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Cell Immobilization with Polyurethane Foam for Retaining *Trichoderma reesei* Cells During Foam Fractionation for Cellulase Collection

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Abstract In situ affinity foam fractionation is a potential powerful tool for continuous, selective removal of products from bioprocesses. When evaluating its applicability to cellulase production by *Trichoderma reesei* fermentation, we encountered the difficulty of significant removal of fungal mycelia along with the cellulase. To solve this problem, cell immobilization using cut pieces of hydrophilic polyurethane (PU) foam was evaluated. Five commercial PU foams with different pore sizes and porosities were tested. Two were found to support good cell growth, cellulase production, and cell loading (about 0.6 g dry cells per g PU). The PU-immobilized mycelia were successfully retained in the foaming process.

Keywords Cell immobilization · Cellulase · *Trichoderma reesei* · Affinity foam fractionation · Hydrophilic polyurethane

Introduction

The filamentous fungus *Trichoderma reesei* is well known for its relatively high productivity of cellulolytic and xylanolytic enzymes [1]. The cellulolytic enzymes cellulase are important for the hydrolysis of cellulosic wastes to usable organic substrates for most of the microbes in food, fuel, and chemical production [2]. To improve the cellulase productivity and process economics, we proposed to use a coupled fermentation and in situ foam fractionation process to continuously remove the produced cellulase from the fermentor while retaining the cells [3, 4]. We also developed an affinity foam fractionation approach that would enable selective, highly enriched collection of cellulase and/or its constituent enzymes (endoglucanase, exoglucanase, and β-glucosidase) from the fermentation broth [3–5]. Unfortunately, in the previous foaming studies, the fungal cells were found to be significantly removed with the foam. Consequently, the design of coupled fermentation and foam fractionation could not be implemented.

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To address the latter challenge of avoiding cell removal by foaming, a procedure using a countercurrent liquid stream in the foam column to wash down the entrapped cells was evaluated. A similar approach was reported to be useful in minimizing the removal of bacteria and/or yeast in the foaming processes [6]. Unfortunately, the morphology of *T. reesei* is quite different from those of yeast and bacteria. The large filamentous mycelia could not be effectively washed down through the thin liquid layers in the foams even at relatively high washing rate (1 mL/min, for foaming of 100 mL of the fungal broth) [7].

In this study, the cell immobilization method was examined for the feasibility of retaining fungal cells during the foam fractionation operation. Several additional advantages might be also achievable with the cell immobilization. For example, intensive agitation was reported to be harmful for cellulase production, leading to shear damage to *T. reesei* mycelia and resulting in low enzyme productivity [8]. The produced cellulase could also be inactivated/denatured by intense shaking or agitation because of the high shear stress involved [9]. A cell immobilization system that keeps the cells and portions of enzymes inside support matrixes could help protect them from the shear damages.

Polyurethane (PU) has been used for cell immobilization by entrapment or adsorption method according to the form of the material, i.e., PU foam or PU bead, since the late 1970s. PU is attractive for this application because of its very stable chemical and mechanical properties [10]. Another advantage of the PU foam is the simplicity of the immobilization method: It does not need to use spores as the inoculum and the filamentous vegetative cells can be used directly. Hydrophilic PU foams have been studied for immobilization of several microbial species, for instance, *Aspergillus niger* [11], *T. reesei* [12–20], *Candida shehatae* [21] and *Penicillium citrinum* [18]. All of the above studies were done in small, gently shaken flasks (up to 500 mL of flask volume). The results of these studies showed that these cells could be immobilized on the surface of PU foams by adsorption/entrapment, and the immobilized cell layers could sustain certain shear stress.

In this study, seven kinds of hydrophilic PU foams from Lendell Manufacturing, Inc. (St. Charles, MI, USA) were evaluated for their suitability as immobilization support for *T. reesei* Rut-C30. The foams that performed the best, in terms of low free cell concentrations and high cellulase (Filter Paper Unit, FPU) production, were selected for further study of cell loading capacity. Finally, the foaming behavior of the fermentation broth containing the cell-immobilized PU-foam particles was investigated.

