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Agarose-coated anion exchanger prevents cell-adsorbent interactions

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Abstract

A common problem during recovery of bioproducts by adsorption from particulate broths is fouling of the adsorbent material as a result of the interaction of cells and cell debris, which present negative charges, with the positively charged anion exchangers commonly used in bioprocesses. The effect of shielding an adsorbent with a layer of agarose on reducing the binding of cells while still allowing the low-molecular-mass bioproducts to be adsorbed was studied. Coating the anion-exchange resin Amberlite IRA-400 with agarose followed by cross-linking the agarose layer effectively prevented the binding of *Escherichia coli*, *Saccharomyces cerevisiae*, and *Lactobacillus casei* cells but allowed binding of lactic acid to the adsorbent. The cross-linked agarose layer was stable during recycling of the adsorbent.
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Keywords: Fluidized bed adsorption; Fouling; Agarose coating; Amberlite IRA-400; Cells; Adsorbents; Lactic acid

1. Introduction

Adsorption is the most common means used for purification of bioproducts. A trend during recent years has been to adapt the separation technique to recover target products directly from the crude feedstock so as to reduce the number of unit operations and process costs, and also increase the overall process yield. Adsorption in a fluidized or expanded bed mode is now used for recovery of both low molecular weight products and proteins from culture broths [1,2]. For this, the feed is pumped in from the bottom of the column at a flow rate which makes the particulate adsorbent rise from its settled state and creates space between the adsorbent beads allowing cells and cell debris to pass through unhindered. The separation efficiency is in principle thus determined by the selectivity of interaction between the product and the matrix.

This attractive concept is however often prone to one major problem, i.e. fouling of the adsorbent due to binding of cells, cell parts and other components present in broths. The problem has been found to be most severe with anion exchange matrices that bind the negatively charged cell surfaces easily [1,3]. Different cell types vary in their tendency to bind to the adsorbents, e.g. *Escherichia coli* has the least effect [1] while hybridoma cells attach indiscriminately to all the adsorbents [3]. Moreover, the extent of adsorption is determined by cell concentration, and age [1,4]. Challenging an adsorbent with cell homogenates increases further the complexity of interactions due to release of other proteinaceous and nucleic acid material [4,5]. A study on influence of biological feedstock on stability of expanded bed adsorption systems showed that only a partial recovery of the original system performance is achieved after extensive washing [4]. It was proposed that electrostatic interactions between adsorbent particles result in reversible adsorption of biomass, while irreversible fouling of the matrix occurs due to permanent adsorption of particulate content in the feed. These interactions eventually lead to the formation of cell-adsorbent aggregates, and reduce the efficiency of the overall process by affecting the fluidization of the adsorbent bed and also the adsorption of the target molecule [3,6,7]. Furthermore, life expectancy of the matrix is reduced due to fouling and harsh regeneration conditions required for washing the non-specifically bound cellular material [3,6].

A methodological design approach has been proposed to determine the window of operation where the biomass/

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adsorbent interactions are minimal [7]. An alternative strategy has been modification of the adsorbent in a manner that would minimize the adsorption of the cell particles [6].

The aim of this study was to investigate the effect of coating an anion-exchange resin with a porous layer of an uncharged hydrophilic polymer on shielding the interactions with cells while still allowing the low molecular weight products to access the ligand groups.

2. Materials and methods

2.1. Materials

Amberlite IRA-400 was obtained from ICN Biochemicals (Costa Mesa, CA, USA), while D,L-lactic acid, agarose and the enzymatic kit for lactic acid determination were purchased from Sigma (St. Louis, MO, USA). HPLC column, Aminex HPX-87H and the organic acid standards were obtained from Bio-Rad Labs (Hercules, CA, USA). All other chemicals used were of analytical standard.

2.2. Microorganisms

Baker's yeast, *Saccharomyces cerevisiae* in the form of compressed blocks was purchased from a local store.

