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A bioreactor with an internal contactor for primary recovery of bovine serum albumin from yeast suspension

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Abstract−In this paper the internal contactor is a newly developed device for the primary recovery of protein from crude feedstock. Ion exchanges (DEAE-Streamline) are confined inside the internal contactor in a stirred tank. Interactions between the ion exchange in the internal contactor and protein (BSA) in yeast suspension have been studied. For better performance, two strategies are considered: to determine the ion conductivity of a simulated yeast suspension, and to select the optimum process time for adsorption. In this system, advantages of both batch adsorption and expanded bed adsorption were obtained. Furthermore, in denser cell concentration (50 g/L) where EBA cannot be operated, the primary recovery was carried out in 1-2 hr. The efficiency of yield is higher than 80% in this condition.

Key words: Protein Recovery, Adsorption, BSA, Internal Contactor, Yeast Suspension

INTRODUCTION

The downstream process usually accounts for the largest part of the production costs of protein. Fast and efficient recovery steps for further purification of proteins form the basis for a successful downstream process [1]. Primary recovery comprises the first steps of downstream processing where some purification and broth volume reduction occurs. On an industrial scale, primary recovery of proteins from cell suspension by centrifugation and filtration is well established; however, its high operation cost requires other solutions [2]. Adsorption of proteins using several kinds of adsorbents has been considered as an alternative way. Suspended-particle adsorption systems include stirred tank (batch), expanded/fluidized bed etc. [3]. Separation using ion exchangers depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. The presence of charged groups is a fundamental property of an ion exchanger. The type of group determines the type and strength of the ion exchanger; their total number and availability determine capacity. There are a variety of groups that have been chosen for use in ion exchangers. The purification and recovery of bio-molecules is performed via a cascade of batch adsorption and desorption stages using stirred tanks and related filtration devices [4,5]. Although batch procedures are less efficient than column techniques, they may offer advantages in particular cases. Expanded bed adsorption (EBA) is a favorite unit operation using ion - exchange for recovering proteins directly from crude samples. A segregated or stratified bed has been achieved by the particle size distribution of the ion exchange, which leads to lower the back-mixing [6]. Crude feed from the fermentor containing the desired product and undesired cells, cell debris and particulates is applied to the expanded bed. EBA can get these advantages by the adsorbents, which are well-defined size and density distribution, and the balance between upward flow rate and adsorbent particle sedimentation velocity. Thus fermentation can be used intensified with an external loop EBA system as a direct product protein recovery, and many researchers are trying to optimize and modify these operations [7]. In this paper, new equipment was designed for the primary recovery of target protein from simulated yeast solution. The main purpose of this study was to optimize the performance of the mentioned device containing ion exchange. Furthermore, the recovery process was studied to be carried out in denser cell solution (up to 50 g/L Dry cell density) with anion exchange.

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MATERIALS AND METHODS

1. Anion Exchanger

DEAE-STREAMLINE (Sepharose based ion exchanger, Pharmacia Biotech AB, Uppsala, Sweden) is a weak anion exchanger [8]. Particle size is 100-300 (μm) and dynamic binding capacity is 55 mg BSA/mL gel. This sepharose ion exchanger can be used at temperatures up to 70 °C and can be sterilized repeatedly in the salt form by autoclaving at 121 °C, pH 7, for 30 min. Working pH range is between 2 and 9. This anion exchanger was cleaned with distilled water and equilibrated with buffer before experiments.

2. Model Protein

The model protein was bovine serum albumin (BSA) (Sigma Chemical Co., St Louis, U.S.A) of 99.9% purity. BSA has a molecular weight of 66,300 Dalton, iso-electic point (pI) 4.7-4.9 and hydrodynamic radius of 3.20 nm. This was used as a model protein.

3. Simulated Solution/Suspension

A simulated solution in this paper was meant a buffer solution dosed with BSA. This solution was made by Tris(hydroxymethyl) aminomethan (Sigma Chemical Co., St Louis, U.S.A) by adjusting pH by 1 M HCl. In some experiments, cells were suspended in this solution. Then ionic conductivity was adjusted with NaCl by ion conductivity meter. (CON5 METER, LaMOTTE company, USA).

