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Semicontinuous Production of Lipase by *Acinetobacter radioresistens* in Presence of Nonwoven Fabric

SHENG-CHOU LI,[1] JAU-YANN WU,[2] CHUH-YUNG CHEN,[1] AND TEH-LIANG CHEN*,[1]

[1]*Department of Chemical Engineering, National Cheng Kung University, Tainan 70101, Taiwan, E-mail: t62660@mail.ncku.edu.tw; and* [2]*Department of Chemical Engineering, I-Shou University, Ta-Hsu Hsiang, Kaohsiung County 84008, Taiwan*

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Abstract

The effect of a hydrophobic nonwoven fabric on the lipase production by *Acinetobacter radioresistens* was investigated with semicontinuous culture. The fermentation medium contained *n*-hexadecane as the carbon source. The nonwoven fabric was made from nylon 6 and coated with an acrylic resin. Equipping the nonwoven fabric around the baffles of a 2.5-L agitated fermentor could provide a fine dispersion of *n*-hexadecane, thus enhancing lipase production. The improvement on lipase yield by using the nonwoven fabric was found to be comparable to that of using an emulsifier (gum arabic). Compared with the corresponding culture in the absence of nonwoven fabric, the employment of the nonwoven fabric could significantly enhance both lipase yield and volumetric productivity.

Index Entries: Lipase production; *Acinetobacter radioresistens*; semicontinuous culture; nonwoven fabric; dispersion of oil.

Introduction

In a previous study *(1)*, the semicontinuous mode of fermentation (or batch fill-and-draw culture) was shown to be effective for the production of lipase from *Acinetobacter radioresistens* using *n*-hexadecane as the carbon source. Compared with batch fermentation, the improvement in lipase production has been attributed to two factors. First, the semicontinuous culture provides an environment for continuous cell growth,

*Author to whom all correspondence and reprint requests should be addressed.

thus depressing the formation of protease; protease has been shown to be responsible for the decrease in lipase yield in the stationary phase of growth *(2)*. Second, the cells become adaptable to growing on *n*-hexadecane during the repeated culture, which results in an increase in lipase yield. However, in addition to the formation of protease and the lag for the cells to adapt themselves to the medium, lipase productivity is also limited by the contact surface of *n*-hexadecane (substrate availability). Although using emulsifiers is a common approach to improve substrate availability *(3)*, it may not be ideal because of the induction of complexity in downstream processing.

The aim of the present study was to investigate the effect of a nonwoven fabric on the production of *A. radioresistens* lipase by semicontinuous culture. Nonwoven fabrics have been applied for immobilization of microbial cells *(4–6)*, but, in this study, the role of the nonwoven fabric was designated to disperse *n*-hexadecane. The nonwoven fabric used was hydrophobic and had a strong tendency to adsorb hydrocarbons. In a stirred-tank fermentor, hydrocarbons are dispersed through agitation. If the same fermentor is equipped with a hydrophobic nonwoven fabric, hydrocarbons will first be adsorbed on the fabric and then be detached by the shear force to form tiny droplets; the substrate availability could thus be enhanced *(7)*.

Materials and Methods

The strain used was *A. radioresistens* CMC-1, which was a Gram-negative bacterium isolated from the sludge of wastewater *(8)*. The nonwoven fabric used was made of nylon 6 fiber (15 denier), coated with a hydrophobic acrylic resin, and had a unit weight of 110 g/m^2 (Fehrer Enterprise, Taiwan).

Lipase production was carried out in a 2.5-L laboratory tank fermentor (Model M-100; Tokyo Rikakikai, Japan) with a working volume of 1.2 L. The fermentor had three baffles. The fermentation medium contained: 10 g/L of tryptone (Difco), 5 g/L of yeast extract (Difco), 10 g/L of NaCl, 1 g/L of NH_4Cl, 1 mL/L of olive oil, and 20 mL/L of *n*-hexadecane. The pH of the medium was adjusted to 7.0 using 1 *N* NaOH. When using gum arabic to emulsify *n*-hexadecane, the emulsion was prepared outside the fermentor and sterilized separately. The cells were cultivated at 30°C with an inoculum size of 100 mL. The inoculum was prepared in a rotary shaker operating at 120 rpm and 30°C for 10 h, using the fermentation medium excluding olive oil and *n*-hexadecane. For tank fermentations, the agitation speed and aeration rate used were 400 rpm and 1 vvm, respectively. The pH was maintained at 7.0 by the use of 1 *N* NaOH/1 *N* HCl, and the antifoam agent used was KM-72 (Shin-Etsu, Japan).