Escherichia coli strain RR1 provided by Dr. G. Feller, University of Liege, Belgium, was cultivated in 200 ml of Luria-Broth medium in a 1 l flask at 37 °C with a constant agitation of 150 rpm. The cells were harvested after 12 h of cultivation by centrifuging at 6000 × *g* in a Sorvall RC-5D centrifuge.

Lactobacillus casei subsp. *rhamnosus* (DSM 20021) was cultivated in 200 ml MRS-medium in 1 l flask at 37 °C. The cells were grown for 12 h and harvested as described above for *E. coli*.

All the microbial cells were suspended at concentrations of 10–100 mg (wet mass) ml^{-1} in 150 mM NaCl solution.

2.3. Preparation of agarose-shielded adsorbent

Ten grams of Amberlite IRA-400 was mixed with 10 ml of 0.2–1.0% (w/v) agarose solution. The mixture was incubated at 40 °C during 15 min with gentle and constant agitation. Subsequently, the agarose solution was drained and the resin was rinsed with 100 ml of distilled water. For cross-linking of the agarose layer, the treated resin was suspended in 7.5 ml of 0.6 M NaOH containing 15 mg of sodium borohydride in a round-bottom flask. The mixture was incubated at room temperature with shaking at 130 rpm while 7.5 ml of 1,4-butanediol-diglycidyl ether was slowly added. After further incubation for 6 h, the agarose-coated resin was washed three times with alternate volumes (50 ml each) of acetone and water. The resin was resuspended in 30% (v/v) ethanol for storage.

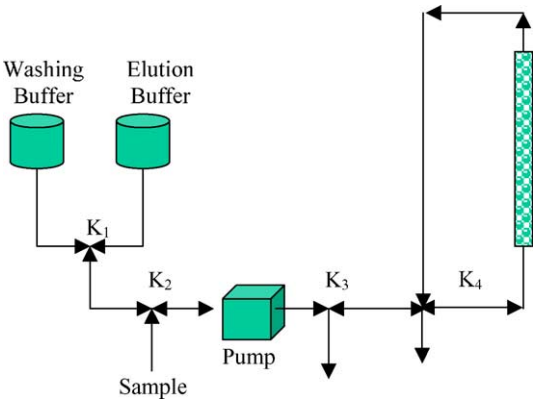

Fig. 1. Experimental set-up of expanded bed chromatography. K represents three way valves, which allow changing the direction of the flow.

2.4. Equipment and conditions for fluidised bed experiments

The experimental setup for adsorption studies is schematically shown in Fig. 1. Two grams of native or agarose-coated Amberlite IRA-400 suspended in 150 mM NaCl, pH 7 was packed in a column (5 cm × 1 cm) and washed with 5 bed volumes of 1.25 M NaOH in order to replace Cl^- groups on the resin with OH^- groups. The column was then rinsed with water until the pH of the effluent was neutral, and finally equilibrated with 150 mM NaCl pH 7, pumped in an upward fashion at a linear velocity of 66 cm h^{-1}.

The capacity of the agarose-coated Amberlite IRA-400 for binding lactic acid was determined by applying 10 mg ml^{-1} D,L-lactic acid solution until breakthrough was achieved. The dynamic binding capacity coefficient was calculated from the breakthrough curves and compared with that of native adsorbent.

The studies on cell adsorption to the matrix were done using following cell suspensions: (a) *E. coli* [10 and 100 mg (wet mass) ml^{-1}, respectively], (b) *S. cerevisiae* [10 and 100 mg (wet mass) ml^{-1}, respectively], and (c) *L. casei* [100 mg (wet mass) ml^{-1}]. After passing the respective microbial suspension, the bed was treated with 150 mM NaCl followed by 1 M NaCl in order to ensure the elution of any bound cells.

