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Full Length Research Paper

Application of a high density adsorbent in expanded bed adsorption of lipase from *Burkholderia pseudomallei*

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Accepted

The application of STREAMLINE Direct HST adsorbent in expanded bed adsorption of lipase from *Burkholderia pseudomallei* was explored in this study. Scouting of optimum binding and elution condition was performed in batch binding mode. The addition of 0.2 M salt in acetate buffer (pH 5) during adsorption has increased the specificity and quantity of lipase binding onto the adsorbent. The addition of 0.4 M salt in phosphate buffer (pH 7) achieved the highest purification fold (2.5) in elution. The high density of the adsorbent allowed the EBA to be operated at linear velocity as high as 657 cm/h with feedstock containing 4.5% (w/v) wet biomass. The Richardson-Zaki correlation obtained for this EBA system at the presence of 4.5% (w/v) wet biomass is 5.14, a value closed to the laminar flow regime of 4.8, demonstrated that a stable bed is achieved under this operating condition. Meanwhile, a flow velocity of 343 cm/h with bed expansion of 3.2 gave highest dynamic binding capacity (4979.28 U/ml) and productivity (61.52 U/ml.min) for this EBA operation. It also demonstrated that biomass concentration up to 4.5% (w/v) wet weight showed slightly drop of sorption efficiency (0.82) compared to lower biomass concentration (0.94). Further increase of biomass concentration above 4.5% (w/v) wet weight has greatly decreased the equilibrium and dynamic capacity. Application of high density adsorbent tolerated to high density and biomass has reduced the processing time and increased the productivity.

Key words: Expanded bed adsorption, lipase, adsorbent, *Burkholderia pseudomallei*, high density.

INTRODUCTION

Microbial lipases are widely used as biocatalysts to catalyze a wide variety of reactions in aqueous and non-aqueous media due to its ability to utilize wide spectrum of substrates, stable at extreme temperature, pH and organic solvent (Koeller and Wong, 2001). Industries have employing lipases for the biocatalytic production of fine chemicals, pharmaceuticals and cosmetics, detergent and degreasing formulation, paper manufacture and accelerated the degradation of fatty waste and polyurethane (Kazlauskas and Bornscheuer, 1998; Masse et al., 2001; Rubin and Dennis, 1997; Takamoto et al., 2001). The biocatalysts properties of lipase also used to synthesis value-added product like biodegradable lubricants (Brenneis et al., 2004) and biodiesel from vegetable oil (Noureddini et al., 2005).

Traditionally, adsorption chromatography is often addressed as packed bed adsorption, which required clarification of crude feedstock before being applied to the

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packed bed column. The removal of insoluble particle from the feed is critical to assure no particulate material was being trapped in the column as this could result in severe operation problem (Chase and Draeger, 1992). Centrifugation and filtration are techniques that widely used for the separation of insoluble particles. However, these clarification methods are time-consuming, high in operation and capital cost and may result in significant loss of desired product (Cheigh et al., 2004).

In some cases, the supernatant has to be concentrated through precipitation and even dialyzed before sample can be applied to column (Tan et al., 2006). Hence, these conventional purification steps are very costly and labor intensive. Recent advanced in expanded bed adsorption (EBA) has shown its promised to be benefits as a direct recovery process by combining the clarification, concentration and initial purification into a single, integrated operation procedure for the recovery of protein from crude feedstock (Fernández-Lahore et al., 2000). The integration of several processes into a single step operation is not only contribute to the economic of the purification operation by reducing the overall processing time, operating cost and capital cost (Anspach et al., 1999), it also produced concentrated partially purified product which is ready for next purification steps to obtain purified product.

In EBA process, upward fluidized stationary phase with particle feedstock result in increased void fraction, which allowed the passage of cells or cell particle with concomitant adsorption of target protein (Jahic et al., 2006). Stable expanded bed is critical to the overall process performance (Jahic et al., 2006), a well performing adsorbent will prevent bed instability and give high breakthrough capacity (Anspach et al., 1999; Lei et al., 2003; Tong and Sun, 2002). By using high density adsorbent, the EBA process can be performed at high flow velocity or high particulate containing feedstock without losing of adsorbent in flow-through (Charoenrat et al., 2006).

STREAMLINE Direct HST is a type of multi modal adsorbent that designated to have high density (1.8 g/cm^3) inert stainless steel core. This high density bead tolerated higher fluid velocity and minimized dilution due to biomass or viscosity. Besides, the beads are attached with ligands of multi-functional groups, which make the adsorbent more tolerate to the high ionic strength and salt concentration and give high binding capacity. This adsorbent has been used for purification of lipase fused with cellulose-binding module from *Neocallimastix patriciarum* cellulose 6A (Jahic et al., 2006) and β-glucosidase (Charoenrat et al., 2006) which gave satisfying result compared to other cation adsorbent. Mechanical analyses of STREAMLINE Direct HST adsorbent also were done by (Gao et al., 2007) for understanding the interaction involved in this adsorbent. Therefore, the aim of this study was to develop an EBA process for direct recovery of lipase from unclarified feedstock using STREAMLIME Direct HST adsorbent. The binding and dissociation conditions of lipases onto and from the STREAMLIME Direct HST adsorbent and the influence of superficial flow velocity and biomass concentration on breakthrough capacity of the EBA process were investigated in the present study.

MATERIALS AND METHODS

Sample preparation

Burkholderia pseudomallei producing lipases was grown in batch culture of the liquid medium constituted of nutrient broth, 0.325% (w/v), $CaCl_2$, 0.1% (w/v), olive oil, 2.5% (v/v), and gum Arabic, 1% (w/v), at 37°C, pH 9.0 in shake flasks agitated at 250 rpm. Culture was harvested after 72 h as unclarified feedstock. Clarified sample was prepared by collecting supernatant from the centrifugation of unclarified feedstock at 16000 xg (Universal 32 R, Hettich Zentrifugen, Germany) for 20 min.

Method Scouting

Optimization of Binding

Scouting experiment was carried out in batch binding mode by using clarified feedstock. Prior to the batch binding experiment, 0.5 ml settled volume of STREAMLINE Direct HST adsorbent was pre-equilibrated overnight with 5 ml buffer of corresponding binding condition. Binding condition of various pHs from pH 4 to 11, and effect of the addition of additive (glycerol and NaCl) were examined. Clarified feedstock with corresponding buffer condition was added to the pre-equilibrated adsorbent and was well mixed in incubator shaker at 30 rpm for 2 h at room temperature. After 2 h incubation, the adsorbent was allowed to settle, and the supernatant was collected for lipase activity assay and total protein quantification. The amount of lipase and total protein bound was estimated based on the mass balance between initial and equilibrium lipase activity and total protein, respectively. Specific activity (S) was calculated as Equation 1:

$$S = \frac{T_1}{T_p} \quad (1)$$

Where T_l is total lipase activity (U) and T_p is total protein (mg).

Equilibration Isotherm

Adsorbent was pre-equilibrated with acetate binding buffer. After equilibration, clarified feedstock with different ratio of sample to acetate buffer was added to the adsorbent and incubated at 30 rpm for 2 h at room temperature. Supernatant was assayed for lipase activity and the adsorption capacity was calculated as Equation 2:

$$Q_e = \frac{(C_o - C_{eq})V_1}{V_{ad}} \quad (2)$$

Where Q_e is adsorption capacity, C_0 is initial lipase activity, C_{eq} is equilibrium-lipase activity and V_l and V_{ad} are the total volume in the batch binding (ml) and the volume of adsorbent (ml), respectively.