Materials and Methods

Materials

T. reesei Rut C-30 (NRRL 11460) was obtained from the US Department of Agriculture (Agricultural Research Service Patent Culture Collection, Peoria, IL, USA). The microorganism was maintained at 4 °C on slants of potato dextrose agar (Sigma; 39 g/L, as recommended), with regular sub-culturing every 3 to 4 weeks. The culture medium used in this study had the same composition as that reported by Mandels et al. [22].

Seven hydrophilic PU foams from Lendell Manufacturing Inc. (St. Charles, MI, USA) were evaluated in this study. Before being used in the cell culture, the PU foams were socked in deionized water and preheated at 121 °C for 20 min to test the heat stability and to remove any possible residues left from the manufacturing processes. Two of the PU foams were found to be not stable under the high temperature. Subsequently, only the

remaining five PU foams were studied for cell immobilization. The commercial names of these five PU foams were Nola-Tex, HSS, Nola-Sponge, Media-Sponge, and Sensa-Temp.

Initial Screening of PU Foams for Cell Immobilization

As described before, five hydrophilic PU foams were chosen for cell immobilization study. The study was conducted in shake flasks at 200 rpm and room temperature (approximately 25 °C). Each flask contained 20 g/L of one of the tested PU foams. The amount of foam used was chosen according to a preliminary study for being enough to hold the cells inside the matrixes. (The culture medium had 10 g/L lactose. At about 50% cell yield, the maximum cell concentrations were expected to be about 5 g/L.) The flasks were inoculated with 10% (*v*/*v*) of the same inoculum culture. Daily samples were taken from each flask for measurements of the free cell concentration, cellulase activity (in FPU), pH, extracellular protein concentration, and reducing sugar concentration. Better immobilization support should have lower free cell concentrations and higher FPU production. Accordingly, two PU foams, Nola-Tex and HSS, were chosen for further study of cell loading capacity. In this study, the PU foams were cut into cubes of 5–6 mm in each dimension.

Loading Capacity Study

Nola-Tex and HSS foams were examined for their cell loading capacity, i.e., the amount of *T. reesei* cells immobilized per unit amount of foams. Each foam material was studied at four levels, i.e., 2, 5, 10, and 15 g/L. The PU foams were cut into smaller cubes (estimated size, 3×3×3 mm) to reduce the inevitable mass transfer limitation associated with such immobilization supports. Periodical samples were taken for duplicate analyses of free cell concentration, reducing sugar concentration, extracellular protein concentration, and cellulase FPU activity.

Foaming Study

The study was done with a foaming column (diameter, 3.5 cm; height, 1 m) having multiple side ports for sample collection if necessary. Fine bubbles were created by pumping air through a sintered disc mounted at the bottom of the column. The behaviors of the PU cubes before and after being used in the immobilized-cell fermentation, i.e., cell-free and cell-loaded foams, were observed during the foaming process.

Analytical Methods

The reducing sugar concentration was measured by the non-specific dinitrosalicylic acid (DNS) method, based on the color formation of DNS reagent when heated with reducing sugars [23]. The total activity of cellulase was measured by the standard filter paper assay [22]. The extracellular protein concentration was measured by the standard Coomassie blue method [24], with the resultant absorbance measured at 595 nm using a UV/VIS spectrophotometer (Perkin-Elmer Lambda 3B). The cell concentration was estimated from intracellular protein concentration, as follows.

By centrifugation, the free cells in broth samples (5 mL) were collected and washed twice with deionized water. The cell pellets were then lysed in 5 mL of 0.2 N NaOH, at 100 °C for 20 min. The protein concentration of the lysate was then measured by the standard Coomassie method, just as the extracellular protein concentration. The determined

intracellular protein concentration (IP) was then converted to cell dry-weight concentration (CDW) using the predetermined relationship: CDW (g/L)=IP (g/L)×8.0 (±0.5) [3, 4].

The immobilized cell concentration (X_{im}, per liter of medium) in the PU foams was estimated from the difference between (1) the total cell concentration (X_T) calculated from the average cell yield ($Y_{X/S}$) and the consumed lactose concentration (ΔS) and (2) the measured free cell concentration (X_f); that is

$$X_{im} = X_T - X_f = Y_{X/S}(\Delta S) - X_f$$

The estimation was made with the assumption that the PU foams did not affect the cell yield, which would be questionable if oxygen limitation occurred inside the foam cubes or if the maintenance requirement differed substantially between the free and immobilized cells. The value of $Y_{X/S}$ was taken as 0.5 in this estimation, based on the results of the study conducted under similar cultivation conditions in the free cell system.

