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Fabrication and Characterisation of a Novel Pellicular Adsorbent Customised for the Effective Fluidised Bed Adsorption of Protein Products

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Abstract A dense pellicular solid matrix has been fabricated by coating 4% agarose gel on to dense zirconia-silica (ZS) spheres by water-in-oil emulsification. The agarose evenly laminated the ZS bead to a depth of 30 μm, and the resulting pellicular assembly was characterised by densities up to 2.39 g/mL and a mean particle diameter of 136 μm. In comparative fluidisation tests, the pellicular solid phase exhibited a two-fold greater flow velocity than commercial benchmark adsorbents necessary to achieve common values of bed expansion. Furthermore, the pellicular particles were characterised by improved qualities of chromatographic behaviour, particularly with respect to a three-fold increase in the apparent effective diffusivity of lysozyme within a pellicular assembly modified with Cibacron Blue 3GA. The properties of rapid protein adsorption/desorption were attributed to the physical design and pellicular deployment of the reactive surfaces in the solid phase. When combined with enhanced feedstock throughput, such practical advantages recommend the pellicular assembly as a base matrix for the selective recovery of protein products from complex, particulate feedstocks (whole fermentation broths, cell disruptates and biological extracts).

Keywords: pellicular adsorbent, adsorption equilibrium, adsorption kinetics, fluidised bed hydrodynamics

INTRODUCTION

Fluidised or expanded bed adsorption has received considerable attention in recent years in the field of bioseparation. Compared to a fixed bed contactor, the fluidised bed possesses a large interparticle porosity which facilitates the passage of micron-sized particulates through the bed. Fuidised bed adsorption (FBA) has therefore been widely considered and adopted as a primary recovery step for protein purification from crude feedstocks containing particulate materials such as cells, cell debris and other bioparticulates [1-6]. Moreover, it has been demonstrated that the FBA can be integrated into operations of batch fermentation [7,8] and cell disruption [9] to facilitate direct product sequestration to the benefit of product quality, purity and yield. In general, FBA technologies enable a reduction in the number of process steps consequent with advantages of reduced processing time, increased process yield and overal cost-effectiveness.

The development of suitable adsorbent solid phases is an essential element in the successful application of FBA for the recovery of proteins from particulate feedstocks. Stable operation at industrial scales demands adorbents having particle densities which exceed those designed for conventional fixed bed chromatography. Such materials are commercially available in limited supply, including cross-linked agarose densified by the incorporation of fine crystalline quartz or metal particles and marketed as STREAMLINE (Amersham Pharmacia Biotech, Uppsala, Sweden [10,11]) and polymer-filled polystyrene and zirconia macroporous particles (BioSepra, Cergy-Saint Charles, France [9,12]). Other adsorbents based on silica or kielselguhr [7,13,14], controlled pore glass [5,15] and custom-assembled perfluorocarbon polymers [16] or polysaccharide-ceramic composites formulated by fluidised bed granulation [17] have also been developed. Such solid phases have been characterised by densities mostly in the range of 1.15 to 1.4 g/mL, with mean particle diameters of 60 to 600 μm which make them suited for stable FBA operations at relative low flow velocities (200-300 cm/h). However, industrial processes will generally benefit from operations at higher linear flow rates (300-600 cm/h), and in such circumstances, particle elutriation and/or intraparticle diffusion of macrosolutes may constrain the achievement of the highest bed efficiencies with established solid phases.

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In order to achieve efficient and effective FBA operations, a small diameter, porous adsorbent having a high density or a dense pellicular design (*i.e.*, a porous laminate cast on a solid core) is indicated (discussed in [18-20]). The present work describes the development of a water-in-oil emulsification technique for the fabrication of a pellicular solid phase by laminating agarose gel onto a dense, silica-coated zirconia sphere. After chemical cross-linking and dye-ligand modification with Cibacron Blue 3GA, the adsorbent was characterised in terms of protein adsorption equilibrium, adsorption kinetics and flow hydrodynamics in the batch and fluidised bed capture of lysozyme. A direct comparison of the performance of the the pellicular adsorbent has been made with custom-assembled agarose and commercially sourced STREAMLINE composite adsorbents derivatised in a similar manner.