Cell concentration was measured by turbidimetry (600 nm) and correlated with dry cell weight. To avoid the interference of the remaining *n*-hexadecane on the turbidity measurement, samples were centrifuged twice in a Taitec CR-12 centrifuge at 5000*g* for 5 min, and the separated cells were resuspended in deionized water. Lipase activity was determined by the pH-stat method using olive oil as the substrate *(1)*. One lipase unit was

defined as the amount of enzyme required for liberating 1 μmol of fatty acid/min at 37°C and pH 10.0. Lipase productivity of the semicontinuous culture was defined as the total activity obtained in one replacement divided by the total working volume and the time interval. Protease activity was measured by proteolysis of azocasein (Sigma), and the procedure of assay was according to Leighton et al. *(9)*, except the definition of enzyme activity; one unit of protease was defined as the amount of enzyme required for increasing 0.01 of absorbance at 440 nm/min.

Results and Discussion

To obtain the basic information for the production of *A. radioresistens* lipase, batch fermentation was first performed as shown in Fig. 1. The maximum lipase activity (24 U/mL) occurred at the beginning of the stationary phase of growth (15 h), and the formation of lipase mainly followed a growth-associated pattern. Lipase was produced most rapidly at the middle of the exponential phase. Protease activity was detected since the end of the exponential phase, which was considered to be responsible for the decrease in lipase yield. According to the growth curve in Fig. 1, exchanging one third (400 mL) of the medium seems to be adequate for operating the semicontinuous culture. This exchange led to a dilution of cell density to two thirds of the original and could be visualized as resetting the system back to the 6-h status, where the high lipase production rate occurred.

Based on the knowledge we obtained from Fig. 1, we then used a free-cell, semicontinuous culture of *A. radioresistens*, shown in Fig. 2, in which the fill-and-draw operation started at 15 h and the interval of reset was 8 h. Steady values of cell concentration and lipase yield were obtained since the second reset. The beneficial effects of semicontinuous culture shown were that protease formation was suppressed and lipase yield was increased to 30 U/mL. Lipase productivity thus obtained was 1250 U/(L·h). Experiments with various reset intervals were also examined, and their results are given in Table 1. By reducing the reset interval to 4 h, cell concentration and lipase yield decreased gradually until a new equilibrium (after four resets) was established. Four hours is apparently not sufficient for the cells to return from the 6- to the 15-h status. However, although lipase yield decreased to 21 U/mL, lipase productivity increased to 1750 U/(L·h). The increase in lipase productivity is attributed to the system being operated in the interval of higher lipase production rate. By increasing the reset interval to 12 h, both cell concentration and lipase yield increased gradually to new higher levels. The significant increase in lipase yield with the length of reset interval indicates that the time intervals used were not sufficient for the cells to complete the utilization of *n*-hexadecane. The insufficiency could be owing to an inadequate supply of the substrate. Although lipase yield increased with increasing reset interval, the volumetric productivity of lipase decreased (*see* Table 1); the increase in the yield was at the expense of the productivity. Therefore, to improve the production of *A. radioresistens*