Optimization of elution

Screening of the elution condition was performed with various pH buffers with and without glycerol and NaCl. Clarified feedstock was added to 0.5 ml of adsorbent and incubated in incubator shaker at 30 rpm for 2 h at room temperature. After equilibration, adsorbent was allowed to settle and supernatant was collected. Washing step was carried out with washing buffer (same buffer bused for binding) to wash out loosely or unbound protein. Following washing steps, elution buffer was added and well mixed at 30 rpm for one hour at room temperature. Supernatant from the feedstock, after binding and after elution were assayed for lipase activity and total protein to calculate the purification factor (P) as Equation 3:

$$P = \frac{S_e}{S_b} \quad (3)$$

Where S_e is specific activity of elution (Umg^{-1}) and S_b is specific activity of binding (Umg^{-1}).

Operation of EBA

Bed expansion characteristic

Expanded bed system consisted UpFront FastLine 10 system (i.d.10 mm) was packed with STREAMLINE Direct HST adsorbent. Column was filled with 5 cm sedimented bed height of adsorbent. The bed was flowed with buffer and unclarified feedstock containing 4.5% (w/v) wet biomass at different superficial velocity and allowed to stable for 5 min. The height of the stable expanded bed was measured. Degree of bed expansion was determined from the ratio of expanded bed height (H) to settle bed height (H_o).

Dynamic binding capacity

UpFront FastLine 10 packed with 5 cm STREAMLINE Direct HST adsorbent was connected to a peristaltic pump, UV detector and a fraction collector. The adsorbent in column was equilibrated with 50 mM sodium-acetate buffer, pH 5 for 10 column volume at corresponding flow rate (from 157 cm/h to 657 cm/s). The sample was then feed to the column at the flow rate similar to that used for equilibration. Sample from the outlet was collected and each fraction of the sample was assayed for lipase activity and total protein quantification. Breakthrough of the lipase and protein at $C/C_0 = 0.1$ was determined from plot C/C_0 versus volume of sample collected. Dynamic capacity (Q_B) of adsorbent was calculated as Equation 4:

$$Q_B = \frac{C_0 V_0}{V_s} \quad (4)$$

Where C is the concentration of sample at outlet, C_0 is the initial concentration, V_b is the volume at 10 % breakthrough, V_s is the settle volume of adsorbent (ml).

The EBA recovery process

Throughout the EBA study, effect of different flow rate (157 to 687 cm/h) and biomass concentration (1 to 35% (w/v) wet biomass) was studied. UpFront FastLine 10 column packed with 5 cm sediment bed height of STREAMLINE Direct HST adsorbent was performed at different superficial velocity and biomass concentration at constant room temperature.

The equilibrium, washing and elution condition for purification of lipase from unclarified feedstock by EBA was chosen from the method scouting experiment. All the purification steps involved above including sample application were carried out at upward flow with corresponding superficial velocity to be examined. Prior to feedstock application, equilibration was carried out with approximate 5 column volume of 50 mM sodium-acetate buffer, pH 5 with 0.2 M NaCl until the pH at inlet equal to outlet, then washing was performed after feedstock application at flow velocity similar to that during equilibration and sample feeding steps. Approximate 10 column volume of buffer was applied to remove all the cell particle and unbound or loosely bound protein. The washing steps can be assisted by the UV detector. Absorbency value at 280 nm returned to base line indicated no unbound and loosely bound protein present in the column. Elution was performed using 50 mM phosphate buffer, pH 7 with addition of 0.4 M NaCl in expanded mode. Regeneration of the adsorbent was performed using 1 M NaCl in 0.5 M sodium hydroxide, distilled water, 25% acetic acid in 20% ethanol and distilled water again as recommended by manufacturer (Charoenrat et al., 2006).

Lipase activity assay

The reaction mixture consisted of 25 µL sample, 200 µL ml 0.05 M phosphate buffer (pH 6.5), 0.1 ml 25 µL *p*-nitrophenil-laurate (pNPL) in ethanol and 2% (v/v) Triton X-100. The amount of *p*-nitrophenol released in the hydrolysis of pNPL at 25°C in 30 min was measured using microplate reader (Tecan Sunrise) at absorbance 405 nm. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 umol *p*-nitrophenol per minute.

Total protein assay

Protein quantification was determined by using Bradford assay (Bradford, 1976) Sample of 160 µl was added with 40 µl of Bradford reagent (Bio-Rad) and incubated for 30 min. The amount of protein was determined by measuring the absorbency of the mixture at 595 nm and the standard curve that has created from a range of know amount of protein.

SDS-PAGE analysis

Electrophoresis was performed in Bio-Rad vertical slab gel apparatus as described by Lammelli (1970). Acrylamide of 12% (w/v) resolving gel and 4.5% stacking gel was used in present study. Sample was prepared by mixing supernatant and 4x sample buffer containing mercapethanol in ratio of 1:1 and incubated for 10 min. at boiling water. Sample of 15 µL was loaded to the well and run at 110 V for 75 min. The gel was stained with Coomassie Blue R-250 for 30 min. and destained by 25% methanol and 10% acetic acid until clear bands appeared.

RESULTS AND DISCUSSION

Effect of pH

Optimization of binding condition

Amount of lipase bound onto the a dsorbent was calcu-

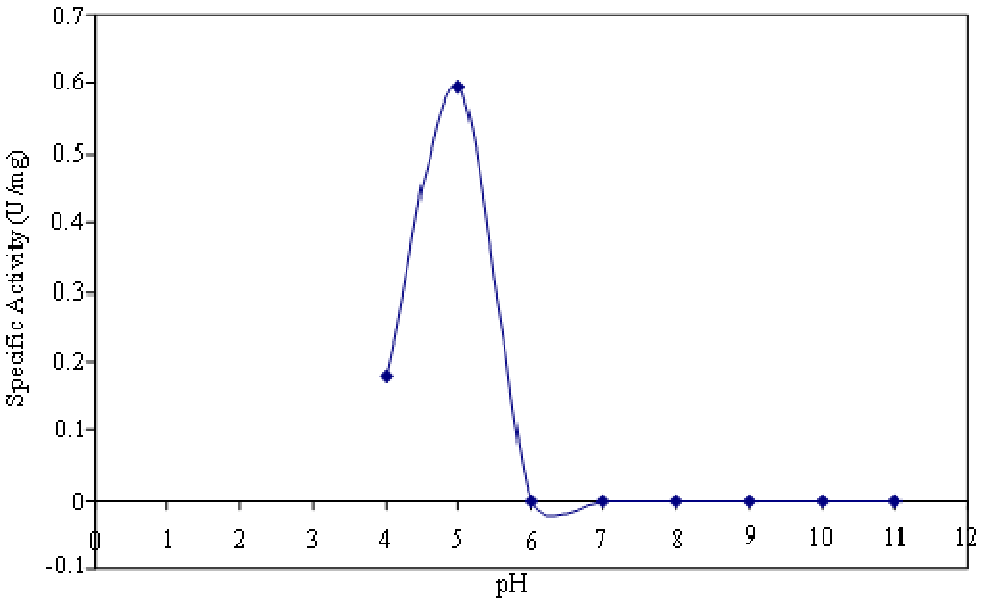

Figure 1. Effect pH on specific adsorption of lipase. The pH scouting for optimal adsorption of lipase onto STREAMLINE Direct HST adsorbent was carried out in batch binding mode at various pH ranged from pH 3 to 11 (Glysine-HCl for pH 3, Sodium acetate-acetic acid for pH 4 and 5, Na_2HPO_4-NaH_2PO_4 for pH 6, 7 and 8, Glysine-OH for pH 9 and 10, Potassium-OH for pH 11). All the buffers were prepared at constant ionic strength of 50 mM. After 2 h incubation of clarified sample with pre-equilibrated adsorbent at corresponding buffer condition, supernatant was assayed for lipase activity and total protein. The amount of lipase and total protein bound was estimated based on the mass balance between initial and equilibrium lipase activity and total protein, respectively. The specific activity was determined according to Equation 1.