MATERIALS AND METHODS

Materials

Low gelling temperature agarose (A 4018), chicken egg white lysozyme (L 6876) and Cibacron Blue 3GA (C 9534) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dense silica-coated zirconia (ZS) particles were sourced from Glen Creston Ltd, London, UK and comprised a white spherical bead with a mean density of 3.8 g/cm^3 and a particle size distribution of 63 to 125 μm (mean diameter 98.9 μm). Edible sunflower oil was obtained from a local superstore. Span 80 (sorbitan monooleate) was purchased from Aldrich Chemical Company (St. Louis, MO, USA). Distilled water was utilized to make up agarose solutions and to prepare buffers. STREAMLINE base matrix was provided by Amersham Pharmacia Biotech (Uppsala, Sweden) and comprised cross-linked 6% (w/v) agarose densified by the incorporation of fine crystalline quartz particles.

Fabrication of Pellicular Agarose Gel

Pellicular agarose gel was prepared by an oil-in-water emulsification method. A 2 litre glass tank (12 cm I.D.) with a moveable baffle frame (4 baffles sized at 10% vessel diameter), a 83 mm diameter impeller and a water jacket around the tank were employed for the emulsification experiments. Initially, 1.5 litres of sunflower oil containing 12.8 g/L Span 80 were fed to the tank and heated to 80°C by circulating thermostated water to the water jacket. The impeller was driven by an electric power unit (Fluid FL200L, Labortechnik, Germany) at 1,160 rpm.

Under separate agitation, agarose powder was dissolved in distilled water at 4% (w/v) in a vessel arranged on a heating plate. In a typical preparation, a defined quantity of washed ZS beads was subsequently added to the agarose solution to achieve a liquid-solid slurry having a volume ratio of 7:1. The suspension temperature was transiently increased to 85-90°C, before it was pumped into the oil phase at 20 mL/min. The physical behavior of the suspension within the oil phase was observed with an stereo microscope connected to a camera and recorded on video for purposes of retrospective qualitative and quantitative analysis of bead formation. After agitation at 80°C for 30 min, cold water was supplied to the water jacket to cool the suspension to 20-24°C whereupon the agitation was terminated and dense composite assemblies were allowed to settle for a 30 minute period. The particles were recovered from the oil phase and washed thoroughly in acetone in order to displace entrapped oil and further polished in distilled water. The residual oil phase was allowed to stand overnight before residual particulate agarose (herein described as a homogenous continuum) was recovered and washed following the procedures outlined above.

Cross-linking and Cibacron Blue Immobilization

The two types of custom-assembled particle (characterised as a pellicular composite and the homogeneous continuum) were wet-screened on a 212 μm standard sieve and those passing through the sieve were collected for chemical cross-linking. The particles were suspended in an equal volume of 1.0 M NaOH containing 5 g/L sodium borohydride. After 30 min, epichlorohydrin was introduced to a final concentration of 2% (v/v) and the reaction continued for 4 h at 25°C. After thorough washing with distilled water, the particles were reacted with Cibacron Blue 3GA (CB) as described by He and colleagues [20]. The STREAMLINE base matrix was modified by a similar procedure. In order to determine the concentration of covalently coupled CB dye, a settled volume (0.2 mL) of exhaustively washed particles [21] were suspended in 15 mL of 1.0 M HCl solution at 80°C. Following neutralisation by titration with 1.0 M NaOH, the CB concentration in solution was estimated spectrophotometrically (A_{620}) against a pure dye standard and expressed relative to the settled volume of particles.

Lysozyme Adsorption Equilibrium and Kinetics

A batch adsorption system was adopted to determine the adsorption isotherms for lysozyme contacted with dye-ligand adsorbents [21,22]. Particles drained of excess fluid (0.2 mL were suspended in various concentrations of lysozyme (10 mL) in 10 mM Tris-HCl buffer pH 7.5. Adsorption was carried out at 20°C on a blood mixer operated at 200 rpm for 24 h. In dynamic adsorption experiments, 0.85 to 1.6 mL of the drained adsorbent was added to 200 mL of 0.5 mg/mL lysozyme solution and incubated as described above. Supernatant samples (1 mL) were taken at timed intervals for spectrophotometric estimation (A_{280}) of protein concentrations, and the time course of decreasing protein concentration was determined.