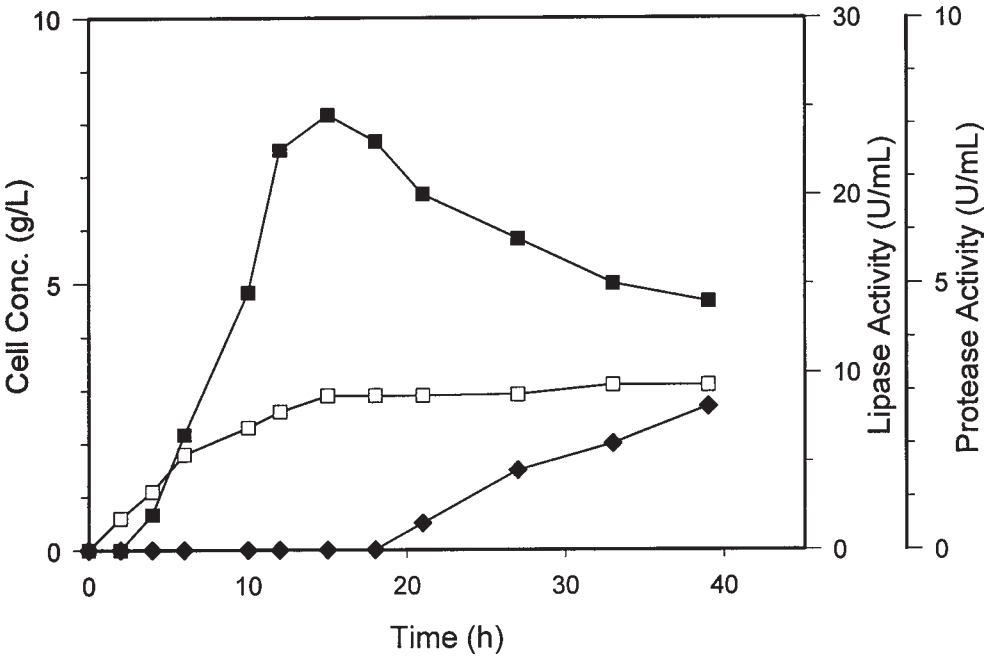

Fig. 1. Batch culture of *A. radioresistens*. □, cell concentration; ■, lipase activity; ◆, protease activity.

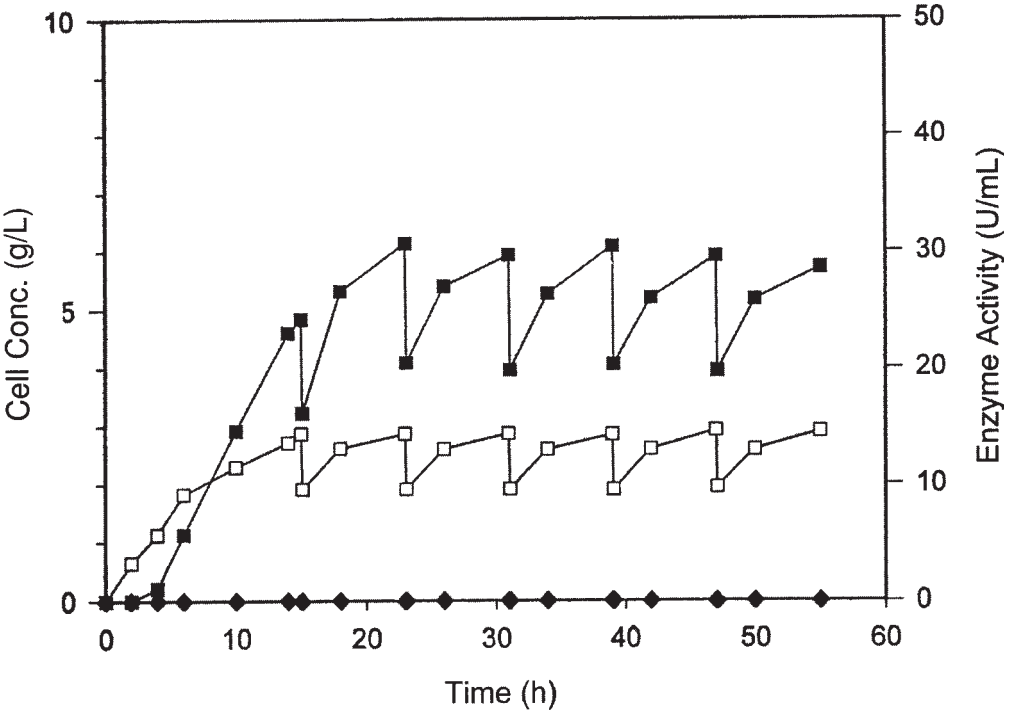

Fig. 2. Semicontinuous culture of *A. radioresistens*: free-cell, no emulsifier, 400 mL exchanged per 8 h. □, cell concentration; ■, lipase activity; ◆, protease activity.

