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PRODUCTION OF LIPOPEPTIDES BY BIOREACTION
SEPARATION COUPLING

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Abstract. The lipopeptide Surfactin produced by *Bacillus subtilis* is one of the most widely studied biosurfactants because of its high biotechnological and therapeutical significance. Surfactin has several advantages over the chemically synthesized surfactants such as lower toxicity, higher biodegradability and biocompatibility. In view of Surfactin production, we propose a new approach integrating the fermentation process with micro-filtration and adsorption. The adsorption column, designed as a fixed bed activated carbon reactor, recovers the Surfactin and eliminates the foam formation. Moreover, the adsorption step reduces the concentration in the effluent so that it does not exceed permissible limit.

Key-words. surfactin, adsorption, fermentation, integrated process

Surfactin is a bacterial lipopeptide produced biologically by *Bacillus subtilis*. It has the advantages of biodegradability, low toxicity and biocompatibility. As a result, it has been found an attractive alternative or supplement to chemically synthesized surfactants that have a detrimental effect on the environment and successful application in areas such as bioremediation [1-2] and oil recovery [3-4]. However, biosurfactants are not widely available because of their high production cost, which result primarily from low strain productivities and high recovery expenses. Moreover, under the aerated conditions, surfactin accumulation generates foam that is apt to flow out of the reactor; which decreases the valid reactor volume and makes the process difficult to control. Process integration is an attractive approach to foam elimination and to produce valuable products of higher quality at reduced costs. Typical example is the fermentation process integration with liquid isolation of a specified reaction product [5].

In this study, in view of surfactin production, we propose a new integrated process comprising fermentation and adsorption as shown in Fig. 1. A micro filter and a heat-exchanger are added for cell recovery and temperature control before the adsorption column. The permeate flow containing no cell enters the adsorption column whereas the retentate flow containing cells returns into the bioreactor. The adsorption column is designed as a fixed bed of activated carbon, which is applied for surfactin recovery and foam elimination.

The column is designed based on the adsorption experience obtained in our preliminary work [6-7]. Both adsorption equilibria and kinetics show that the activated carbon acts as a very effective adsorbent for surfactin adsorption. In view of using an integrated process, in this work the adsorption of surfactin from real medium solutions is studied in both batch and continuous fixed bed conditions. Fixed bed adsorption experiments were performed to study the adsorption dynamics and give accurate data for scale up. Main objective of the fixed bed adsorption is to reduce concentration in the effluent so that it does not exceed permissible limit. The most important criterion in the design of fixed bed adsorption systems is the prediction of column breakthrough or the shape of the adsorption wave front, which

determine the operation life span of the bed and regeneration times. Some experiments were necessary to determine the adsorption model. The effects of parameters of fixed bed process, such as operation temperature, flow rate and adsorbent dose on the shape of breakthrough curves were investigated using the predicted model. Finally, more than 94 % of the surfactin adsorbed onto the fixed bed column was eluted by desorption with methanol indicating that an activated carbon fixed bed could be used for surfactin recovery effectively in the integrated process. Moreover, a production kinetic model was determined in batch culture. Adding the breakthrough curve model, this study was the preliminary work of the global process optimization.