Fluidised Bed Operation

A glass column (1.0 cm i.d., 100 cm height), fitted with a a stainless steel mesh (77 μm) as the liquid distributor, was employed as the fluidised bed contactor. Solid phase was introduced to achieve a settled bed height of 15 ± 0.3 cm for all experiments. The voidage of the settled bed was measured separately in a 10-mL measuring cylinder, and calculated using the estimated mass and density of drained particles. A movable adapter was used to adjust the position of the liquid outlet at the top of the fluidised bed whilst the liquid phase (10 mM Tris-HCl, pH 7.5) was introduced to the base distributor of the contactor by peristaltic pump. Vertical alignment of the contactor was confirmed by plumb-line in all experiments.

The liquid dispersion in the operating contactor was determined by the F-curve method [23] with acetone (1% v/v) employed as a tracer. The bed effluent was monitored in a flow UV spectrophotometer set at 280 nm. Individual experiments were performed for the complete experimental rig in the presence and absence of adsorbent particles in order to identify the contribition of the volume of fittings. Response to the step input of acetone tracer was recorded by chart recorder, and the variance and plate number was determined as described by Barnfield Frej and colleagues [24].

Analytical Methods

Particle size distribution was measured with a Mastersizer S unit (Malvern Instruments Ltd., UK). A volume-weighted, mean particle diameter was utilized to express the particle size. The density of drained particles was measured by a water displacement method. Cibracron Blue 3GA (CB) was estimated in solution by spectrophotometer at 620 nm exploiting a molar extinction coefficient of 1.30×10^4 L/mol/cm determined by preliminary experiments with standard preparations. Lysozyme concentration was determined by spectrophotometer at 280 nm exploiting a published extinction coefficient of 2.37 mL/g/cm [25].

RESULTS AND DISCUSSION

Physical Properties of the Pellicular Adsorbent

Composite particles produced by coating 4% agarose onto zirconia-silica beads are referred to herein as 4AZS, while the homogeneous agarose gel beads recovered from the same batch reaction are denoted as 4AB. Table 1 depicts physical properties of CB-4AZS, CB-4AB and CB-Streamline adsorbent particles. The average gel volume fraction (f) of particles was calculated by mass balance using the density data as follows:

$$f = \frac{\rho_1 - \rho_s}{\rho_1 - \rho_{gel}} \quad (1)$$

where ρ_1 represents the density of the inner solid particles (*i.e.* 3.8 g/cm^3 for zirconia-silica in 4AZS and 2.6 g/cm^3 for crystalline quartz in STREAMLINE), ρ_2 is the mean density of the particles and ρ_{gel} is the desnity of agarose (1.03 g/cm^3). The composite matrix 4AZS was assembled with a density of 2.39 g/cm^3, which was greater than the commercial fluidised bed adsorbent. Optical microscopy revealed that most of the 4AZS beads were composed of a single ZS sphere, entrapped evenly by a transparent agarose gel layer of approximately 10 to 30 μm (data not shown) to form a pellicular particle assumed to be spherical in the following quantitative modelling analyses.

Table 1. Physical properties of dye-ligand adsorbents

Adsorbent	d (μm)	ρ_s (g/cm^3)	f (-)	CB [a] (μmol/mL)
CB-4AZS	136	2.39	0.504	9.8
CB-4AB	132	1.03	1.0	14.5
CB-Streamline	254	1.17	0.894	16.8

d is defined as particle diameter, ρ_s as particle density, f as the volume fraction of agarose, and CB as the concentration of immobilised dye-ligand expressed on the basis of settled bed volume of particles.

Adsorption Equilibrium and Kinetics

Adsorption isotherms of lysozyme to the dye-ligand adsorbents correlated well with the Langmuir equation:

$$q = \frac{q_m c}{K_d + c} \quad (2)$$

where q and c are equilibrium binding and aqueous phase non-bound protein concentrations, respectively, expressed in mass units per unit volume, q_m is the maximum adsorption capacity expressed in mass units per unit volume of settled adsorbent, and K_d is the equilibrium dissociation constant for the interaction. Estimates of the q_m and K_d for the three adsorbents are presented in Table 2. It was found that the estimated maximum adsorption capacity of the pellicular adsorbent CB-4AZS was somewhat lower than that of the adsorbent (CB-4AB) prepared in base matrix form (a homogeneous continuum) as an unreacted by-product of pellicular assembly. However, the reduction in capacity was not proportional to the decrease of the effective gel volume. The effective capacity of CB-4AZS expressed relative to the estimated volume of the agarose pellicle (volume fraction equal to 0.50, Table 1) was estimated to be 30% lower than that of CB-4AB.