Results and Discussion

Screening of PU Foams for Cell Immobilization

In this study, a relatively high PU foam content (20 g/L) was used to make sure that, if significant growth of free (not immobilized) cells occurred, it was not because of insufficient amount of foams available for immobilization. The experimental results obtained for the five PU foams tested are described in the following.

As shown in Fig. 1, the free cell concentrations in the HSS and Nola-Tex systems dropped rapidly and remained at very low levels (below 0.5 g/L) until the end of the experiments. As lactose was actively consumed in these systems (Fig. 2), the low free cell concentrations observed were not caused by cell death. Instead, the free cells inoculated rapidly adsorbed onto the foams, faster than their rate of growth as free cells. Presumably, the subsequent cell growth occurred on and/or in the porous foams. In the Media-Sponge and Nola–Sponge systems, the cells could also adsorb onto the foams, but the adsorption rates were lower than their initial free-cell growth rates; as a result, the free cell concentrations increased initially. Later, the growth of free cells slowed down, and the adsorption caught up and caused the free cell concentrations to decrease to lower levels. The free cell concentration profile observed in the Sensa-Temp system was rather similar to those in the Media-Sponge and Nola-Sponge systems, except that the free cell concentration in the Sensa-Temp system did not increase initially. Nonetheless, together

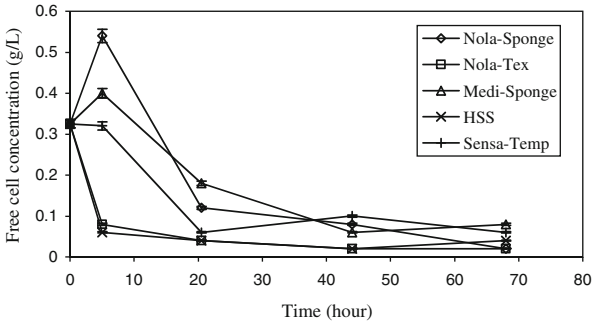

Fig. 1 Free cell concentration profiles observed in the initial screening study with 20 g/L of each of the five hydrophilic PU foams

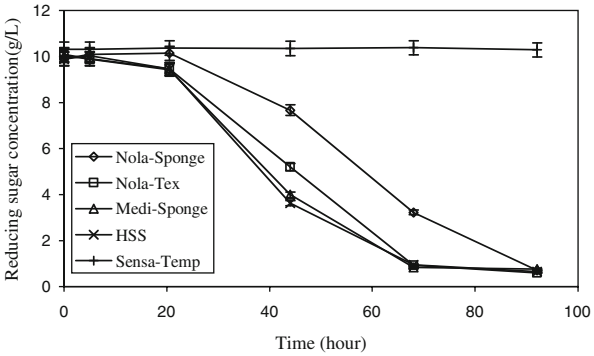

Fig. 2 Reducing sugar consumption profiles observed in the initial PU foam screening study

with the profiles of pH change and lactose consumption observed (as described in the later paragraphs), it could be concluded that the Sensa-Temp foam seriously inhibited cell growth and might have caused cell death.

One of the other properties measured in this study was the broth pH. pH was easy to measure and could be a qualitative indicator for cell growth or metabolic activity. This study was conducted in shake flasks without pH control. When cells grew actively on sugars, pH of the broth would drop because the cells produced some acidic metabolites. In addition, ammonium sulfate was used as the primary nitrogen source. The consumption of basic ammonium for growth would also lead to decreasing pH. The pH profile was followed in each system (Fig. 3). In the systems of HSS, Nola-Tex, Nola-Sponge, and Media-Sponge, pH all dropped similarly, indicating cell growth. (pH in all of these systems increased slightly after the lactose was depleted and the culture entered into the stationary phase. The pH increase was a result of the ammonium release due to endogenous metabolism.) On the other hand, pH in the Sensa-Temp system increased slightly in the beginning and did not drop at all, which implied no or very low cell activities. Presumably, there were some inhibitory or toxic effects associated with this foam material even after the pretreatment.