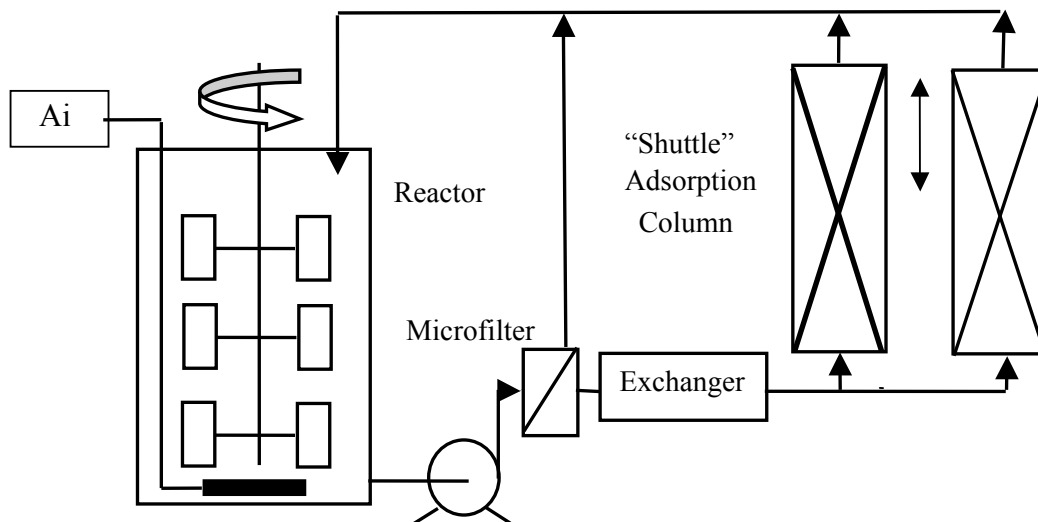


Fig.1 Schematic of the integrated process

EXPERIMENTAL

Fermentation procedure

The surfactin was produced by *Bacillus subtilis* ATCC 21332. The strain selected could produce only one kind of lipopeptide - surfactin. The cultures from late exponential growth phase were harvested as the inoculum for surfactin fermentation experiments. Optical density of the culture was determined at 600 nm and culture was washed and concentrated 10 times by centrifugation. The seed cultures were inoculated into 1000 mL flasks containing 200 mL of Landy modified medium [6] in order to obtain 0.5 as initial optical density. Batch cultures were incubated at 37 °C and pH at ~7 with an agitation rate of 200 rpm. The cell growth and surfactin concentration were monitored with respect to time. After the fermentation was ended, the culture medium was centrifuged. The supernatant was collected for the following adsorption experiments.

Adsorption of surfactin from culture medium onto the activated carbon

Merck activated carbon (Ref. 1.02514. 1000) with spherical geometry was used. In our previous work, only the adsorption of pure surfactin from aqueous buffer solution was studied, and the sorption capacity of the adsorbents was obtained using the adsorption isotherm technique [6]. In this work, the surfactin was adsorbed directly from the culture medium in continuous fixed bed conditions. Continuous fixed bed adsorption was also studied to evaluate the column performance for surfactin adsorption from culture medium. Experiments were conducted in a 1.3 cm diameter and 30 cm length glass column packed with 0.9 mm particle-size of the activated carbon with of 0.9 mm diameter between two supporting layers of glass balls with 2 mm diameter. Nine grams of activated carbon were added to produce 110 mm length column. The influent culture medium was put in an overhead feed reservoir of 3.0 L capacity and kept agitation during the experiment process to make the culture medium homogeneous. The influent feed flowed vertically through the column from down top. The flow rate was supplied and controlled by a peristaltic pump. Sampling of effluent was done at the chosen time, and the surfactin concentrations in the samples were measured. Effects of initial adsorbate concentration (350 mg/L and 550 mg/L) were investigated on the performance of breakthrough for the adsorption of surfactin directly from the culture medium.

RESULTS AND DISCUSSION

Dynamics of cell growth and surfactin production

B. subtilis ATCC 21332 was grown in Landy modified medium [6]. The time courses of cell growth and surfactin production are shown in Fig. 2, which indicates that surfactin production growth is apparently associated with cell growth. After about 45 hours fermentation process, both cell growth and surfactin production reach the maximum and then level off. Generally, the surfactin production in such a fermentation process can reach up to 500-600 mg/L. The surfactin concentration of the culture medium used for adsorption studying was controlled to be $C_0 = 550$ mg/L by water dilution or vacuum distillation concentration.

To modeling the experimental point of the biomass and the surfactin production, we propose the equation:

$$\begin{cases} \frac{dX}{dt} = \alpha X \left(1 - \frac{X}{X_{\max}} \right) \\ \frac{dP}{dt} = \gamma \frac{dX}{dt} \end{cases} \quad (1)$$

where α is the kinetic constant (h^{-1}), X_{\max} the maximal biomass concentration, and γ the productivity ($\text{mg}\cdot\text{L}^{-1}\cdot\text{OD}^{-1}$). The best fit of the above equations to the experimental data yielded $\alpha = 0.277$ h^{-1} , $X_{\max} = 16.72$ OD unit and $\gamma = 29.7$ $\text{mg}\cdot\text{L}^{-1}\cdot\text{OD}^{-1}$.