lated by measuring the mass balance between the amount of lipase in the binding buffer before binding and after binding. From Figure 1, it is clearly shown that about 80% and 10% of lipase was bound onto adsorbent at pH 5 and 4, respectively and no lipase was bound onto the adsorbents at pH > 5. For further confirmation of the binding of lipase onto the adsorbent, washing step by binding buffer and elution by corresponding pH buffer with 0.5 NaCl was carried out. The elution result showed that only the adsorbent from pH 4 and 5 found to have lipase. Meanwhile, no lipase was present in the elution fraction of adsorbents from other binding pH. This indicated that no binding of lipase onto the adsorbent was occurred from pH 6 to pH 11.

Dissociation of COOH group, a weak cation group of STREAMLINE Direct HST adsorbent to COO^- and H^+ make the adsorbent charged negative at acidic pH (Chang et al., 2007; Gao et al., 2007). According to Ishimato et al. (Ryo Ishimoto et al., 2001), lipase from *Burkholderia sp* have *pI* value of 6.3, lipase charged negative when pH > *pI*, therefore, repulsion between lipase and adsorbent occurred at pH > 6. However, pH 4 also showed lesser lipase binding compared to pH 5. This can be explained through Henderson–Hasselbalch equation where higher pH value represent higher ratio of COO^- concentration to COOH concentration. Since pK_a of STREAMLINE Direct HST adsorbent was 1.95 (Chang et al., 2007), thus, by referring to Henderson–Hasselbalch equation:

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \quad (5)$$

Where pK_a is the negative log of the thermodynamic dissociation constant. Therefore, pH > 3, the association of COOH group will make the adsorbent negatively charged and no dissociation of COOH group occurred when pH < 3. This indicated the dissociation of carboxylic group at pH 4 was lesser compared to pH 5. In other words, there are more ligands available for lipase binding at pH 5.

Effect of glycerol and salt on the lipase binding

Adsorbent used in this study is STREAMLINE Direct HST, which is a cation multimodal functional adsorbent. The interactions that might appear included electrostatic interaction by carboxylate group, hydrophobic interaction by phenyl group and hydrogen bonding by hydroxyl group, carbonyl group and amide group (Chang et al., 2007). To increase the specificity of lipase binding, additive like sodium chloride (NaCl) and glycerol were commonly added to reduce the electrostatic interaction and hydrophobic

bond present.

Glycerol was chosen because lipase remained active in glycerol compared to other additive like urea, potassium cyanate, SDS and acetone. The present of glycerol reduce the hydrophobic interaction by decreased the polarity of liquid (Chang et al., 2007). However, the presence of glycerol also increased the viscosity of the liquid medium which affecting the mass transfer and hence decreased the protein binding.

Therefore, glycerol additions to the buffer at high and low ionic strength were examined to exclude the effect of viscosity on lipase binding. Indeed, Porath (1990) and Gao et al. (2007) have reported that the presence of hydrophobic interaction was not significant under low ionic strength condition.

From Figure 2a, addition of glycerol onto low ionic strength buffers have decreased the binding of lipase onto adsorbent. The decreased of lipase binding might due to the mass transfer resistant in the viscous buffer, because hydrophobic effect was not significant under low ionic strength (Gao et al., 2007; Porath, 1990). Under high ionic strength buffer, lower amount of lipase was found to bind at each glycerol concentration compared to low ionic strength buffer. This may further suggested that the decreased of lipase binding was due to the decreased of hydrophobic interaction. Presence of 50% glycerol at high ionic strength buffer has reduced 30% of the lipase binding compared to buffer without glycerol addition. This indicated that either hydrophobic agent used was not strong enough to dissociate hydrophobic bond presence or hydrophobic interaction was not played much role on the binding of lipase onto STREAMLINE Direct HST adsorbent (Gao et al., 2007).

To further clarify this observation, different concentrations of salts were added during the binding process and the results are shown at Figure 2b. High salt concentration gave high ionic strength of buffer, which loosen the ionic and hydrophilic interaction and disrupted all the non-hydrophobic interaction (Chang et al., 2007). As a result, the electrostatic interactions was shielded but the hydrophobic interaction was somehow strengthened (Gao et al., 2007).

However, the results showed that the increase of salt concentration has decreased the relative lipase binding to 40% at 1 M NaCl compared to buffer without salt addition. This indicated that electrostatic interaction is more pronouns to hydrophobic interaction in contributing lipase binding to STREAMLINE Direct HST adsorbent.

Since the addition of glycerol and salt had affected the binding of lipase onto adsorbent, specificity of binding of lipase at different concentration of salt and glycerol were studied and the results are shown at Figures 2c and d. Addition of 0.2 M of NaCl in acetate buffer pH 5 give most ideal binding of lipase in term of low amount of lipase loss (10%) and high specificity (0.97 U/mg). Addition of salt gave higher specificity of lipase binding because higher conductivity or ionic strength of buffer prevented contaminant protein bound to adsorbent through non-specific interaction especially at low ionic strength condition (Ng et al., 2007). Tolerant to high salt condition for binding is only applicable to salt-tolerant adsorbent which is one of the special characteristic of STREAM-LINE Direct HST adsorbent.

Adsorption equilibrium

Langmuir model (Equation 6) was used to describe the equilibrium adsorption isotherm of lipase:

$$q = \frac{q_{\max} C}{K_d + C} \quad (6)$$

Linearized form of the Langmuir equation gives Equation 7:

$$\frac{C}{q} = \frac{K_d}{q_{\max}} + \frac{C}{q_{\max}} \quad (7)$$

The q_{max} and K_d can be calculated by least squares linear regression analysis. The q_{max} indicated the maximum binding capacity of adsorbent and K_d is the apparent dissociation constant which is a measure of the binding affinity or selectivity of the adsorbent for protein. The experiment data of the present study was well fitted to the Langmuir adsorption model.

As shown in Figure 3, addition of salt and glycerol significantly decrease the maximum binding capacity to 138 U/ml and 162 U/ml, respectively, which is almost 50% lower than that of the buffer without any additive (266 U/ml). The dissociation constant for lipase in buffer without additive is 1.46, meanwhile, buffer with salt and glycerol have dissociation constant of 13.63 and 10.78, respectively. The constant was related to the slope of the early part of the curve (Sarmento et al., 2000).