Table 1
Production of *A. radioresistens* Lipase by Semicontinuous Culture[a]

Reset interval	Yield (U/mL)	Productivity (U/[L·h])
Free-cell, no emulsifier		
8 h	30	1250
12 h	43	1190
4 h	21	1750
Free-cell, with gum arabic		
8 h	50	2080
12 h	50	1390
NWF-1		
8 h	25	1040
NWF-2		
8 h	50	2080
12 h	52	1440
4 h	48	4000

[a]The fill-and-draw operation started at 15 h and the volume of exchange was 400 mL.

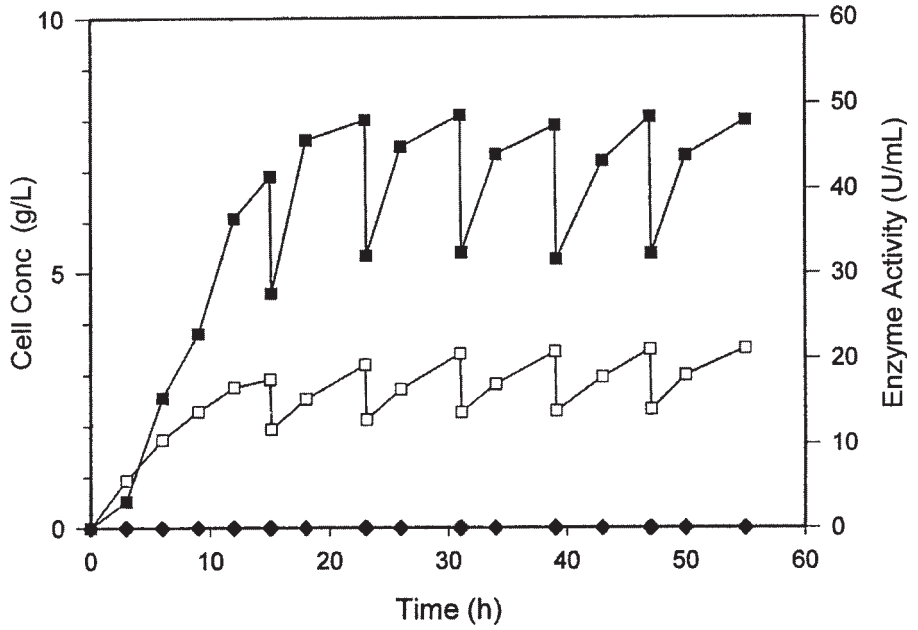

Fig. 3. Semicontinuous culture of *A. radioresistens*: free-cell, gum arabic as emulsifier, 400 mL exchanged per 8 h. □, cell concentration; ■, lipase activity; ◆, protease activity.

lipase by semicontinuous culture, increasing lipase yield and the productivity simultaneously is a dual goal to achieve.

Because lipase production by *A. radioresistens* is related to the rate of consumption of *n*-hexadecane, using *n*-hexadecane in an emulsified form would be beneficial owing to the improvement in substrate availability. Figure 3 and Table 1 indicate the performance of the free-cell, semi-