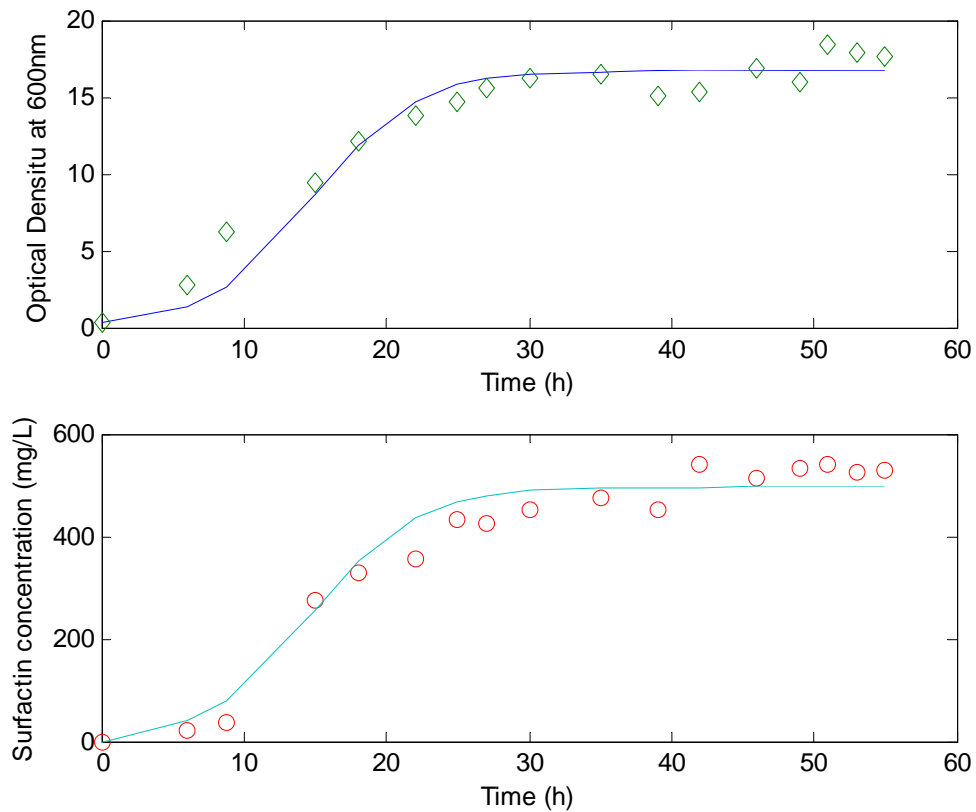


Fig. 2 Time-course of cell growth and surfactin concentration during *Bacillus subtilis* ATCC 21332 fermentation (line = model, symbol =experimental point)

Breakthrough curve analysis

In a fixed bed adsorption system, the adsorbent located close to inlet where maximum adsorption takes place initially. This adsorption zone moves further as time passes and then approaches the exit of the bed. When the adsorption zone has moved through the column, the concentration of the adsorbate at the exit becomes equal to the feed concentration. Adsorption involves interface accumulation or concentration of an adsorbate species at an adsorbent surface or interface. A plot of exit concentration as a function of lapse time or volume throughput reacted is known as breakthrough curve. The

characteristic shape of the breakthrough curve depends on the inlet flow rates, concentration, adsorption equilibria and kinetics, and other properties such as column diameter and bed height. The area under the breakthrough curve gives the total quantity of adsorbate adsorbed for a given feed concentration.

The model used in the present work to fit the experimental data takes account of the effect of both intraparticle and external- film mass-transfer resistances. This model was used in different works in particular for big molecule like α -Amylase [8]. Simultaneously, instantaneous local adsorption equilibrium on the pore surface was considered. Furthermore, the following assumptions were made to set up the model equations:

- 1) The system operates under isothermal conditions.
- 2) The equilibrium of adsorption is described by an isothermal equation in the form $q=f(c)$.
- 3) Intraparticle mass transport is due to Fickian diffusion, and it is characterized by the pore diffusion coefficient, i.e. D_p . Mass transfer across the boundary layer surrounding the solid particles is characterized by the external-film mass transfer coefficient k_f .
- 4) The linear velocity of the liquid phase along the column, and the mass-transfer parameters are independent of the solute concentration in the bulk liquid phase.
- 5) Axial dispersion is considered to account for nonideal flux along the longitudinal axis of the column.
- 6) The macroporous adsorbent particles are spherical and homogeneous in size and density.