The apparent lower value of Kd represent as higher value of slope, which indicated sooner the maximum capacity was achieved (Sarmento et al., 2000) and higher binding affinity of lipase onto adsorbent (Chang et al., 2007). The curve of buffer without additive demonstrated an abrupt initial slope indicated the adsorbent was highly selective for adsorption of protein (Chang et al., 2007) compare to the other two curves in the present study.

Optimization of elution conditions

The lipase binding studies showed that the lipase binding onto the adsorbents were decreased as the increase of pH values and the addition of NaCl and glycerol. Therefore, elution efficiency under various pH and the addition of salt and glycerol was investigated. From Figure 4,

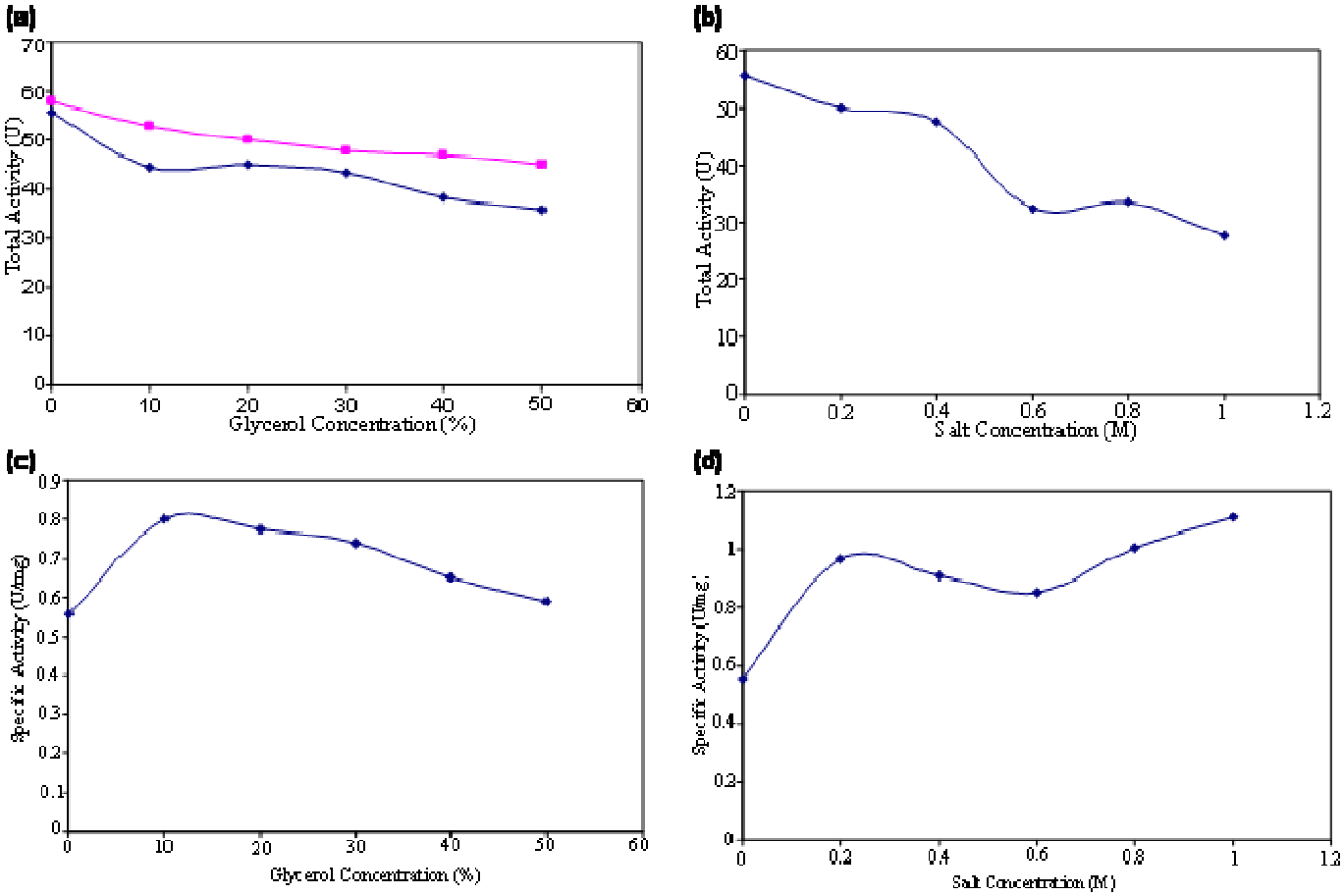

Figure 2. Effect of additives on specific adsorption of lipase. In this scouting experiment, all the studies were performed in batch binding mode at acetate buffer of constant pH 5. Graph of total activity versus different concentration of additives was plotted to study the effect of different concentration of a) glycerol at low ionic strength of 50 mM (■) and high ionic strength of 500 mM (♦) and b) NaCl to the binding of lipase. One unit of enzymatic activity was defined as the amount of enzyme needed to release 1 umol *p*-nitrophenol per minute. For further investigation, effect of different concentration of c) glycerol and d) NaCl to the specific binding of lipase was tabulated. The specific activity was determined according to Equation 1.

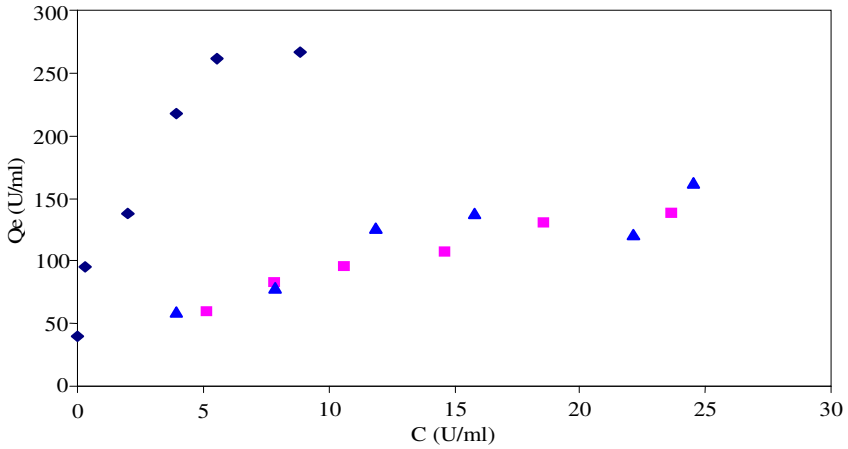

Figure 3. Equilibrium adsorption isotherm of lipase. Clarified sample with buffer pH 5 without any additive (♦) was compared to the both buffer pH 5 with the addition of 1M NaCl (■) and 50% glycerol (▲) on the adsorption equilibrium of lipase onto STREAMLINE Direct HST adsorbent at room temperature. Adsorption capacity (Q_e) was calculated according to Equation 2.