4. Strain and Media

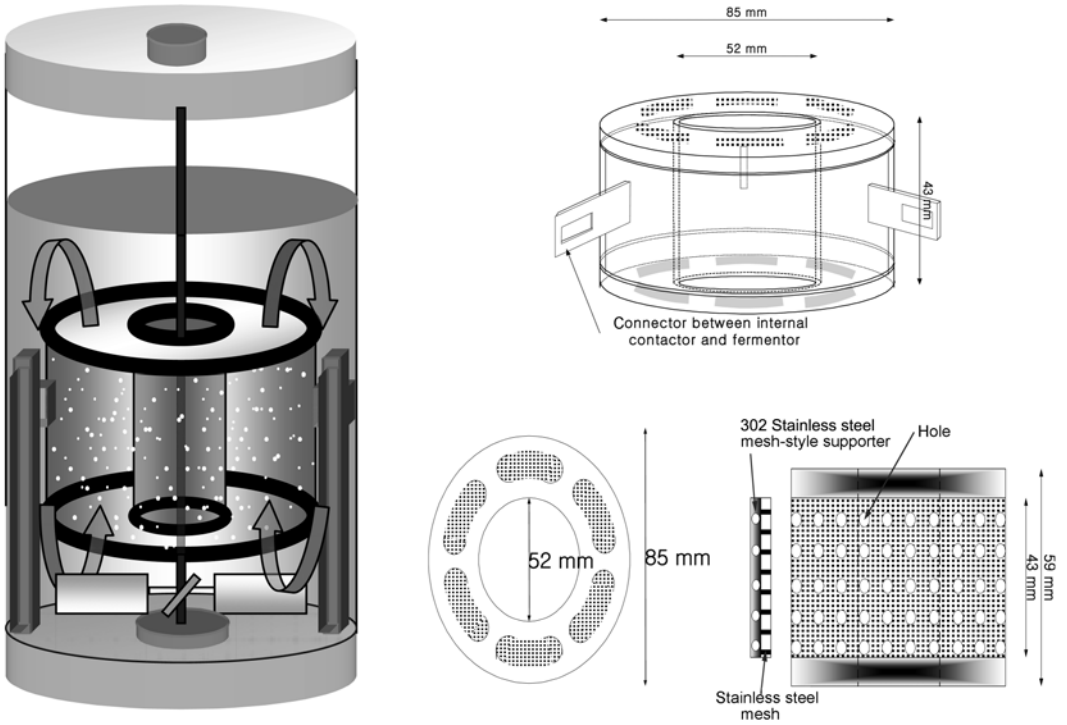

Fig. 1. Schematic diagram of stirred tank reactor with an internal contactor.

Feed stock of yeast cell suspensions are relatively common starting materials, because yeast usually exports the expressed recombinant protein efficiently into the culture medium. Two kinds of yeast were used in these experiments. *Saccaromyces cerevisiae* was purchased from Sigma-Aldrich, and *Hansenula polymorpha* was cultivated. The strain was stored at −70 °C in 30% (w/w) glycerol solution. To obtain subculture, the frozen strain was transferred to YPD agar plates containing 10.0 g/L yeast extract, 10.0 g/L peptone, 20.0 g/L glucose and 10.0 g/L agar. These were incubated at 30 °C for 24 hrs in an incubator (K.M.-8480SF, Vision Scientific Co., Korea), and then stored at 4 °C.

5. Fed-batch Cell Culture of *Hansenula polymorpha*

It was impossible to get a high cell density (over 3%) in a batch culture because of a substrate inhibition. Fed-batch techniques can be used to achieve concentrations greater than 50 g DCW/L. In microbial processes, the exponential feeding of a growth-limiting substrate resulted in a constant substrate concentration and control of the specific growth rate [9]. The fed-batch cultures were performed in the described 2.5 L fermentor. Subcultures were transferred to the seed culture medium (50 mL) in a 500 mL Erlenmeyer flask, containing 1% yeast extract, 2% peptone and 2% glycerol. The medium was incubated at 37 °C, 300 rpm for 24 hr to become 3.0 g/L DCW. Yeast was grown in batch phase until depletion of glycerol (50 g/L). Following the batch phase, a concentrated glycerol feeding phase was started by using a computer controlled peristaltic pump (Masterflex L\S computerized drive, Cole-Parmer Instrument Co., Barrington, U.S.A.). The feed solution contained 200 g/L glycerol, 50 g/L yeast extract and 100 g/L bacto peptone. The pH of culture broth was controlled to 5.0 with 2 N H_2SO_4 and 2 N NaOH. Antifoam 204 (Sigma Chemical Co., St Louis, U.S.A) was added prior to feeding solution. The process was finished after the cell density in broth became about 70 g DCW/L.