The characteristic structure of the pellicular adsorbent CB-4AZS suggested that the protein uptake might differ from those adsorbents characterised by possession of a porous continuum of agarose (eg 4AZS and STREAMLINE). Therefore the kinetics of lysozyme adsorption was studied and interpreted exploiting a homogeneous diffusion model (HDM). Here, the available adsorbent volume was considered as a homogene-

Table 2. Lysozyme adsorption equilibrium and kinetic parameters of dye-ligand adsorbents

Adsorbent	q_m (mg/L)	K_d (mg/mL)	k_f (×10^{3}cm/s)	D_e (×10^{9}cm^2/s)	$D_{e,a}$ (×10^{9}cm^2/s)
CB-4AZS	86.2	0.012	1.88	1.10	3.46
CB-4AB	122.1	0.013	0.66	1.06	-
CB-Streamline	108.0	0.029	0.95	1.26	-

q_m is defined as the estimated maximum capacity of adsorbents, K_d is the equilibrium dissociation constant for lysozyme interactions, k_f is the rate constant for lysozyme adsorption, D_e is the effective diffusivity of lysozyme and $D_{e,a}$ is the apparent effective diffusivity for the pellicular particle (4AZS).

ous network, and the driving force for mass transport was the total protein concentration gradient within the adsorbent. The intraparticle material balance for the model has been described [26] as:

$$\frac{\partial q}{\partial t} = D_e\left(\frac{\partial^2 q}{\partial r^2} + \frac{2}{r}\frac{\partial q}{\partial r}\right) \quad (3)$$

where D_e is the effective diffusivity, r is the radial distance, and t is the adsorption time. From the mass balance for a protein per unit volume of batch adsorption system, the change of the liquid phase concentration due to adsorption can be expressed as :

$$\frac{dc}{dt} + F\frac{d\bar{q}}{dt} = 0 \quad (4)$$

where $\bar{q}$ is the average concentration adsorbed into the adsorbent, F is the volume ratio of solid to liquid phases. The second term in the left-hand side of Eq. (4) represents the uptake rate of protein by the particle, which can be described by a modified form of Fick's law:

$$\frac{d\bar{q}}{dt} = \left(\frac{3}{R}\right)D_e\left(\frac{\partial q}{\partial r}\right)_{r=R} \quad (5)$$

where R is the mean particle radius. By combining Eqs. (4) and (5), the following equation is given,

$$\frac{dc}{dt} + \frac{3FD_e}{R}\left(\frac{\partial q}{\partial r}\right)_{r=R} = 0 \quad (6)$$

The initial and boundary conditions for Eqs. (3) and (6) are

$$c = c_0,\ q = 0,\ t=0 \quad (7)$$

$$\frac{3D_e}{R}\left(\frac{dq}{dr}\right)_{r=R} = k_f(c - c_s),\ r=R \quad (8)$$

$$q = \frac{q_m c_s}{K_d + c_s},\ r=R \quad (9)$$

$$\frac{\partial q}{\partial r} = 0,\ r=r_i \quad (10)$$

where r_i is the inner solid core radius, d is the particle diameter, c is the bulk liquid concentration, c_s is the concentration at the adsorbent surface, and k_f is the external liquid-film mass transfer coefficient.