The effect of the presence of cells on lactic acid binding to the agarose-coated adsorbent was then studied by passing individually: (a) 5 ml solution containing 20 mg ml^{-1} *E. coli* cells and 20 mg ml^{-1} lactic acid, and (b) 2 ml solution containing 50 mg ml^{-1} *L. casei* and 50 mg ml^{-1} lactic acid. After rinsing of the column with sodium chloride solutions (150 mM and 1 M, respectively) in an upward direction, the lactic acid was eluted with 1 M HCl in a downward fashion.

The amount of cells in the effluent was monitored by continuous absorbance at 620 nm while lactic acid content was determined by HPLC after centrifuging the eluant fractions (2 ml) to remove the cells.

2.5. Lactic acid determination

The concentration of lactic acid was quantified by HPLC (Shimadzu) on an Aminex HPX-87H column and a UV detector (Shimadzu) at 220 nm. A solution of 4 mM sulfuric acid was used as mobile phase with a constant flow rate of 0.6 ml min^{-1} at 35 °C. Pure lactic acid in a concentration range of 0–100 mg ml^{-1} was used to prepare a standard curve.

2.6. Electron microscopy

For scanning electron microscopy (SEM) the resin (uncoated and 1% agarose-coated, respectively) was packed in a column and a suspension of 1% *L. casei* in 150 mM NaCl solution was pumped from bottom to top until the absorbance at 620 nm reached the breakthrough. After this, the column was washed with the same mobile phase until the absorbance returned to the baseline.

A sample of the beads from each column was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 and kept overnight. The samples were rinsed with buffer and incubated in 1% OsO_4 at room temperature for 1 h. After rinsing with buffer the samples were dehydrated in ethanol. The fixed and dehydrated beads were critical point dried and sputtered with gold–palladium (80:20). The preparations were examined under a Joel T330 scanning electron microscope (JEOL, MA, USA).

3. Results and discussion

Surface modification of an adsorbent with charged polymers has been reported recently as a means for avoiding the binding of cells by creating a shield that allows the diffusion of small molecules to the porous stationary phase and prevents the unspecific binding of cellular material to the matrix [6,8]. In the present work, use was made of agarose, an uncharged hydrophilic polymer, for coating an adsorbent. Agarose is a well-proven polymer for macromolecular separations; agarose based gels being among the most commonly used for protein and nucleic acids chromatography.

3.1. Effect of agarose coating on binding of cells to Amberlite IRA-400

Coating of chromatography gels with agarose using a bead polymerisation method has been reported earlier [9,10]. The method is effective providing a well-defined layer, but is rather expensive. Here, we have made use of a simple procedure for agarose coating of Amberlite IRA-400 to evaluate the effect on cell binding. Amberlite IRA-400 is a standard anion-exchange chromatography resin normally used for the adsorption of low-molecular-mass products [11–13]. It is a strongly basic gel-type resin with quaternary ammonium functionality and a particle size in the range of 16–50 mesh,

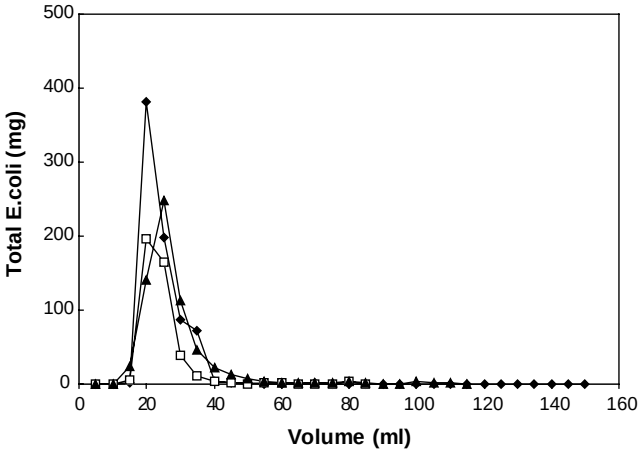

Fig. 2. Adsorption of *E. coli* cells to Amberlite IRA-400 coated with 1% agarose solution. A suspension of 5 ml of *E. coli* cells (100 mg ml^{-1}) was injected into the column. The column was washed with 0.9% NaCl, pH 7. After elution of the cells the column was regenerated and the procedure was repeated during 3 consecutive cycles. (◆) Cycle I, (□) cycle II, (▲) cycle III.