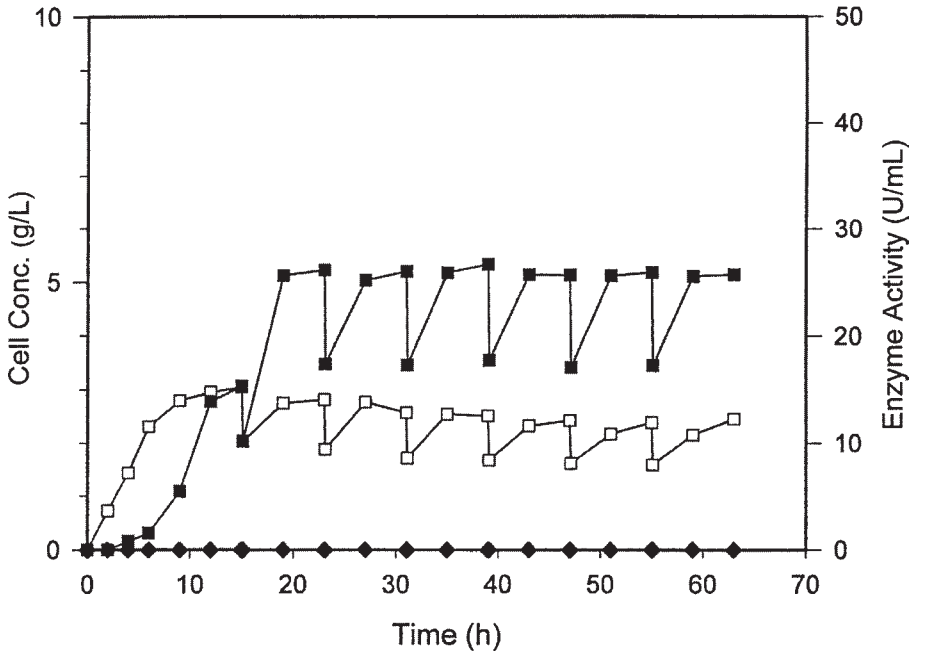

Fig. 4. Semicontinuous culture of *A. radioresistens*: NWF-1, no emulsifier, 400 mL exchanged per 8 h. □, cell concentration; ■, lipase activity; ◆, protease activity.

continuous production of *A. radioresistens* lipase, in which 1% (w/v) of gum arabic was used as the emulsifier. For consistency of comparison, the fill-and-draw operation also started at 15 h and the amount of medium exchanged was 400 mL. It was found that lipase yield reached 41 U/mL at 15 h, a reset interval of 8 h resulted in a lipase yield of 50 U/mL, and a further increase of the reset interval to 12 h did not increase the lipase yield. In both experiments, steady values of cell concentration and lipase yield were obtained since the second reset. Although the improvement in lipase yield is qualitatively anticipated, these two experiments reveal an interesting result: lipase yield could reach a level of 50 U/mL if the cells could efficiently utilize *n*-hexadecane. In other words, a lipase yield of about 50 U/mL could be taken as an indication of good substrate availability.

Although using emulsifiers can enhance lipase production, it also complicates the downstream processing, which is why we propose the use of a hydrophobic nonwoven fabric. It was found that *n*-hexadecane has a strong tendency to be adsorbed by the present nonwoven fabric owing to hydrophobic interaction, the adsorption capacity determined in the shake flask being 9 mL of *n*-hexadecane/g of nonwoven fabric. To equip the nonwoven fabric in the fermentor, a 9 × 54 cm sheet of the fabric was fixed at the three baffles of the fermentor, forming a circular curtain between the baffles and the tank wall. The manner of equipping the nonwoven fabric is referred to hereafter as NWF-1. Figure 4 shows semicontinuous production of *A. radioresistens* lipase in the NWF-1 fermentor. The fill-and-draw opera-