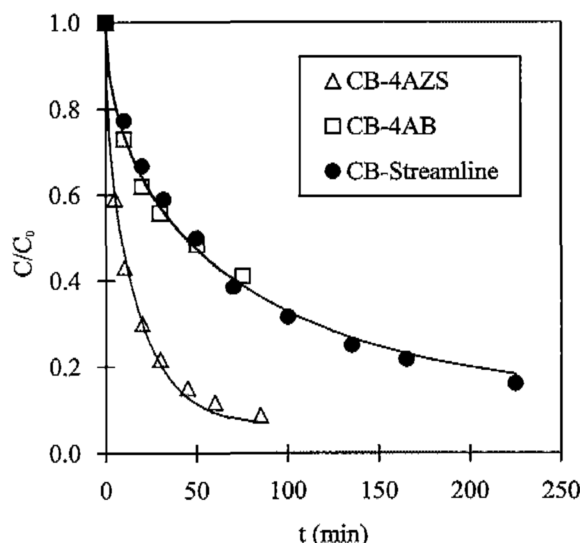

Fig. 1. Dynamic adsorption profiles of lysozyme adsorption to dye-ligand adsorbents. *C/Co* represents the ratio of unbound free lysozyme (*C*) to the initial concentration (*Co*) at time *t* minutes. Symbols represent experimental determinations and solid lines represent calculations from the homogeneous diffusion model (HDM) discussed in Results and Discussion.

On the basis of the microscopic observation of the CB-4AZS (data not shown), it was assumed that a single ZS sphere exists at the centre of the 4AZS particle and thus the inner solid core radius r_i was set to be equal to the mean radius of the ZS sphere (*i.e.* 49.5 μm). However, this value was assumed to be zero for the homogenous agarose (4AB) and STREAMLINE. In the stirred batch adsorption system, the film mass transfer coefficient can be estimated from the following correlation of Geankopolis [27]:

$$k_f = \frac{2D_m}{d} + 0.31\left(\frac{\mu}{\rho D_m}\right)^{-2/3}\left(\frac{\Delta\rho\mu g}{\rho^2}\right)^{1/3} \quad (11)$$

where D_m is the diffusivity in free solution taken as 11.8 × 10^{-7} cm^2/s for lysozyme [28], μ is the liquid phase viscosity, ρ is the liquid density and Δρ is the density difference between the solid and liquid phases in a fluidised bed.

Using the k_f values presented in Table 2, and the initial and boundary conditions, Eqs. (3) and (6) were numerically integrated by an orthogonal method [21] and fitted to the adsorption dynamic data to determine D_e. Fig. 1 confirms that the HDM closely predicts the experimental uptake kinetics for lysozyme upon the dye-ligand adsorbents. From Table 2, it can be seen that D_e values for the three adsorbents were approximately equal which invites the following conclusions: (i) single zirconia-silica spheres exist at the centre of the 4AZS particles and (ii) lysozyme displays a similar mass transport behaviour in the agarose gel of both customised assemblies and commercial STREAMLINE products.

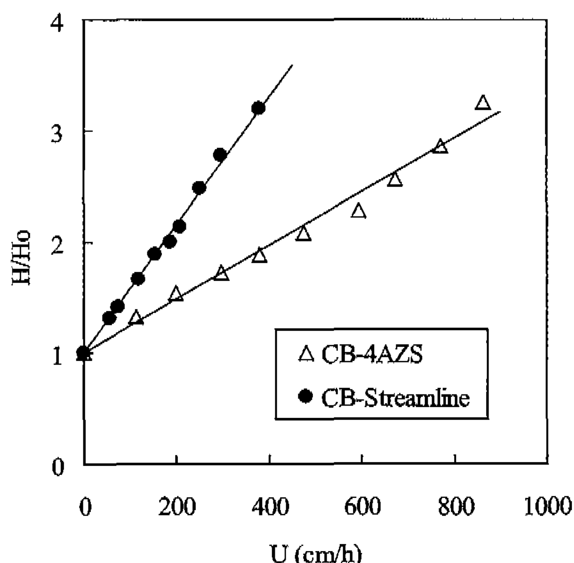

Fig. 2. Bed expansion behaviour of fluidised beds of CB-4AZS and CB-Streamline. H_0 represents the settled bed height (~ 15 cm) and H the fluidised bed height recorded at a superficial linear velocity of U cm/h (described in Materials and Methods).