exhibiting a very low expansion capacity ($H/H_o = 1.25$ at 300 cm h^{-1} linear velocity). Initial studies on coating the adsorbent with agarose were performed by simply incubating the beads in 0.2–1.0% (w/v) polymer solution at a temperature (∼40 °C) at which the polymer is soluble [14]. Subsequent washing at room temperature retains a layer of agarose around the beads. These beads when exposed to *E. coli* cell suspension, revealed no cell adsorption. All the cells introduced into the column were recovered on washing with 150 mM NaCl. However, when the beads were recycled after treatment with 1.25 M NaOH, the amount of cells recovered from the column was reduced (Fig. 2). Microscopic analyses of the beads subsequent to the regeneration procedure showed cracking of the agarose layer, explaining the increase in the interaction between the resin and the cells on repeated use (Fig. 3).

Stabilization of the agarose layer was thus required which was done by covalent cross-linking of the polymer by means of epoxy activation [15], a procedure normally used for preparing cross-linked agarose beads for chromatography. The micrographs of Amberlite IRA-400 beads coated using 1% agarose solution and cross-linked, showed the presence of a 0.3–0.8 μm thick polymer layer.

The performance of the coated Amberlite IRA-400 beads was compared with that of the native beads. Coating with agarose reduced the expansion ability of the matrix to zero, nevertheless allowing fluidization of the matrix. Increased expansion of a matrix modified chemically with charged polymers was reported, which was attributed to increased superficial charge of the support that produces repulsion forces between the beads [6].

Adsorption of different cell types, *E. coli*, *S. cerevisiae* and *L. casei* to the native and coated resin was compared. Microbial suspensions containing about 100 mg ml^{-1} cells

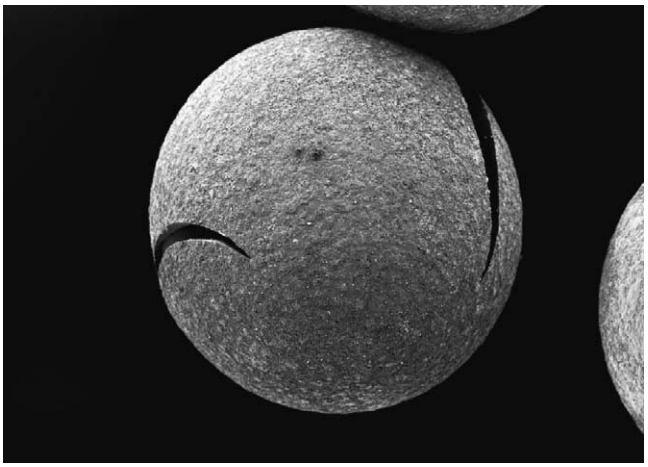

Fig. 3. Scanning electron micrograph showing coated Amberlite IRA-400, using 1% agarose solution but without subsequent cross-linking of the polymer, after 3 cycles of chromatography and regeneration. Magnitude 150, scale bar 100 μm.

(equivalent to a total of 10^9–10^{10} cells) were passed through the column; the column was washed with 150 mM NaCl and finally any bound cells were eluted using 1 M NaCl, followed by regeneration of the column using 1.25 M NaOH before repeating the adsorption cycle. Fig. 4 shows a decrease in the recovery of cells from the native Amberlite IRA-400 column during 3 consecutive cycles after which the column was clogged and could not be regenerated. On the other hand, 86–100% of the cells were recovered during 5 cycles from the resins coated using 0.2–1% agarose solutions. Cell recovery was consistent at 100% in the case of the matrix coated using higher concentrations (0.5–1%) of the polymer solution. Effect of the polymer coating on preventing the aggregation of the adsorbent beads mixed with a concentrated bacterial cell suspension (10% w/v *E. coli* suspension) can be appreciated from Fig. 5a and b. The effect of the polymer coating on preventing the cell adsorption to the resin was also confirmed by electron microscopy of the uncoated and coated beads exposed to 10% (w/v) *L. casei* suspension during 5 cycles of adsorption and regeneration (Fig. 6a and b).