The profiles of sugar consumption are shown in Fig. 2. Most notably, the lactose was essentially not consumed in the Sensa-Temp system, clearly confirming the inhibitory/toxic effect of this foam material to *T. reesei*. The sugar profiles also suggested that the cells might need longer time to adapt to the new environments containing PU foams. The sugar concentrations almost did not change in the first day while the sugar concentration would

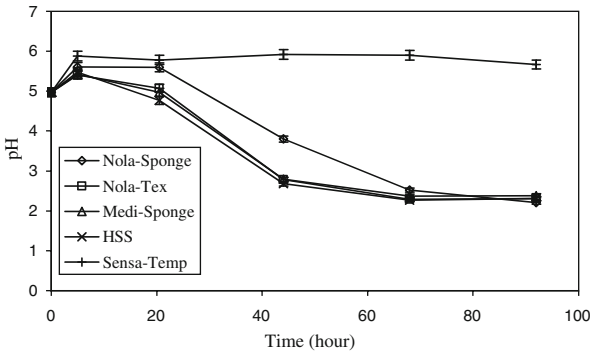

Fig. 3 pH change profiles observed in the initial PU foam screening study

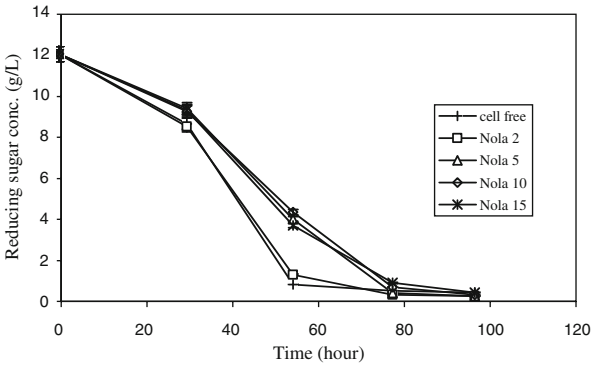

Fig. 4 Reducing sugar consumption profiles observed in systems with different concentrations of Nola-Tex foam

typically decrease more in the free cell systems without PU foams (as shown in Figs. 4 and 5 for the later cell loading capacity study). The cells adsorbed onto the foam surface might require longer adaptation for the new environment. In addition, the adsorption lowered the free cell concentrations, which might have caused a longer lag phase for free-cell growth, similar to the prolonged lag phase commonly observed in the cultures with low inoculum sizes. Nonetheless, certain cell growth did occur during the first day (although no sugar consumption was apparent), evident from the increased free cell concentrations during this period in the Nola-Sponge and Media-Sponge systems (Fig. 1). This initial growth was supported by the consumption of the peptone in the medium. Peptone is an organic carbon and nitrogen source. Consumption of peptone as carbon source typically led to pH increase, as seen in Fig. 3.

The profiles of cellulase (FPU) production observed in these foam systems are shown in Fig. 6. In the HSS system, FPU increased at the highest rate and reached the highest maximum level among all systems. FPU in this system, however, decreased to lower levels subsequently, perhaps caused by adsorption on the foam surface or by enzyme deactivation. The cellulase production profiles were similar in the Nola-Tex and Media-Sponge systems. Poorer cellulase production was obtained in the Nola-Sponge system. As expected, negligible cellulase production occurred in the inhibitory/toxic Sensa-Temp system.

Loading Capacity Study

Because of their ability to give fewer free cells and higher FPU production, HSS and Nola-Tex were further evaluated for the cell loading capacity and the effects on the foaming

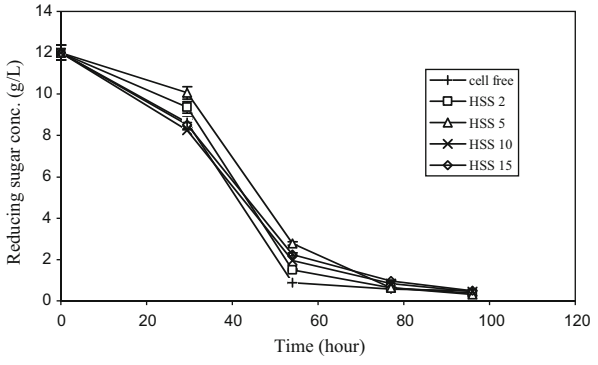

Fig. 5 Reducing sugar consumption profiles observed in systems with different concentrations of HSS foam