6. Cell Washing

After cell cultivation, cells had to be cleaned to remove possible cell debris and the remaining proteins of the medium. Cells were precipitated by using a centrifuge at 3,000 rpm for 10min, then were suspended with distilled saline water. These steps were repeated several times until a supernatant of cells had no proteins.

7. Internal Contactor

An internal contactor was installed to recover protein in a bioreactor. The schematic is shown in Fig. 1 and 2, which is similar to an original model [10]. This model was transformed to get holes in both the top and bottom and to have larger holes than the earlier one. This led to more expansion of resin in the axial direction. All material of internal contactor was made with stainless 302 steel, which was known to be endurable and best in high salt concentration. There were two compartments in the internal contactor. A frame was made as a supporter. Inside stainless steel mesh, which was 200 mesh with 76 μm diameter, was installed to prevent DEAE-Streamline, diameter of which is between 100-300 μm, from releasing to outside of the internal contactor.

8. Plastic Pot for Lab-scale Desorption Process

A desorption pot was made of acrylic plastic. The internal contactor was fit compactly inside it. The schematic is shown in Fig. 2. After an adsorption step was finished, the internal contactor was uninstalled from the stirred tank and was moved to set in the pot. A washing buffer or elution buffer which was 200 mL each was added. The pot was put in the shaker at 80 rpm room temperature. The optimum process time for those steps would have been determined. The conductivity of the washing buffer was fixed as same as the simulated solutions and the elution buffer was selected by experiments.

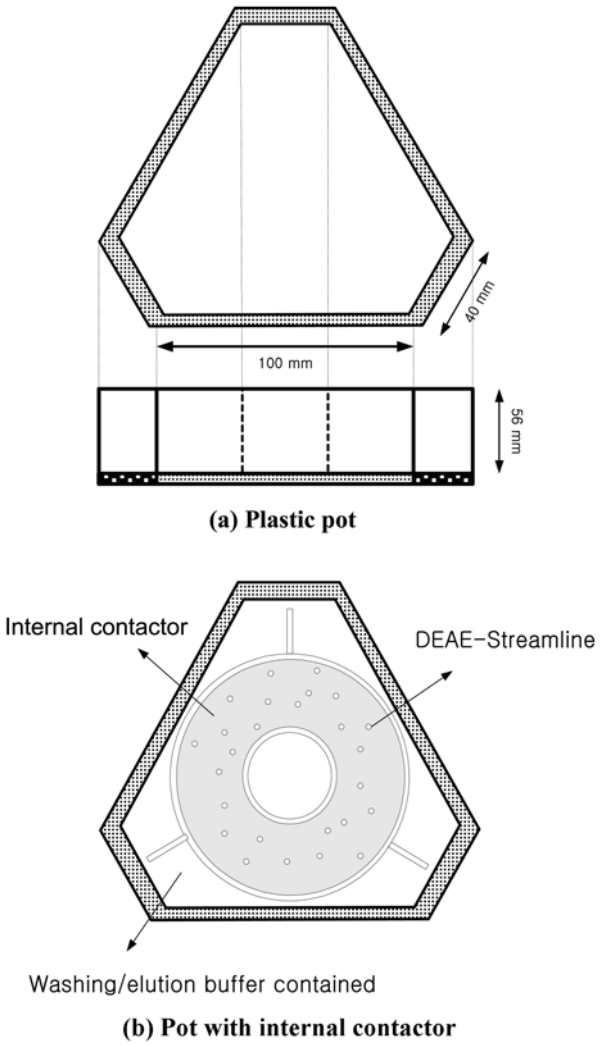

Fig. 2. The Shape of plastic pot with internal contactor.

9. Adsorption Experiment

In the finite batch adsorption experiment, bovine serum albumin (BSA) was incubated with the ion exchange and a diluted suspension of yeast cell under investigation of pH and ion conductivity as to establish a proper ratio of BSA adsorption to ion conductivity in cell suspension [11]. Prior to the experiment, the ion exchanges were equilibrated with 50mL Tris-HCl (pH 7.6). A 1.0 g/L BSA, 3% (w/w) yeast were suspended. Then each simulated solution was adjusted to have different ion conductivities from 4 to 10.2 mS/cm. Finally, 1 mL DEAE-Streamline was added to each shake flask. The flasks were incubated with gentle agitation (70 rpm). After 60 min process, the amounts of BSA in the flasks were check by Lowry method. EBA is a favorite unit operation using ion - exchange for recovering proteins directly from crude samples. A segregated or stratified bed was achieved by the particle size distribution of the ion exchange, which led to lower the back-mixing. In a stirred tank with an internal contactor containing DEAE-Streamline, BSA adsorption was carried out under controls of pH and ion conductivity. Prior to the experiment, the ion exchange was equilibrated with Tris-HCl (pH 7.6) for 1 hr. Then, it was poured into the installed internal contactor. Then simulated solution was fed into the stirred tank. The pH was controlled to be 7.6 with 2 N H_2SO_4 and 2 N NaOH. The ionic conductivity was also controlled with ion conductivity meter. The experiments were done at room temperature.