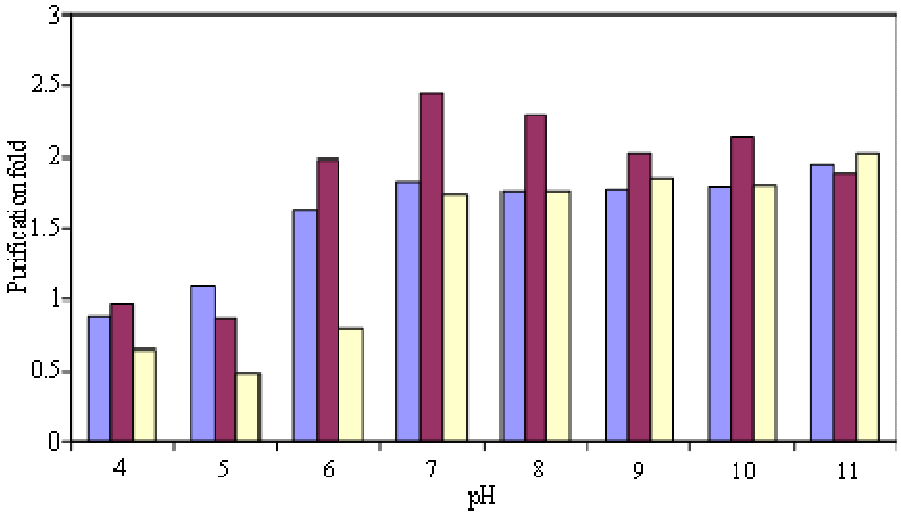

Figure 4. Effect of pH and additives to the specific elution of lipase. All the scouting experiment was carried out after binding with buffer pH 5 and washing were performed. Effect of various pH condition and additive to the elution of lipase was tabulated on the bar chart of purification fold versus elution condition. Purification fold was determined according to the Equation 3. Elution condition: buffer of various pHs without any additive (■), buffer of various pH with 1 M NaCl (■), buffer of various pH with 50% glycerol (■).

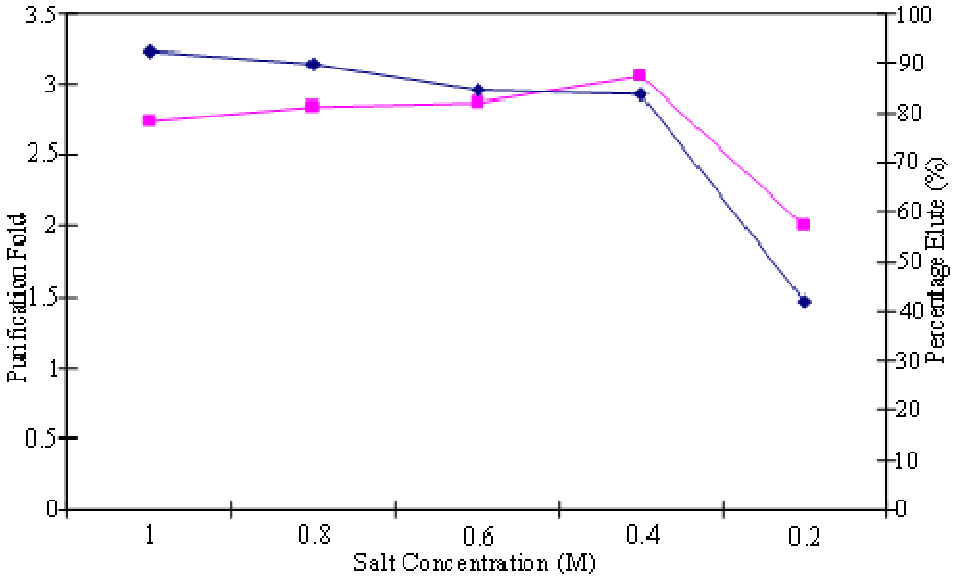

Figure 5. Effect of different salt concentration at pH 7 to elution of lipase.
Further optimization of elution condition at phosphate buffer pH 7 with addition of salt was performed. All the scouting experiment was carried out after binding with buffer pH 5 and washing were done. Purification fold (■) was determined according to Equation 3 and percentage of lipase (♦) being eluted from STREAMLINE Direct HST was calculated using the follow equation:

$$Percentage\ (\%) = \frac{total\ activity\ of\ lipase\ eluted}{total\ activity\ of\ lipase\ bound}\ x\ 100$$

phosphate buffer of pH 7 with addition of 1 M NaCl gave highest purification factor compared to other elution conditions. In this purification condition, 92% of lipase being eluted and a purification fold of 2.5 were achieved. In order to increase the specificity of lipase elution, phosphate buffer of pH 7 with different molarities of salt was studied as shown in Figure 5.

It is found that, when the salt concentration was

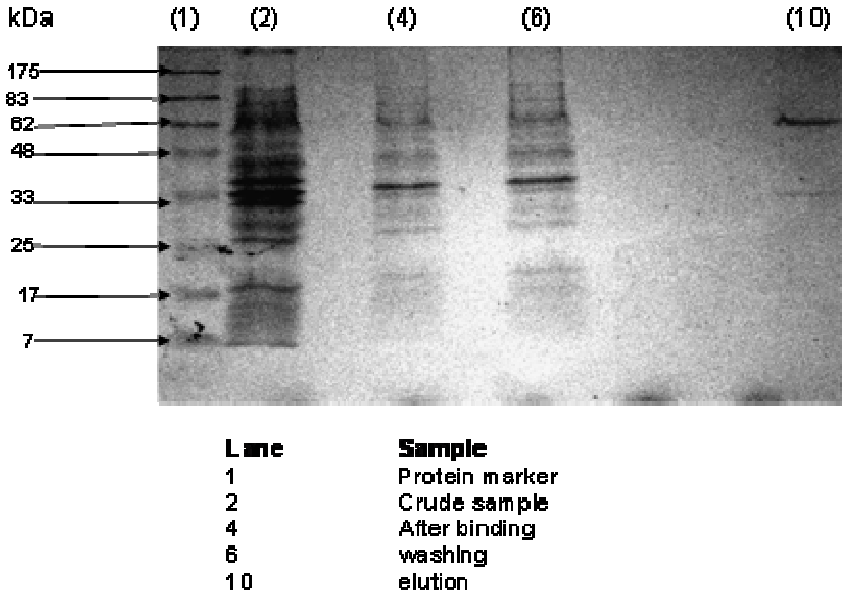

Figure 6. SDS-PAGE analysis of lipase recovery in batch binding mode.
SDS-PAGE of 12% gel was performed to assess the purity of eluted lipase (Laemmli, 1970). Proteins were precipitated by TCA and the pellets were dissolved with sample buffer constituted of 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 100mM Tris-HCl pH 6.8, 20% (v/v) glycerol and bromophenol blue. Molecular weight of standard protein marker ranged 7 - 175 kDa. Gel was staining with Coomassie® Brilliant Blue G-250.

decreased from 1 M NaCl to 0.4 M NaCl, the percentage of lipase eluted was lesser but the purification factor of the lipase increased to 3.05 fold as less contaminant protein was eluted together with lipase under this condition. By considering both the purification fold and percentage of lipase being eluted, phosphate buffer of pH 7 with 0.4 M NaCl was selected as elution buffer for further study.

SDS PAGE analysis of the samples showed that there are only two clear protein bands present in the elution fraction that corresponding to 36 kDa and 70 kDa (Figure 6). The MW of the lipase from *Burkaderia pseudomallei* was about 34 kDa in size as reported by Ishimoto et al. (2001). The 70 kDa band may be the aggregated dimer of the lipases. Therefore, condition optimized for the binding of lipase to STREAMLINE Direct HST has improved the purity of recovered lipases substantially.