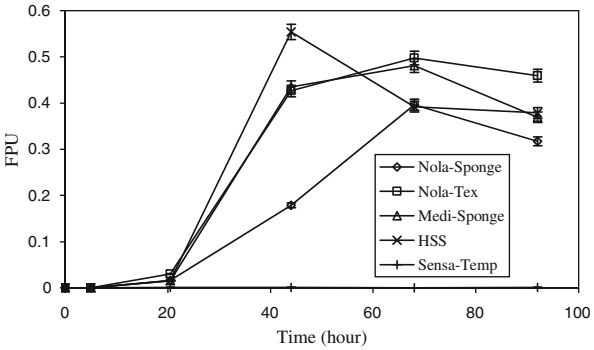

Fig. 6 Cellulase (FPU) production profiles observed in the initial PU foam screening study

process. The cell loading study was done at the same conditions as the previous screening for cell immobilization. One major difference was in the amounts of PU foams used: 20 g/L were used in the previous screening study, while four different amounts, i.e., 2, 5, 10, and 15 g/L, were used in this study.

The cell loading capacity (g cells/g foams) was calculated by dividing the foam-immobilized cell concentration X_{im} (g/L) by the concentration of foams (g/L) employed, where X_{im} was calculated by the equation given in "Analytical methods". Conceptually, cell immobilization might be influenced by the following factors: mixing rate, which affects the collision frequency between the foam pieces and the shear stress experienced by the cells immobilized on the surface of the foams; the ratio of total cell concentration to foam concentration; and the porosity and pore size of the foam.

The free cell concentrations were measured along the fermentation. The results are shown in Figs. 7 and 8 for the Nola-Tex and HSS systems, respectively, each at four different foam contents. It is clear that the maximum free cell concentrations reached were lower in the systems of higher foam contents. For either foam material, the free cell concentrations in the systems with 10 and 15 g/L of foam were never higher than the inoculated concentrations and were about the same. The calculated maximum cell loading reached in the systems of different foam contents are summarized in Table 1. The consistent trend revealed was that the maximum cell loading was approximately the same, at about 0.6 g cells/g foams, in the systems with 2 to 10 g/L of foams, while the loading decreased to about 0.4 g cells/g foams in the systems with 15 g/L of foams. This observation can be well explained as follows.

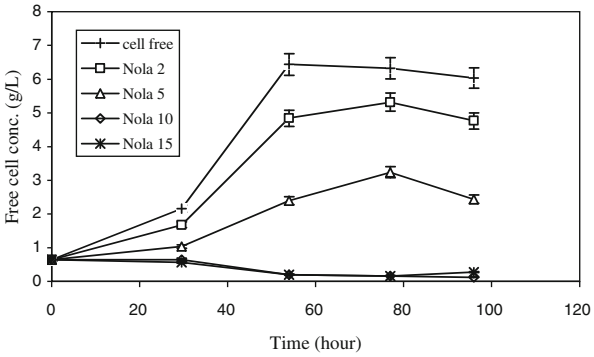

Fig. 7 Free cell concentration profiles observed in the cell loading study using four concentrations of Nola-Tex foam

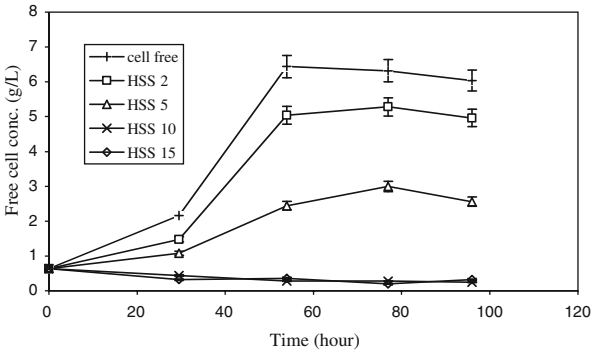

Fig. 8 Free cell concentration profiles observed in the cell loading study using four concentrations of HSS foam

In the systems with up to 10 g/L of foams, the cell-to-foam ratios were high, and the cells could occupy all the surface and/or space of the foam matrixes available for cell attachment and growth, resulting in the constant cell loading capacity of about 0.6 g cells/g foams. In the systems with 15 g/L of foams, the cell-to-foam ratios were not high enough to fill all the available surface and/or space with the immobilized cells. It should be noted that the available space for cell immobilization was not necessarily the whole foam volume. Mass transfer limitation might have caused the cells to occupy only the outer foam matrixes of certain unknown depth. The cell loading capacity is expected to change with the size and shape of the foam pieces. The depth of actual cell immobilization on/in the foam matrixes can be determined in future studies with varying sizes of the foam pieces.