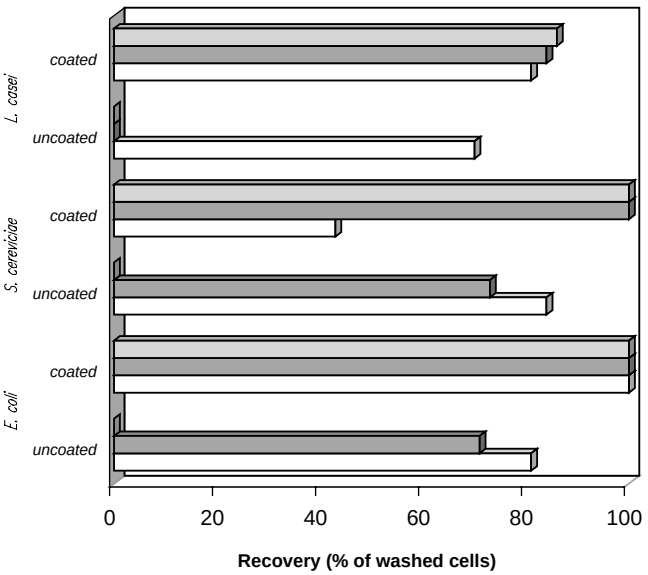

Fig. 4. Recovery (% of amount loaded into column) of *E. coli*, *S. cerevisiae* and *L. casei* cells using 150 mM NaCl solution during repeated use of a column of native and agarose-coated Amberlite IRA-400. The column (2 ml) was challenged with 5 ml of 100 mg ml^{-1} cell suspension before passing 150 mM NaCl, the cells were eluted with 1 M NaCl, and the column was regenerated applying 1.25N NaOH in succession. The procedure was then repeated for 5 cycles. Cycle I (□), cycle III (■), cycle V (■).

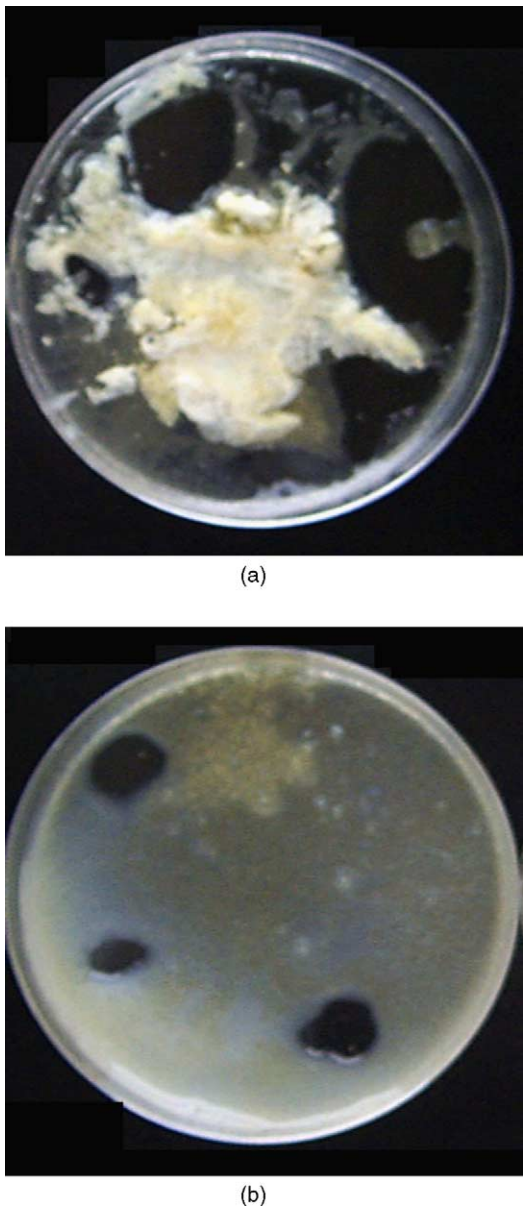

Fig. 5. A photograph showing the effect of mixing *E. coli* cell suspension (10%) with (a) native and (b) 1% agarose-coated Amberlite IRA-400 for 5 min at room temperature.