Operation of EBA

Bed expansion characteristic

Figure 7 showed the relation between the flow rate and degree of expansion of the STREAMLINE Direct HST adsorbent when applied with acetate buffer solution and unclarified feedstock. The bed height increased linearly with flow rate. When STREAMLINE Direct HST applied with buffer solution, the bed was stable up to flow rate of 840 cm/h with bed expansion of 3, meanwhile, feedstock with 4.5% (w/v) wet biomass only stable up to 687 cm/h with bed expansion of 7. Further increase of flow rate cause channeling, leading unstable interface at top of expanded bed, over expansion and even cause the collapse of the expanded bed.

During visual inspection, local well mixed and large circular movements of adsorbent and liquid were observed at the whole expanded bed especially at high flow rate. However, at low flow rate, stable expanded zone can be observed above a well mixed and partially mixed zone. Fluidization of adsorbents bed in this study can be well represented by the empirical Richardson-Zaki correlation (Equation 8),

$$U = U_t \varepsilon^n \quad (8)$$

Where U is superficial velocity (cm/h), ε is the bed voidage, U_t is the terminal setting velocity (cm/h), and n is the Richardson-Zaki coefficient.

The value of terminal velocity (U_t) and coefficient (n) for unclarified feedstock was lesser than those in the buffer. This is because the unclarified feedstock with the present of biomass has a greater viscosity and density than the buffer (Chang and Chang, 2006). The increase of viscosity associated with increasing biomass concentration raised the bed voidage which eventually increased the degree of bed expansion (Chang and Chang, 2006; Ling and Lyddiatt, 2005). The value of terminal velocity and

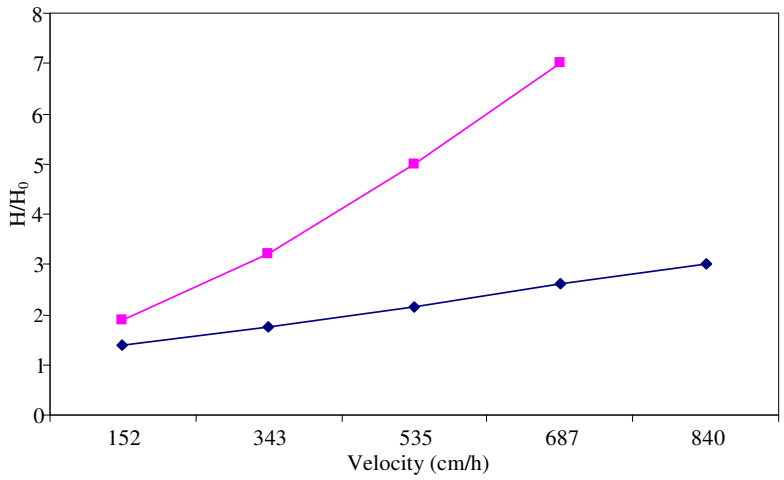

Figure 7. Bed expansion characteristic.
Bed expansion characteristic of STREAMLINE Direct HST adsorbent by acetate binding buffer pH 5 (♦) and 4.5% (w/v) wet biomass unclarified feedstock (■). H denoted expanded bed height and H_0 denoted settled bed height.

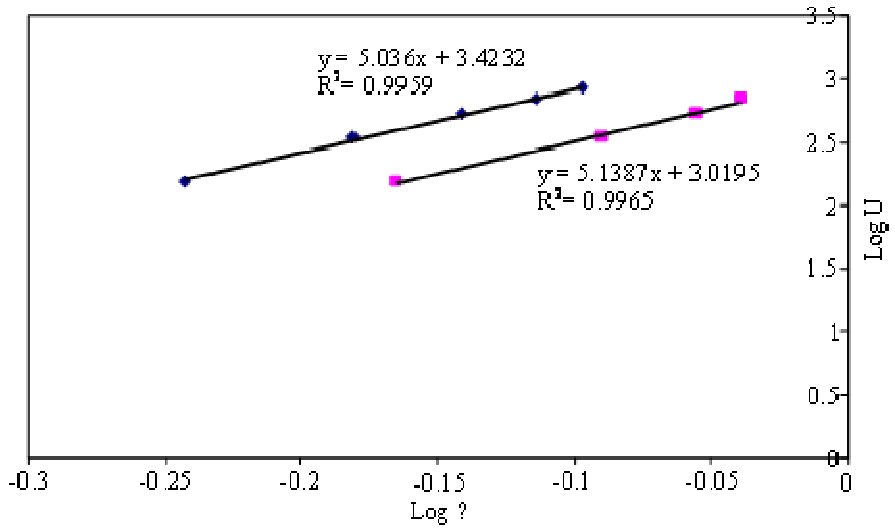

Figure 8. Linear regression plot of Richardson-Zaki correlation.
Linear regression plot of the Richardson-Zaki correlation as described by Equation 8 were demonstrated on the acetate binding buffer pH 5 (♦) and 4.5 % (w/v) wet biomass unclarified feedstock (■). Richardson-Zaki coefficient (n) obtained from the slope of logarithm of superficial velocity (log U) versus logarithm of bed voidage (log ε).

Richardson-Zaki coefficient (n) were determined from the linear plots of log ε versus log U as shown in Figure 8. The value of n for buffer and unclarified feedstock was 5.04 and 5.14 respectively. The n value for unclarified feedstock was reasonable close to the theoretical value of 4.8 (< 10%), which is normally used in the laminar flow regime (Kunii and Levenspiel, 1969), thus the bed was assumed to be stable upon expansion in the presence of 4.5% (w/v) wet biomass (Tan et al., 2006). Besides, terminal velocity of unclarified feedstock (1000 cm/h) is far higher than the flow rate going to be used for study in the effect of flow rate to dynamic capacity, therefore, adsorbent bead was predicted not to flow out from the column if flow rate within the range of to 687 cm/h that being used.

Flow rate

The influence of superficial flow velocity (157-657 cm/h)

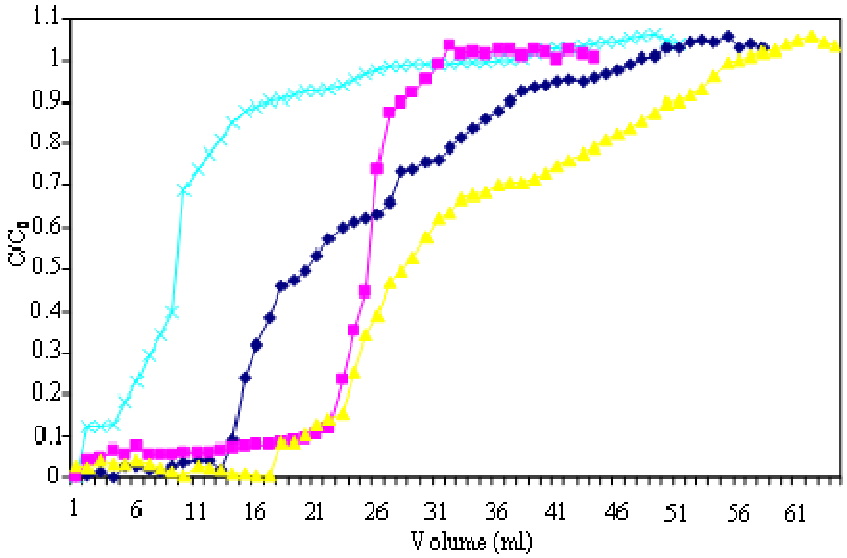

Figure 9. Effect of flow rate to dynamic capacity lipase in EBA
Effect of different flow rate (152 cm/h to 687 cm/h) to the dynamic capacity of lipase was determined at breakthrough point of 10 %. Sample was applied with 4.5 % (w/v) wet biomass unclarified feedstock adjusted to pH 5 by acetic acid and conductivity of 20.5 ms by NaCl solution. Dynamic binding capacity was calculated according to Equation 4. The result was summarized at Table 1. Linear velocity: 152 cm/h (■); 343 cm/h (▲); 535 cm/h (♦); 687 cm/h (x).