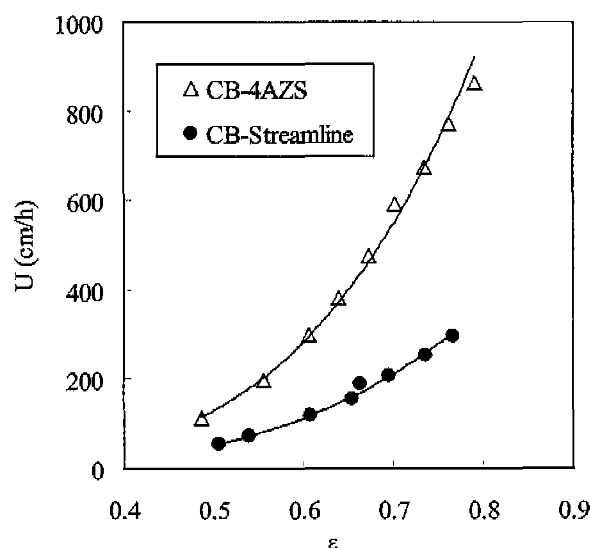

Fig. 3. Relation between bed voidage and superficial liquid-phase velocity. Standard fluidised beds (15 cm settled bed height) were operated as described in Materials and Methods where U and ε represent the superficial linear velocity and bed voidage, respectively. Symbols represent experimental values whilst solid lines are calculated from Eq. (12).

When the inert solid core in the pellicular 4AZS beads is eliminated from a consideration of the boundary condition expressed by Eq. (10) (*i.e.* let $r_i=0$), the *apparent* effective diffusivity, $D_{e,a}$, for the pellicular adsorbent can be estimated and is given in Table 2. It is notable that the value for 4AZS is over three times greater than the corresponding value of D_e. This can be attributed to the presence of the inert solid core, which effectively decreases the length of the diffusion path for lysozyme within the particle (estimates of pellicle depth indicated values of 10 to 30 μm). The significant improvement of the uptake performance in the pellicular adsorbent suggests that fluidised bed adsorption could be operated at greater flow rates than those recommended for STREAMLINE (200-300 cm/h [10]) without compromise to bed efficiency.

Flow Hydrodynamics in Fluidised Bed Operations

Bed expansion behaviour of the 4AZS and STREAMLINE beads are compared in Fig. 2. Clearly, the height of a fluidised bed of 4AZS adsorbent increased more slowly with increasing flow rate when compared directly with CB-STREAMLINE. It was estimated that for a two-fold bed expansion, the flow rate for the 4AZS bed (415 cm/h) was 2.4 times higher than that for STREAMLINE. Moreover, it was concluded by visualisation that the enhanced density of the 4AZS pellicular beads faciliated the formation of a more stable fluidised bed than that achieved with STREAMLINE when *H/Ho* equalled or exceeded a value of 3. When CB-4AZS was employed in bed expansion experiments, a significant colour distribution was observed along the bed axis with the darkest blue colouration apparent at the top and attributed to a variation of core diameter: pellicle depth ratios (decreasing with increasing height in the bed). It was concluded that this indicated a similar distribution of density in the bed of CB-4AZS, as has been reported for STREAMLINE matrices [29] but not apparent here because of the continuum of agarose deployment (and therefore dye) characteristic of that composite adsorbent.

Fig. 3 indicates that the relationship between the bed voidage and superficial liquid velocity was well expressed by the Richardson-Zaki equation [30]:

$$U = U_t \varepsilon^n \tag{12}$$

where U_t is the terminal settling velocity, ε is the inter-particle void fraction of the fluidised bed, and n is the Richardson-Zaki coefficient. Table 3 presents these parameters for CB-4AZS and CB-STREAMLINE, where the theoretical terminal velocity was estimated from the Stokes' equation :

$$U_t = \frac{D^2 \Delta\rho g}{18\mu} \tag{13}$$

The Richardson-Zaki coefficient was found to fall within a range of 4.1 to 4.3, which was less than values reported for STREAMLINE particles [4,31]. In addition, for both fluidisable adsorbents the experimentally determination of terminal velocity from Eq. (12) was only about half the value obtained from the theory of Stokes This observation might be attributed to the relatively

Table 3. Richardson-Zaki parameters and theoretical terminal velocity of the dye-ligand adsorbents in fluidised bed operation

Adsorbent	n	$U_{t\ (exp)}$ (cm/h)	$U_{t\ (theor)}$ (cm/h)
CB-4AZS	4.27	2510	4600
CB-Streamline	4.10	894	1950

n is defined as the Richardson-Zaki coefficient, and $U_{t\ (exp)}$ and $U_{t\ (theor)}$ represent experimental and theoretical estimations of the terminal settling velocity for individual particles.

large distribution of particle size and density which characterised the 4AZS beads. Thoemmes and colleagues [5] reported that terminal velocities calculated by the Stokes' theory were about 10-fold greater than values experimentally determined for the fluidised beds of controlled pore glass. This was attributed to the porous nature and irregular shape of the glass solid phase. In contrast, an opposite trend was observed by Chang and colleagues [4,31] with STREAMLINE adsorbents when contacted with viscous feedstocks. Published data thus confirms that the bed expansion of a fluidised bed may vary substantially with the properties of both the solid and liquid phases as well as the configuration of the contactor.