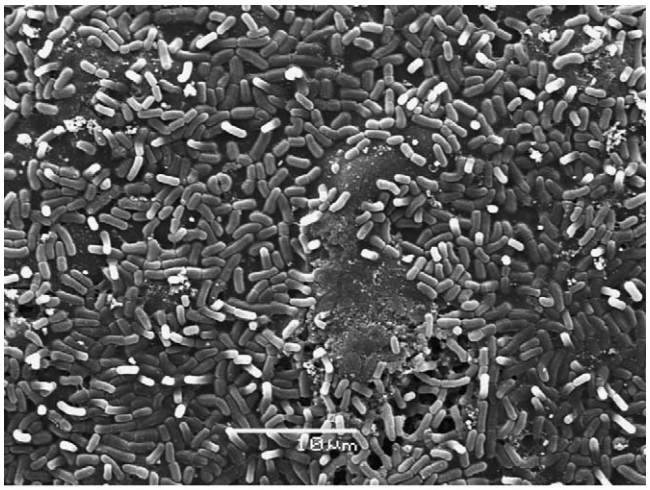

Fig. 6. Scanning electron micrographs showing the surface of (a) native (scale bar 10 μm) and (b) 1% agarose-coated Amberlite IRA-400 (scale bar 5 μm) resin after being exposed to 1% *L. casei* suspension as described in the text.

3.2. Binding of lactic acid to agarose-coated Amberlite IRA-400

The binding capacity of the coated adsorbent for lactic acid at pH 7 was then compared with that for the native resin. As can be seen in Fig. 7, the breakthrough curves for the resin coated using 0.2–0.5% agarose solutions were the same as that of the native Amberlite IRA-400. A slight difference in the shape of the curve can be noticed in case of the resin coated using 1% agarose solution, perhaps indicating diffusional restrictions due to increase in the thickness of the polymer layer. This was seen in about 20% increase in the elution volume of lactic acid. However, the dynamic binding capacity remained unchanged at 100 mg lactic acid per gram of adsorbent.

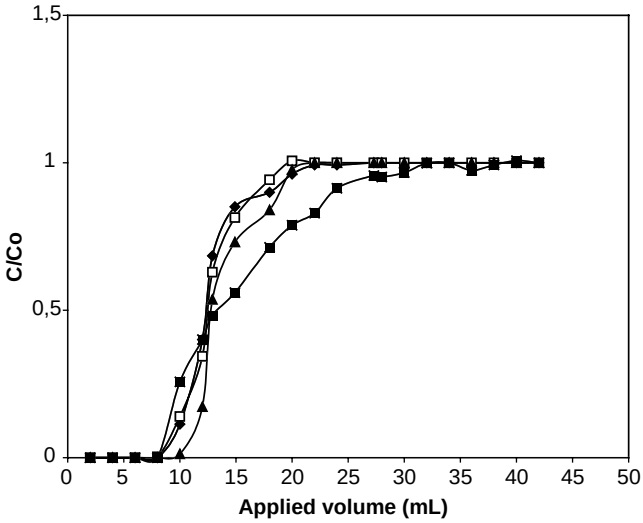

Fig. 7. Breakthrough curves for lactic acid adsorption to native and agarose-coated Amberlite IRA-400. A solution of 10 mg ml^{-1} lactic acid was passed at a flow rate of 1 ml min^{-1} through the column packed with 2 g of the matrix equilibrated with 150 mM NaCl, pH 7. The lactic acid in the effluent was quantified by HPLC. Symbols represent the resin coated using different concentrations of agarose solution: (◆) 0, (□) 0.2%, (▲) 0.5 %, and (■) 1%.