The surfactin diffusion coefficient with molecular dynamics simulation is estimate like the following procedure. Classical molecular dynamics is employed to estimate the diffusion coefficient. Because the surfactin molecule is primarily composed of amino acids, we choose GAFF (generalized AMBER force field) parameter set to model the intermolecular and intramolecular interactions, as well as atomic charges. [9] The cubic simulation cell is setup with only one surfactin molecule (two carboxylic groups are deprotonated), as well as sufficient water molecules, plus 2 K⁺ cation to maintain electric neutrality. We use SPC (Single Point Charge) model for the water molecule and employ standard periodical boundary conditions in all three directions. And the dimension of simulation cell is larger than 6 nm, so that it's enough to encapsulate the surfactin and prevent it from escaping. For both van der Waals and Coulomb potential, a cutoff radius of 1 nm is used, thus each surfactin does not have interaction with its image, so actually it's in an infinite dilute solution.

The initial configuration is prepared targeting an arbitrary density of 1.0 g/cm³, and equilibrated to 0.1 MPa, desired temperatures respectively with NPT ensemble. Then a 400 ps (with a timestep of 2 fs) NVT simulation with standard Nose-Hoover thermostat is carried out to further equilibrate the system, after which 3200 ps production run is performed to estimate the diffusion coefficient. In fact, we first calculate mean square displacement and plot it against time, using the Einstein relation then the diffusion constant could be obtained. For a temperature equal 310 K, the diffusion value is equal to 0.428×10⁻⁵ cm²/s.

The axial dispersion coefficient is calculated with the correlation proposed by [10]:

$$D_L = \frac{D_m}{\varepsilon} (20 + 0.5Sc Re \varepsilon) \quad (2)$$

The external mass transfer coefficient is calculated with the correlation proposed by [11]:

$$k_f = \frac{v}{Sc^{\frac{2}{3}}} \left(\frac{0.765}{(Re \varepsilon)^{0.82}} + \frac{0.365}{(Re \varepsilon)^{0.386}} \right) \quad (3)$$

The D_p value was determined with minimizing the quadratic difference between experimental and modeling points;

The adsorption performance of surfactin from culture medium was studied at two inlet surfactin concentrations, 350 and 550 mg/L. The original culture medium of 550 mg/L was diluted with water to obtain 350 mg/L. Fig. 3 shows the breakthrough curves at the two initial concentrations. The column height was 110 mm, the flow rate was 0.295 ml/min, and temperature was kept at 37 °C. The two breakthrough curves broke through nearly at the same contact time. Moreover, in the first stage of exhaustion, the two columns were exhausted nearly as fast as each other. However, in the second stage, the column got exhausted faster at a higher initial surfactin concentration. As a whole, an increase in

initial surfactin concentration of inlet culture medium increased the adsorbed surfactin quantity at the same contact time on the adsorbent column and the efficiency of the adsorbent column as well, which should also be due to increase of the mass transfer of the surfactin to the adsorbent at a higher initial surfactin concentration.