RESULTS AND DISCUSSION

1. Finite Batch Adsorption

Generally, yeast is known to have a negative charge on its surface in neutral pH condition. In the case of using anion exchanger, yeast cells were shown to be adsorbed onto it, which led to a deteriorated stability of the ion exchanger in EBA and in turn to a reduced sorption efficiency of desired proteins. This is the main rationale to develop a better EBA system or an internal contactor in this work. It is found that the interaction between biomass (yeast) and anion exchangers is dominated mainly by electrostatic force; thus, the conductivity of the cell suspension was chosen as the main operating parameter to modulate cell adhesion [1]. Fig. 3 shows that in lower conductivity than about 7.3 mS/cm, the adsorption efficiency of BSA was reduced in the case of *H. polymorpha*. It was understood that in low conductivity conditions, the significant adsorption of cells to the stationary phase of resin may reduce the available number of ligands for protein binding, thus reducing the equilibrium capacity for the desired protein. In addition, a degree of purification was also worse because of the remaining yeast.

The efficiency was reduced with increasing ionic conductivity of the simulated solution in the result. The reason was that high ionic conductivity hindered the interaction between proteins and functional groups of ion exchange [12]. Thus, this required further work for determining an optimum ion conductivity value higher than 7.3 mS/cm.

2. The Effect of Impeller Type and Speed on Ion Exchange Flow Patterns

In a primary recovery, it is important to reduce operation time

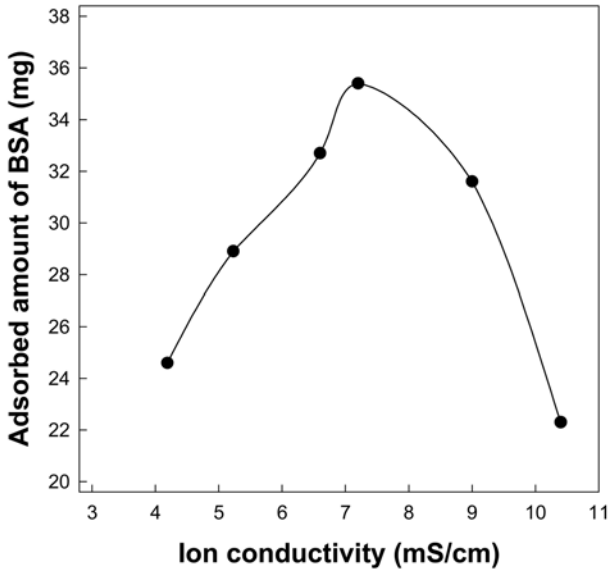

Fig. 3. BSA adsorption at different ion-conductivities in finite batch mode, shaking 100 rpm for 1 hr, room temperature, 3% *Hansenula polymorpha* solution of finite batch adsorption, initial amount of BSA (50 mg, 1 g/L).

not only for preventing protein degradation but also for decreasing purification costs. Accordingly, it is required to have an efficient impeller type for the effective mixing to get a better adsorption between proteins and DEAE-Streamline in the internal contactor. In spite of the existence of intraparticle resistance, film mass transfer resistance, liquid axial dispersion, and solid axial dispersion during expanded bed adsorption, the contribution of BSA effective pore diffusivity is dominant [13]. Two different types of impeller were conducted to determine the better one for an internal contactor. The mixing performance was examined in the screw type impeller and the rushton turbine type impeller. The rushton type impeller is generally used in fermentation, but it has less efficiency to expand DEAE-Streamline in an internal contactor. On the other hand, screw type impeller was shown to form axial flow in the internal contactor. The axial flow was thought to help full expansion of ion exchange inside to have the advantage of EBA. Furthermore, in the case of expanding volume during the process to ion exchange packing volume, EBA was 2-3 and the internal contactor was more than 5. Thus, an adsorption rate in an internal contactor was thought better. It is important to get an optimum impeller rotating speed, which makes axial flow to the internal contactor, for better distributions of ion exchanges by the screw-type impeller. In low axial flow, ion-exchange would remain packed on the bottom of an internal contactor. On the other hand, in high axial flow, they could float to the top. Rotation speed was selected to be 500 rpm in this study.