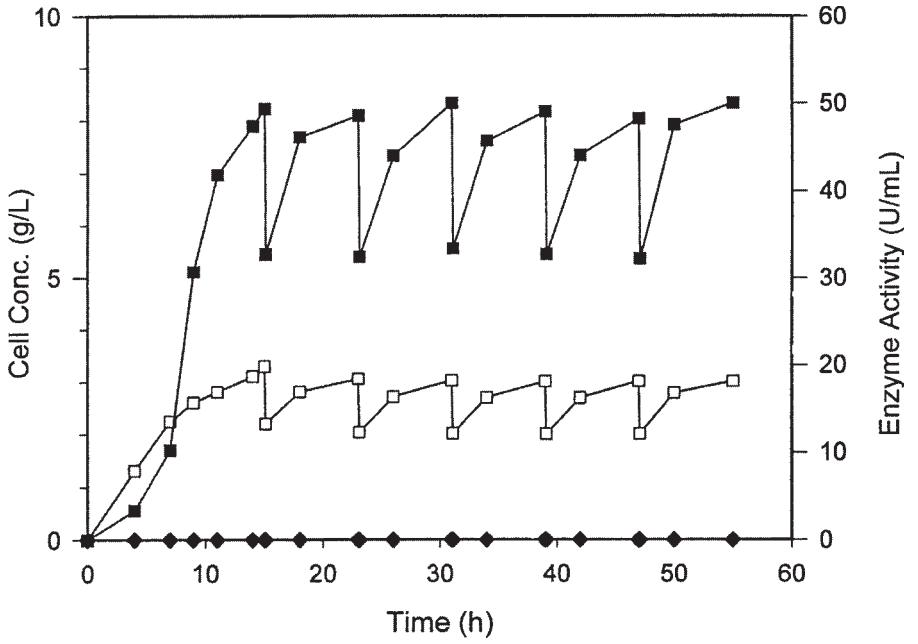

Fig. 5. Semicontinuous culture of *A. radioresistens*: NWF-2, no emulsifier, 400 mL exchanged per 8 h. □, cell concentration; ■, lipase activity; ◆, protease activity.

tion also started at 15 h and the exchange of medium was 400 mL/8 h. The lipase yield at 15 h was 15 U/mL and it reached 25 U/mL after the first reset; the presence of the nonwoven fabric was not beneficial for the lipase production. During the fermentation, we observed that the space between the curtain of the nonwoven fabric and the tank wall was poorly agitated, a large portion of the oil phase floated on the surface of this region. The failure of the nonwoven fabric to enhance lipase production was therefore attributed to the manner of equipping the cloth.

Accordingly, a second manner of equipping the nonwoven fabric (referred to hereafter as NWF-2), was tried. Three sheets of the nonwoven fabric (each one 9 × 13 cm) were tied separately around the three baffles of the fermentor. Good homogeneity was observed in the NWF-2 fermentor. We used a reset interval of 8 h to replace 400 mL of the medium for the semicontinuous production of *A. radioresistens* lipase (*see* Fig. 5). The lipase yield at 15 h was 50 U/mL, and the fill-and-draw operation could maintain the lipase yield at this level. As already mentioned, a lipase yield of 50 U/mL is an indication of good substrate availability; the role of the nonwoven fabric to disperse *n*-hexadecane can thus be suggested. Direct proof of fine dispersion of *n*-hexadecane in the presence of the nonwoven fabric was obtained by microscope. Most of the oil droplets had a diameter of 4 μm in NWF-2, whereas they were about 100 μm in the absence of the cloth and 20 μm when using 1% gum arabic to emulsify *n*-hexadecane. By increasing the reset interval to 12 h, only a slightly higher lipase yield

(52 U/mL) was obtained, indicating again that NWF-2 provides good substrate availability. In addition to high lipase yield, high volumetric productivity represents another requirement for a successful fermentation process. Therefore, the reset interval was reduced to 4 h, and the lipase yield obtained was 48 U/mL, which is equivalent to a volumetric productivity of 4000 U/(L·h). Compared with the values obtained in the corresponding free-cell culture (21 U/mL and 1750 U/[L·h] for lipase yield and productivity, respectively), the improvement in lipase production by using the nonwoven fabric was quite marked.

Conclusion

We have demonstrated that the production of *A. radioresistens* lipase by semicontinuous culture could be enhanced by the employment of a hydrophobic nonwoven fabric; more than twofold lipase yield and/or volumetric productivity could be achieved. Instead of serving as the support for cell immobilization, the nonwoven fabric plays a role in dispersing *n*-hexadecane under the shear force exerted by the agitator. The effect of the nonwoven fabric to disperse *n*-hexadecane was found to be comparable to that of gum arabic. The present approach could be applied to other fermentation processes involving hydrocarbon-degrading microorganisms.

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