The cellulase production profiles in all of the studied systems are shown in Figs. 9 and 10. The maximum FPU reached were similar in all of the foam-containing systems. Furthermore, the cellulase activity in the systems with higher foam contents (10 and 15 g/L) appeared to remain stable longer after reaching the maximum (i.e., with slower decreasing trends). This observation was consistent with the reported protective effects associated with cell and/or enzyme immobilization. By comparing the results obtained in the immobilized cell systems with those in the free cell system, it was easily seen that the cells produced more cellulase when immobilized in the hydrophilic PU foams of HSS and Nola-Tex. The maximum specific cellulase productivity was 70 (FPU/g cells) in the cell free system while that in the Nola-Tex and HSS systems was 120 and 110 (FPU/g cells), respectively. The increase of the specific cellulase production was 71% in the Nola-Tex systems and 57% in the HSS systems. The possible explanations include the shear protection offered by the foams, the changed cell morphology and local microenvironment due to the immobilization, and, less likely, the stimulated cellulase synthesis caused by some surface functional groups of the PU foams or by some compounds released from the PU foams. Note that the maximum cellulase activities in Fig. 6 for Nola-Tex and HSS at 20 g/L were lower than

Table 1 Maximum cell loading obtained in the *T. reesei* Rut C30 fermentation with different concentrations of Nola-Tex and HSS foams.

PU Foam	2 g/L	5 g/L	10 g/L	15 g/L	20 g/L*
Nola-Tex					
Maximum cell loading (g/g)	0.60±0.08	0.62±0.06	0.59±0.03	0.39±0.04	0.24±0.02
HSS					
Maximum cell loading (g/g)	0.59±0.05	0.63±0.06	0.58±0.04	0.38±0.03	0.23±0.03

*Results from the PU foam screening study

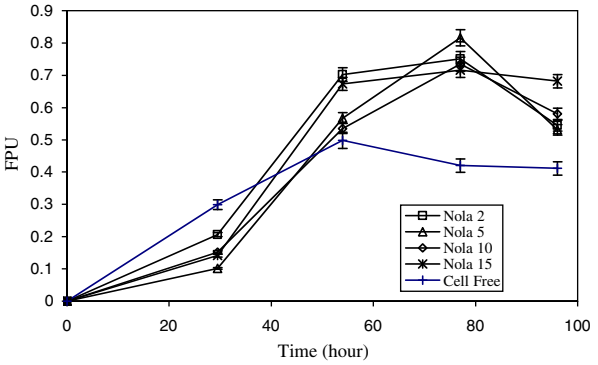

Fig. 9 Cellulase production profiles observed in systems with different concentrations of Nola-Tex foam

those at 2, 5, 10, and 15 g/L in Figs. 9 and 10. The possible reason was the different sizes of the PU foams used in the two studies. Larger PU foams were used in the screening test, and smaller ones were used for the loading capacity study. The larger PU foams were subjected to more serious mass transfer limitation.

The lactose (reducing sugar) consumption profiles are shown in Figs. 4 and 5 for Nola-Tex and HSS systems, respectively. The sugar consumption slowed down with increasing foam contents, presumably due to the mass transfer limitation associated with cell immobilization. The limitation appeared to be stronger in the Nola-Tex systems (except at the lowest foam content of 2 g/L) than in the HSS systems.

Retention of Immobilized Cells in Foaming Operation

The behaviors of Nola-Tex and HSS PU foam pieces with and without immobilized cells were observed in simple foaming experiments. The supernatant/broth harvested from the *T. reesei* fermentation with the lactose-based medium was used as the foaming solution. The cell-free PU foam pieces were soaked in the broth supernatant before the foaming experiment. The volume of the foaming solution used in this study was 70 mL, and the aeration rate was 0.5 L/min, which was about 7 vvm, much higher than the aeration employed in common fermentation processes. The intention was to investigate whether the foams could be retained at even this high aeration rate.