The plate number of fluidised beds of both 4AZS and STREAMLINE adsorbents increased with the increase in liquid flow rate (data not shown). A comparison of plate number as a function of bed expansion indicated that CB-4AZS beds would exhibit comparable chromatographic efficiency as CB-STREAMLINE (Fig. 4) and could accommodate crude and more rheologically complex feedstocks. Reference to Fig. 2, depicting the variation of bed expansion with linear fluid velocity confirms that this chromatographic efficiency should continue to be exhibited at higher feedstock throughput than that possible for the commercial adsorbent.

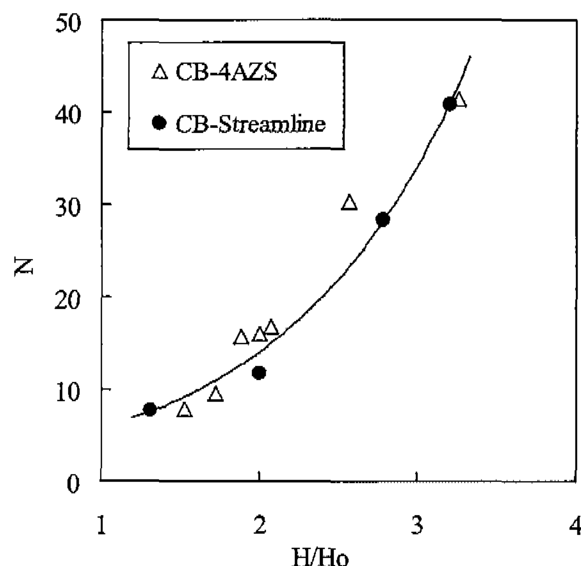

Fig. 4. Variation of plate number of the fluidised bed of different solid phases as a function of bed expansion. N is defined as the plate number (calculated from methods in [22]) and H/Ho represents the degree of bed expansion for a 15cm settled bed volume (Ho) as described in Figure 2.

CONCLUSION

A dense pellicular adsorbent (4AZS) has been fabricated by coating agarose gel on to a zirconia-silica sphere exploiting a water-in-oil emulsification method. The adsorbent had a density of 2.39 g/cm^3, which guaranteed a high terminal velocity and stable flow hydrodynamics during fluidised bed operations. The adsorption capacity for lysozyme protein (expressed in mass units per unit volume of settled adsorbent) for Cibracron Blue 3GA-modifed pelliculates was somewhat reduced when compared with similarly modified, custom assembled agarose particles and commercial STREAMLINE solid phases. This was attributed to the reduced volume fraction of agarose which was characteristic of the pellicular assembly. However, the apparent effective diffusivity of lysozyme was increased over three-fold as a direct result of the decrease diffuson resistance engendered by the discrete pellicle thickness (estimated at 10 to 30 μm) as compared to the particle diameter of the porous agarose continuum of STREAMLINE (150-300 μm). In addition, the chromatographic efficiency of CB-4AZ (as exemplified by plate number) appears to be maintained at flow-rates representing a product throughput greater than two-fold that achievable with the commercial adsorbent by virtue of the greater density of the pellicular adsorbent.

The data presented herein thus indicates the potential applicability of a dense, pellicular adsorbent for the fast adsorption/desorption of protein and macromolecular products contained in complex, particulate feedstocks such as whole fermentation broths, cell disruptates and biological extracts. This potential has been confirmed by the successful operation of homologues of pellicular CB-4AZS in the direct recovery of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) from unclarified disruptates of Bakers' yeast cells (up to 30% wet w/v; Jahanshahi and Lyddiatt, unpublished experiments) in a process where a bead mill was directly intergrated with a pilot-scale fluidised bed contactor [9, 32]. The detailed assembly of such adsorbents, together with a full dissection of the impact of depth of pellicle, and the density and diameter of pellicular assemblies, will be the subject of a further publication.

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