Table 1. Effect of flow rate to productivity and dynamic binding capacity of lipase. Effect of flow rate to the productivity and dynamic binding capacity was determined at breakthrough point of 10%. Dynamic binding capacity was calculated according to Equation 4 and productivity was determined as described by Jahic et al. (2006).

Volumetric velocity (ml/min)	Linear velocity (cm/h)	H / H_0	Dynamic binding capacity (U/ml)	Processing time (min)	Productivity (U/ml.min)
2	152	2.1	3872.8	270	25.3
4.5	343	3.2	4979.3	111	61.5
7	535	5	2581.9	88	12.9
9.5	687	7	553.3	77	6.2

H_0=settled bed height, H= expanded bed height

upon lipase adsorption was measured by establishing the breakthrough curves in the UpFront FastLine 10 System (10 mm i.d.). Dynamic capacity was measured from break-through point of 10% and productivity is defined as the amount of eluted lipase over total processing time of expanded bed (Jahic et al., 2006) (Figure 9 and Table 1). It was demonstrated that there is significant impact of superficial flow velocity upon lipase adsorption. With regard to the dynamic binding capacity at C_1/C_0 = 0.1, there appeared an optimum velocity (343 cm/h) in this study which give highest dynamic capacity and productivity.

A decreased in the dynamic binding capacity at lower interstitial flow velocity (152 cm/h) might have resulted from high resistance in film mass transfer between the lipase and functional group of bead as the viscous feedstock was applied (Cheigh et al., 2004). According to Hostmann (Horstmann and Chase, 1989), film mass transfer has been found to influence the initial breakthrough point of target molecule even though the film mass transfer step is normally move rapid than the intraparticle diffusion and / or surface reaction steps.

In contrast, a significant decrease in the dynamic binding capacity about 535 cm/h might have been due to reduced residence time for diffusion of target protein into adsorbent, which result in early breakthrough of enzyme (Cheigh et al., 2004; Ling and Lyddiatt, 2005). A similar observation was made by Thoemmas et al. (1995) and Ling and Lyddiatt (2005) when adsorptions of target protein were studied at different flow velocity. They suggested that the protein adsorption start to be limited by diffusion as soon as optimum superficial velocity was

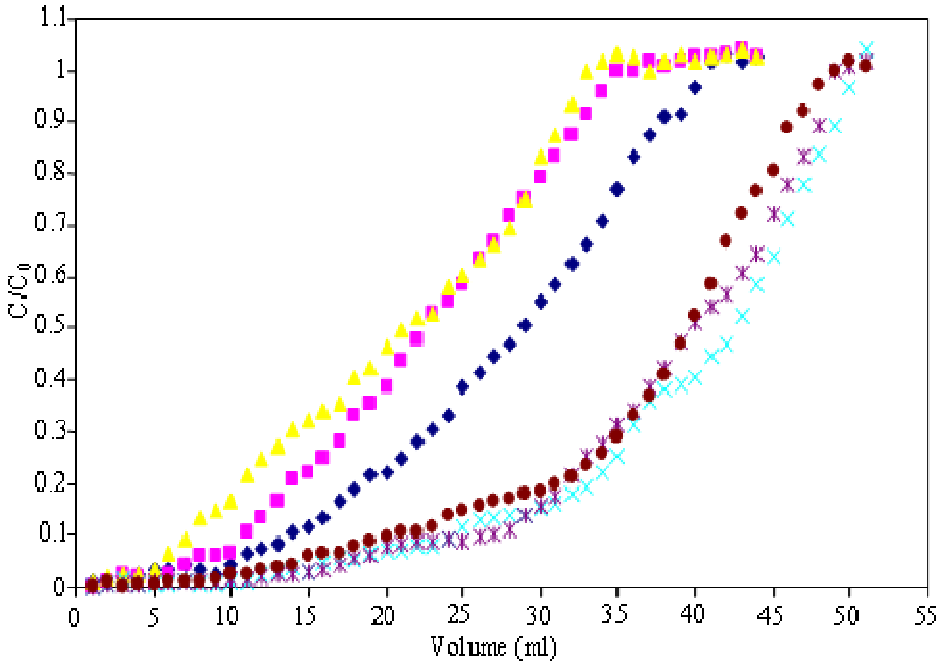

Figure 10. Effect of biomass concentration to binding capacity of lipase in EBA.
Breakthrough curve for the lipase binding onto STREAMLINE Direct HST adsorbent was performed at different biomass concentrations at constant flow of 343 cm/h linear velocity. Sample was adjusted to pH 5 and conductivity of 20.5 ms by acetic acid and NaCl solution, respectively. Dynamic binding capacity was determined at breakthrough point of 10% according to Equation 4 and equilibrium binding capacity was calculated according to equation described by Yamamoto (Yamamoto and Sano, 1992). Biomass concentration: 1.12% (x); 2.25% (ж); 4.5% (●); 9% (♦); 18% (■); 36% (▲).

achieved.

By accounting 10% breakthrough, flow rate at 343 cm/h with bed expansion of 3.1 was found to have highest efficiency in lipase binding compare to bed expansion of 2.1 and 5. This finding was consistent with other reports that maximum binding capacity of adsorbent was achieved with approximately 3-fold bed expansion for other protein (Chang and Chang, 1996; Cheigh et al., 2004; Mullick and Flickinger, 1999). When bed expansion is above 2, the voidage become large enough to neglect the influence of inert bio-particle (Dong-Qiang Lin et al., 2004), however, higher degree of bed expansion (>3) where the bed voidage is near 0.9 cause an axial dispersion in the protein front passing through the bed and the axial mixing was increased (Chang and Chase, 1996). Therefore, it has been suggested that with the employment of adsorbent with high density, degree of bed expansion can be reduced to decrease the bed voidage which eventually decreased the axial dispersion (Bierau, 2001). In addition, the finding of this study might indicated that the superficial flow velocity can improve the adsorption performance as long as there are sufficient time for protein molecule present in the bulk solution diffused into the adsorbent particles (Thommes et al., 1995).

Biomass concentration

The effect of biomass concentration on the binding capacity was examined. Sample was prepared by adjusted unclarified feedstock of different biomass concentration to pH 5 by acetic acid and conductivity of 20.5 ms by NaCl solution. In this study, both equilibrium capacity and dynamic capacity at the 10% breakthrough were measured. The equilibrium capacity was determined by integration of breakthrough curve as described by Yamamoto and Sano (1992) and dynamic capacity was calculated according to Equation (4).