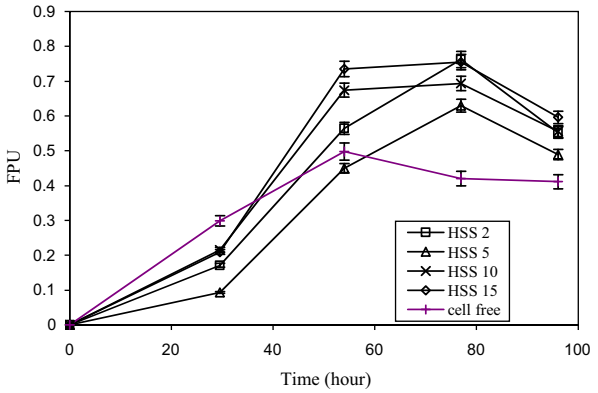

Fig. 10 Cellulase production profiles observed in systems with different concentrations of HSS foam

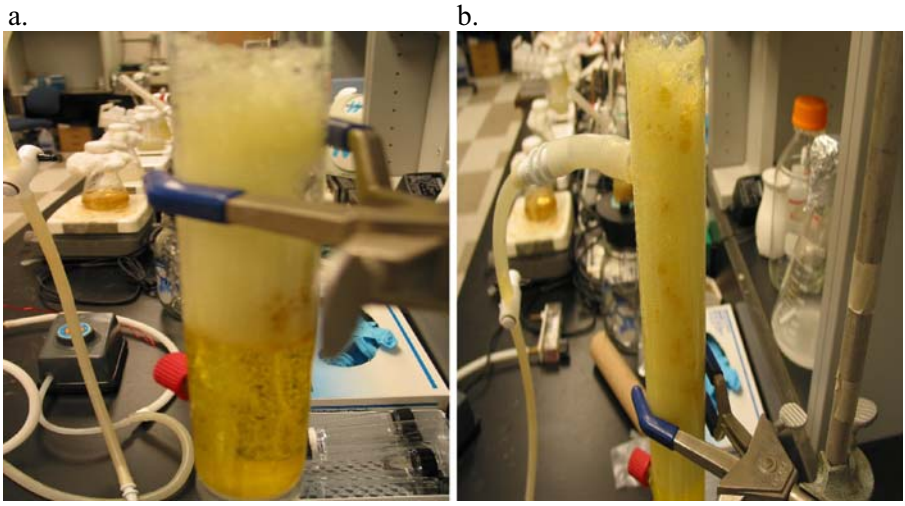

Fig. 11 Foaming behavior studies of the **a** HSS and **b** Nola-Tex PU pieces without being subjected to the cell immobilization fermentation

The cut foam pieces behaved very differently before and after being subjected to the immobilized-cell fermentation. As shown in Fig. 11, up to 25% of the cell-free PU pieces were foamed out, and after the aeration was stopped, the remaining pieces were either trapped in the upper foam layer or floating at the interface of liquid and foam layer. On the other hand, no cell-immobilized PU pieces were carried out by foaming, and all of the PU pieces settled rapidly to the bottom of the foam column after the aeration was turned off, as shown in Figs. 12 and 13. Closer observations of the PU pieces after the foaming study

