted fraction of β-glucosidase showed one band with very high intensity (Fig. 6b) at the same size as for samples during the cultivation (Fig. 2d). Also several bands of much lower intensity and smaller in size were observed (Fig. 6b). According to cell counting by flow cytometry a small amount of cells were observed in the eluted β-glucosidase fraction from both Streamline SP and Streamline Direct HST1 columns. However, still a cell reduction of about $10^6$ times was achieved compared to the feed (data not shown).

The elution recovery of β-glucosidase on Streamline Direct HST1 (74%) was higher than on Streamline SP (48%). However, the total recovery was lower on the Streamline Direct HST1 adsorbent (77%) compared to the Streamline SP adsorbent (93%). This might be explained by complex interaction of the Streamline Direct HST1 ligand that was also observed in the

Table 3
Recovery of β-glucosidase from *P. pastoris* unclarified culture broth using Streamline Direct HST1 expanded bed chromatography

| Purification step | Volume (ml) | Flow velocity (cm $h^{-1}$) | Total activity (U) | Total protein (mg) | Specific activity (U $mg^{-1}$) | Purification factor | Recovery of β-glu (%) | β-Glu concentration (U $ml^{-1}$) | Concentration factor |
|---|---|---|---|---|---|---|---|---|---|
| Feed prepared | 4600 | – | 16630 | 1236 | 13.5 | (1) | (100) | 3.6 | (1) |
| Flow-through | 4600 | 600 | 353 | 756 | – | – | 4.5 | – | – |
| Wash | 1400 | 600 | 121 | 270 | – | – | 0.7 | – | – |
| Elute | 200 | 100 | 12383 | 224 | 55.3 | 4.1 | 74.4 | 61.9 | 17.2 |
| Total recovery (%) | – | – | 77.3 | 101.1 | – | – | – | – | – |

Experimental conditions as in Table 1.

binding and dissociation conditions experiments and discussed above.

In conclusion, the Streamline Direct HST1 adsorbent compared to Streamline SP shown to be much more suitable for the design of an EBA processes for recovery of β-glucosidase directly from high-cell-density and high conductivity *P. pastoris* culture broth. By using the Streamline Direct HST1 adsorbent the dilution of the culture broth feedstock could be significantly reduced. The productivity during loading, washing and eluting was almost 4 times higher on Streamline Direct HST1 ($20.7\,kU\,l^{-1}\,h^{-1}$) compared to Streamline SP ($5.3\,kU\,l^{-1}\,h^{-1}$).

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