Adsorption efficiency was obtained from the ratio of dynamic capacity to equilibrium capacity (Fernández-Lahore et al., 2000). Figure 10 depicted the breakthrough curve of lipase when applied with different concentration of biomass. The breakthrough curve shifted to right when biomass concentration was increased from 1% to 36% (w/v) wet biomass. The equilibrium capacity, dynamic capacity and sorption efficiency calculated from Figure 10 are tabulated in Table 2.

When low biomass concentration was applied to the expanded bed, both equilibrium capacity and dynamic capacity was very similar, in which identical high adsorption efficiency (0.9) was achieved. However, when

Table 2. Effect of biomass concentration to binding capacity of lipase. Effect of biomass concentration to the equilibrium binding capacity and dynamic binding capacity of lipase to STREAMLINE Direct HST adsorbent. Dynamic binding capacity was determined at breakthrough point of 10% according to Equation 4 and equilibrium binding capacity was calculated according to equation described by Yamamoto (Yamamoto and Sano, 1992). Adsorption efficiency was calculated as following equation.

$$Adsorption\ efficiency\ (\%) = \frac{dynamic\ binding\ capacity}{equlibrium\ binding\ capacity} \quad x \quad 100$$

Biomass Concentratio (%)	Equilibrium capacity (U/ml)	Dynamic binding capacity (U/ml)	Adsorption efficiency (%)
1.12	168.8	158.1	93.6
2.25	161.9	151.8	93.7
4.5	162.5	132.8	81.7
9	120.8	82.2	68.1
18	86.0	50.6	58.8
36	74.0	25.3	34.2

biomass concentration increased to 4.5% (w/v), dynamic binding capacity dropped about 12.5% and the adsorption efficiency was dropped slightly to 81%. A further increase of biomass concentration to 9 and 18% (w/v), has resulted a dropped of equilibrium binding capacity to 4780.3 and 3403.8 U/mg respectively.

In addition, dynamic binding capacity for 9 and 18% (w/v) wet biomass concentration was drop to 3253.6 and 2002.2 U/mg, respectively, thus resulted a drop in adsorption efficiency. At very high biomass concentration (36% (w/v)) operation, a slightly dropped of equilibrium binding capacity (2928.3 U/mg) was observed as compared to that of 18% (w/v) wet biomass concentration operation. The dynamic binding capacity of 36% (w/v) wet biomass concentration operation was rather low (1001.2 U/mg) as compared to other operations.

The results indicated that presence of cell at low concentration (< 2.25%) give no effect to the sorption performance in both equilibrium and dynamic binding capacity, when biomass concentration increased to 4.5% (w/v), only dynamic capacity was affected significantly, this might due to the existent of cell at a significant amount cause the presence of mass transfer resistant which eventually affected the binding of lipase to the adsorbent. Greater effect of biomass concentration was observed when 9 and 18% (w/v) of wet biomass were applied to the column, in which both equilibrium capacity and dynamic binding capacity were affected.

This might due to the presence of great amount of biomass have increased the chance of cell particle bound onto the hydrophobic group at STREAMLINE Direct HST adsorbent. Indeed, Fernandez-Lahore et al. (2000) has reported the possibility of interaction between hydrophobic domain on cell surface and ligand of adsorbent in a work evaluating the hydrophobic interaction mode of STREAMLINE Phenyl adsorbent. Cell in high biomass concentration prevented lipase from bound onto the STREAMLINE Direct HST adsorbent by reduced space of beads available for binding and created high mass transfer resistant to lipase.

Further increased of wet biomass to 36% (w/v) was not further affected much the equilibrium capacity but further decreased the dynamic capacity and sorption efficiency. This might due to limited amount of phenyl group that present on adsorbent. Limited amount of phenyl group may limit the amount of cell bound to adsorbent, which eventually allowed more space for lipase bound onto adsorbent. Thus, the equilibrium binding capacity was only affected at biomass concentration as high as 36% (w/v). Instead, the high viscosity condition of the feedstock as a result of the presence of biomass has reduced the mass transfer rate which has affected the dynamic capacity of the adsorbent.

The film mass transfer resistance that existed due to the restricted diffusion of protein onto internal surface of the adsorbent as a result of greater amount of protein and cells in the feedstock can be explained by the equation developed by Liang-Tseng Fan et al. (1960). The equation typically correlated for film mass transfer coefficient (K_f), which consider the bed voidage:

$$K_f = \frac{Dm}{d_p}\{2 + [1.5 \times (1-\varepsilon) \times N_{\mathrm{Re}}]\}^{1/2} \times S_c^{1/3}\}$$

$$N_{\mathrm{Re}} = \frac{\rho_1 U d_p}{\mu} \quad \text{and} \quad S_c = \frac{\mu}{\rho_1 D_m} \qquad 9)$$

Where D_m is the diffusion coefficient, d_p is diameter of the adsorbent, ε is the fluidized bed voidage, ρ_1 is the density of the process liquid and μ and U are the liquid viscosity ε and superficial flow velocity, respectively. This equation was adopted by Chang and Chase (1996) in the study of effect of operating parameter on the adsorption of model protein onto ion exchanger. It was suggested that the increased viscosity of the applied feedstock increased the bed voidage (ε) which eventually decreased the film

mass transfer (Ling and Lyddiatt, 2005; Thommes, 1997).

STREAMLINE Direct HST adsorbent is a multi functional adsorbent. Beside behave as a cation exchanger that gave negative charge, the presence of phenyl group also gave hydrophobic interaction. According to Fernández-Lahore et al. (2000), hydrophobic interaction that presence on STREAMLINE Phenyl adsorbent gave moderate effect to the binding of cell to adsorbent which might due to the present of hydrophobic domains on the cell surface by adopting particular conformation and orientation.

However, the presence of hydrophobic group on the HST adsorbent did not give much effect to the binding of cell; therefore, the binding of lipase was not affected by the presence of cell close 4.5% (w/v) wet biomass. Indeed, higher affinity of lipase to STREAMLINE Direct HST adsorbent, may has reduced binding surface that available to other contaminant proteins, (eg. cells and cells debris.) The interaction of lipase to the hydrophobic ligand has been demonstrated in the experiments of the addition of glycerol and salts during the batch binding process as depicted in Figure 2a and b.

Result from Table 2 also showed that present of biomass concentration until 4.5% (w/v) only slightly affected the dynamic capacity but almost no effect on equilibrium capacity. The results of the effect of biomass concentration on the operation of EBA showed that, there was only slightly decreased in adsorption efficiency for the biomass concentration at 4.5% (w/v) wet biomass. On the other hand, dilution of feedstock make the process more complex and increased the processing time and cost of production, therefore fermentation broth that has biomass concentration about 4.5% (w/v) can be applied directly to EBA operation without prior dilution.

Conclusion

Application of a high density adsorbent in EBA has been performed in the present study. The high density adsorbent tolerate high flow rate up to 687 cm/h and gave dynamic binding capacity of 4979.28 U/ml at 343 cm/h. Application of culture feedstock without prior dilution (4.5% (w/v) wet biomass concentration) did not affected dynamic binding capacity significantly compared to lower biomass concentration. Therefore this study reinforced the perception that high density adsorbent tolerate high flow rate and biomass which reduced processing time and increased productivity of lipase purification.

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