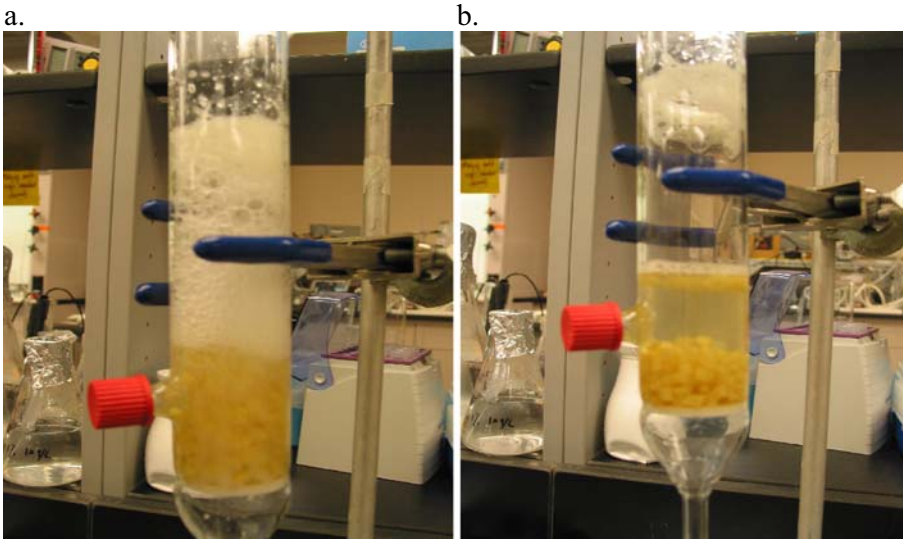

Fig. 12 Foaming study of the broth collected from the fermentation with cell-immobilized Nola-Tex foam: **a** during foaming; **b** after foaming

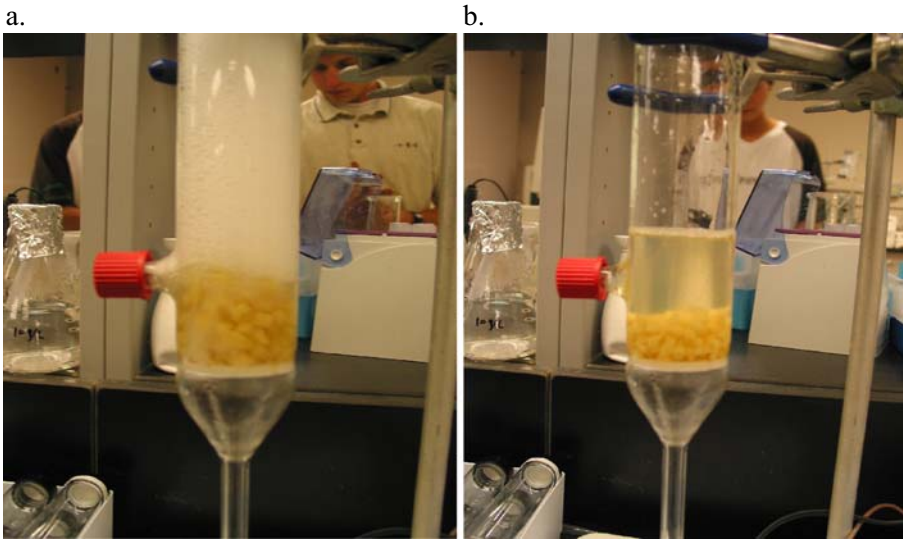

Fig. 13 Foaming study of the broth collected from the fermentation with cell-immobilized HSS foam: **a** during foaming; **b** after foaming

indicated that the cells grown inside the foam pieces occupied the pores and prevented the liquid in the matrixes from being replaced by air during the foaming, which occurred with the cell-free PU pieces. The higher density of the cell (and liquid)-filled pieces kept them from being foamed out and caused them to settle in the broth.

The results successfully demonstrated the feasibility of using small hydrophilic PU foams for immobilization of *T. reesei* cells and for retaining the cells in the fermentor if a foaming process would be introduced to continuously collect the produced cellulase from the broth. A future study to develop the coupled fermentation and in situ foam fractionation process is warranted.

Conclusion

Hydrophilic PU foams, particularly the HSS and Nola-Tex investigated in this study, could be used to immobilize *T. reesei* Rut C-30 for cellulase production. The cells rapidly adsorbed onto the PU foam surface and, presumably, grew into the porous matrixes. The cell loading capacity of the PU foams, when cut into cubes of 3 × 3 × 3 mm, was around 0.63 for HSS and 0.62 for Nola-Tex under the culture condition used. The immobilized cell systems with HSS and Nola-Tex foams gave clearly higher cellulase production than the free cell control. The specific cellulase productivity increased by about 71% in the Nola-Tex systems and 57% in the HSS systems. The behaviors of the PU foams with immobilized cells showed that this immobilization method could be an effective way to prevent cell loss during the foaming process for in situ cellulase collection.

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