3. Determination of Optimum Adsorption Time

In the primary recovery step, reducing the operation time and acquiring most of desired products are most important [12]. The advantage of batch adsorption over EBA was to have less adsorption time. It was known that non-specific layering of cells onto the resin occurred after 3 hr contact times during ion exchange adsorptions in EBA [14]. The internal contactor was derivative of batch adsorption, the ion exchange was confined in an internal contactor, which could be a restriction for recovery performance and lead to need more adsorption time. Thus, it was necessary to check the optimum adsorption time. In Fig. 4, the optimum adsorption time value was chosen to be about 2 hr when adsorption equilibrium was attained. It was a little bit longer than 60-90 min of batch adsorption and less than 3 hr of EBA.

4. Determination of Optimum Ion Conductivity

To get a better adsorption yield during fixed adsorption time, the optimum of ionic conductivity was deduced from previous results. A compromise value was between 7.3 and 7.7 mS/cm in Fig. 5. So,

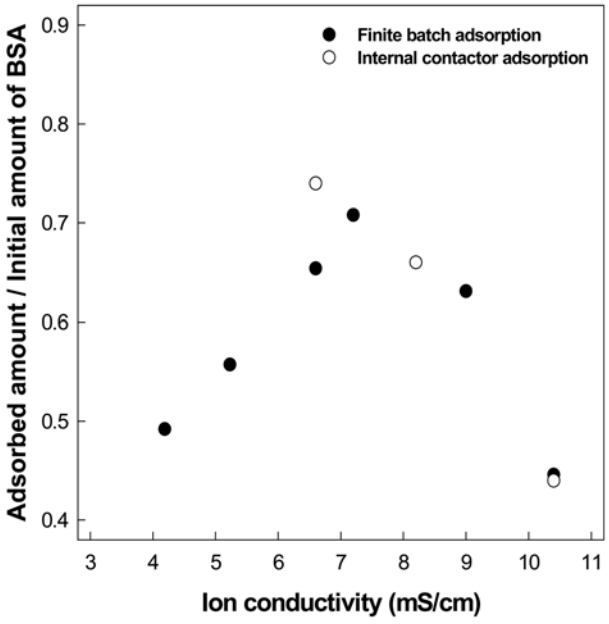

Fig. 5. Efficiency of BSA adsorption to DEAE-Streamline as a function of fluid phase conductivity in cell free solution using an internal contactor (○) and in 3% *Hansenula polymorpha* solution of finite batch adsorption (●).

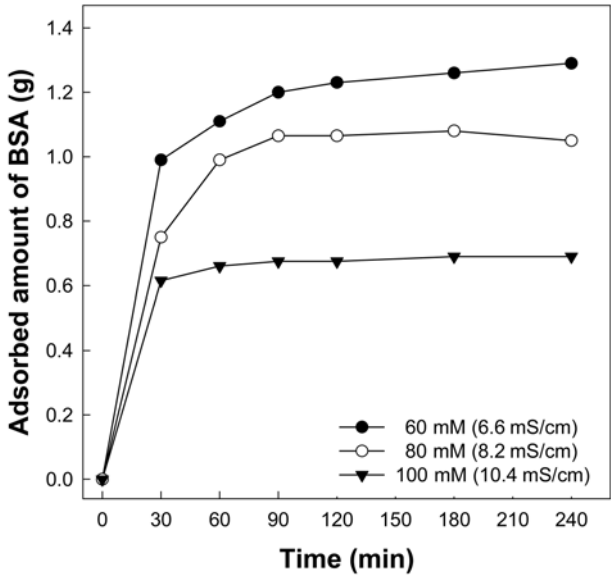

Fig. 4. The determination of processing time in different ion conductivity using an internal contactor in stirred tank, cell free solution, at room temperature.