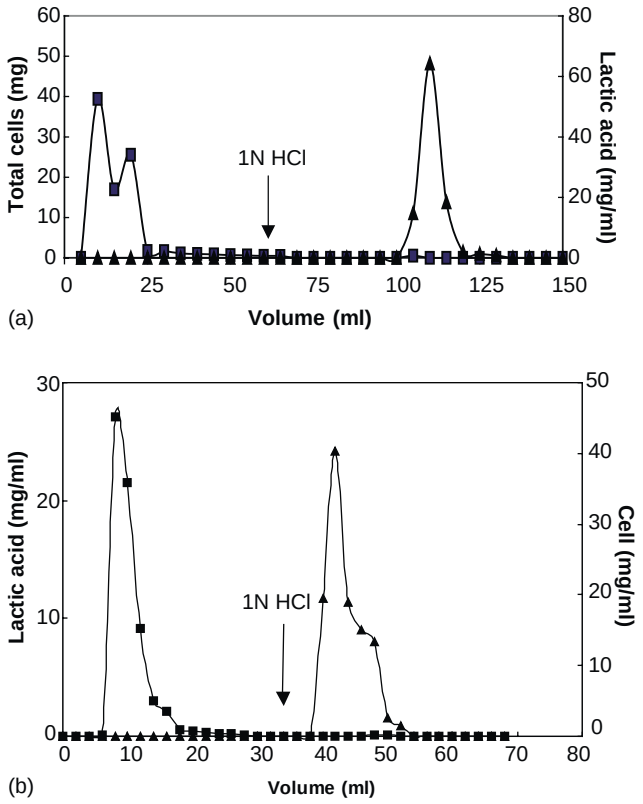

Fig. 8. Elution profile of lactic acid (▲) and *E. coli* cells (■) from Amberlite IRA-400 coated using 1% agarose solution. Sample loaded on 2 ml column was: (a) 5 ml solution of 10 mg ml^{-1} of lactic acid and 10 mg ml^{-1} *E. coli* cells, and (b) 2 ml solution containing 50 mg ml^{-1} of lactic acid (▲) and 50 mg ml^{-1} *L. casei* (■), at a constant flow rate of 1.0 ml min^{-1}. The column was washed with 150 mM NaCl and the bound lactic acid was eluted with 1 M HCl.

Lactic acid binding was then measured in the presence of microbial cells. Cordoba et al. have earlier suggested the need to perform lactic acid purification at very high flow rates (720 cm h^{-1}) to avoid the binding of cells to Amberlite IRA-400 [16]. However, this affects the capacity of the matrix to bind the target molecule due to the reduced time of contact with the stationary phase, hence necessitating re-circulation of the culture broth in order to improve the recovery of lactic acid [13,16]. In the present work, binding of lactic acid to the coated adsorbent was studied in the presence of *E. coli* and *L. casei*, respectively, at a flow rate of 66 cm h^{-1}. The column was washed until the absorbance of the effluent at 620 nm returned to zero, and the bound lactic acid was eluted with 1 M HCl. The elution profiles for lactic acid and the cells are shown in Fig. 8a and b. The recovery values show that 100% of the cells injected were recovered in the flow through, while all the lactic acid was recovered during the elution step. After appropriate regeneration, the column was used for at least 3 more cycles, giving the same results as the first one.

4. Conclusions

This study shows that the use of an uncharged polymer such as agarose to shield the adsorbent beads avoided the interaction between different types of cells and the support itself (Amberlite IRA-400) allowing the target molecule to bind without affecting the binding capacity of the resin. This simple coating method can provide a very useful tool for modification of supports used in bioprocesses and also bioanalysis, which involve the separation (capture) of bioproducts from cellular broths. Further investigations on applicability of this approach on recovery of other small molecules as well as proteins from crude systems are in progress.

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