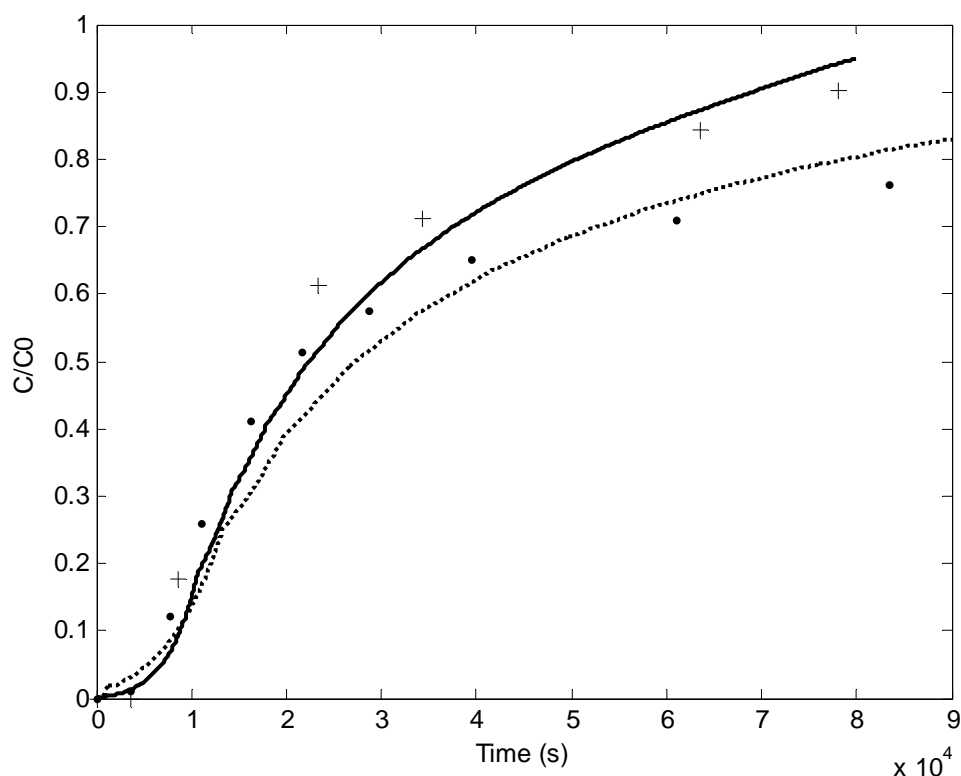


Fig. 3 Experimental (dots) and model (lines) breakthrough curve for adsorption of Surfactin [column height = 110 mm, flow rate = 0.295 ml/min, $T = 37\text{ }^{\circ}\text{C}$, C_0 (mg/L): (■)350; (+)550].

The D_p value were respectively $1 \times 10^{-11}\text{ m}^2 \cdot \text{s}^{-1}$ and $1.5 \times 10^{-11}\text{ m}^2 \cdot \text{s}^{-1}$ for an initial concentration equal to 350 and 550 mg/L.

Recovery of surfactin by methanol elution

It has been shown that the adsorption of surfactin onto the activated carbon is an irreversible process, during which the adsorbed surfactin can not be desorbed with water solutions. Therefore, methanol was used as eluent to recover surfactin adsorbed in the activated carbon. The adsorbent column was operated for 30 h under the following conditions: column height = 110 mm, flow rate = 0.295 mL/min, $T = 37\text{ }^{\circ}\text{C}$ and $C_0 = 550\text{ mg/L}$. The total quantity of surfactin adsorbed, which is about 82.0 mg. Then, after washed with water, the adsorbent column was eluted by methanol with a flow rate of 0.295 ml/min at $25\text{ }^{\circ}\text{C}$. Fig. 4 shows the evolution of surfactin concentration in effluent as a function of the throughput volume of eluent. The area under the curve gives the total quantity of surfactin recovered in effluent methanol, which is about 77.0 mg. Therefore, more than 94 % of surfactin adsorbed onto the activated carbon could be recovered by using the adsorption and subsequent desorption processes.

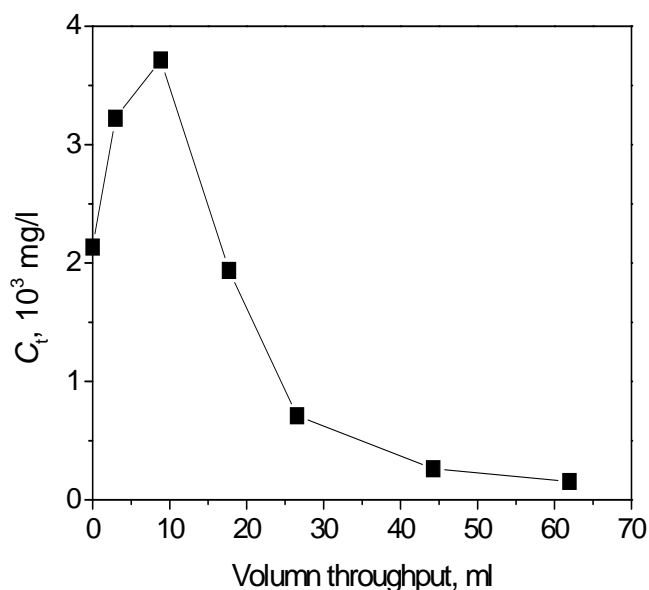


Fig. 4 Evolution of surfactin concentration in effluent as a function of the throughput volume of eluent (column height = 110 mm, flow rate = 0.295 ml/min and $T = 25\text{ }^{\circ}\text{C}$)

CONCLUSION

Surfactin productivity of up to 600 mg/l could be obtained by *B. subtilis* ATCC 21332 in the Landy modified medium. Surfactin adsorption capacity from culture media confirms that the activated carbon could be used as a good adsorbent for recovery of surfactin in an integrated process. Finally, more than 88 % of the surfactin adsorbed was eluted by desorption with methanol indicating that an activated carbon fixed bed could be used for surfactin recovery effectively in the integrated process.

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