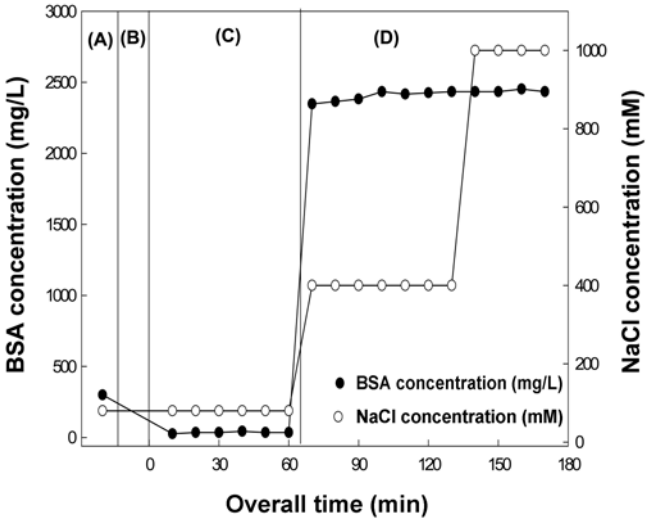

Fig. 6. Desorption steps and process time in an internal contactor, concentration of NaCl was raised stepwise 80 mM, 400 mM and 1 M, BSA solution initially (A), BSA solution during adsorption process (B), BSA solution during washing step (C), and BSA solution during elution step (D).

experiments after this were performed under ion conductivity of 7.6 mS/cm. In addition, the binding capacity was also determined to be 1.0-1.1 g BSA/30 mL DEAE-Streamline or 33-37 mg (BSA/mL DEAE-Streamline).

5. Desorption Process

To resolve the difficulty of a solution-concentration in an internal contactor, a plastic pot was introduced. Fig. 6 shows that BSA was not desorbed during the washing step, in which the remaining yeast cells would be detached from DEAE-Streamline. As an elution buffer, 0.4 M NaCl was selected to desorb all BSA or desired protein from ion exchange. The 1 M NaCl solution was used for further elution and the result verified that a 0.4 M NaCl solution was good enough. The time to each step (washing\elution) was determined to be 30 min each from the result. It showed that 300 mg/L BSA solution was concentrated to nearly 2.3 g/L, as the volume was reduced from over 1.5 L to 0.2 L after desorption. Thus, it is reliable to use a plastic pot for this step.

6. Adsorption of BSA from Cell Suspension

The conditions in an internal contactor were concluded from adsorption step to desorption step through previous experiments. BSA adsorption was done in a simulated solution with various cell suspension conditions. Expanded bed adsorption had no problem under a 2-3% cell suspension. In an expanded bed adsorption with anion ion exchanges, it was difficult to operate a primary recovery of protein from high yeast density broth. In the case of *Hansenula polymorpha*, 50 g DCW/L density was the upper limit and in the case of *S. cerevisiae*, 45 g DCW/L was a known optimum value for EBA with DEAE-streamline. BSA adsorption was done in the simulated solution with about 5% cell suspension (*S. cerevisiae*) as shown in Fig. 7. The result showed that in this cell concentration, BSA adsorption was done greatly. However, some proteins from autolyzed yeast could be serious contaminants after a long (over 1 hr) process. Cell autolysis was thought to occur because there were no nutrients in the simulated solution. After consideration of cell autolysis, the yield was over 80%. Although a short adsorption process (about 1 hr) may have lower primary recovery performance, it was considered better, judging from a point of view like purification.

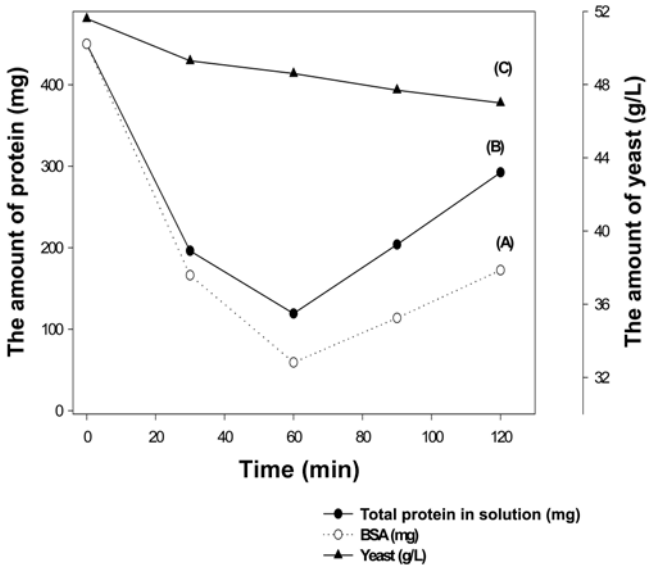

Fig. 7. BSA adsorption in high cell density (5%) solution, only BSA present at first as protein (initially 450 mg), estimation of BSA adsorbed when considering protein by cell autolysis (A), experimental result (B) cell degradation (C).

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