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Lipase Production by *Acinetobacter radioresistens* in a Batch Fill-and-Draw Culture

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ABSTRACT

Alkaline lipase production by *Acinetobacter radioresistens* was performed in a batch fill-and-draw culture, and the results were compared with a batch culture. In the batch culture, the lipase yield was 18 U/mL, which was restricted by the occurrence of protease forming and excessive foaming as cell growth ceased. Because the formation of lipase was found to be chiefly growth-associated, the fill-and-draw culture, which provided an environment for continuous growth, could surpass the limitation encountered in a batch culture and increase the lipase yield to 30 U/mL. The improvement in the lipase yield was suggested to be caused by an adaptation of the cells to the medium during the repeated culture. Although the increase in the lipase yield was accompanied by a decrease in lipase productivity, the fill-and-draw culture could be a better mode for lipase production.

Index Entries: Lipase fermentation; *Acinetobacter radioresistens;* batch culture; fed-batch culture; fill-and-draw; protease activity.

INTRODUCTION

Lipase (EC 3.1.1.3) are important industrial enzymes; their applications include food, chemical, pharmaceutical, and detergent industries. Many reports on the production of microbial lipase by batch fermentation indicate that, as the fermentation proceeded, lipase activity increased to a maximum, and then fell off. A few examples of microbial sources showing

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this feature have been *Acinetobacter lwoffi (1), Aspergillus wentii (2), Bacillus subtilis (3),* and *Acinetobacter calcoaceticus (4).* The decrease in lipase yield has been attributed to a proteolytic degradation of the enzyme, as in the cases of *Aspergillus flavus (5),* and *A. calcoaceticus (6,7).* In addition to causing a decrease in lipase yield, the presence of protease also complicates downstream processing.

As summarized from previous reports, lipase is mostly a growth-associated product *(1,3–7),* and protease formation began at the end of the exponential phase *(6,7).* Accordingly, it is expected that the problems resulting from the formation of protease can be avoided, if the cultivation is controlled, so that the cells continue growth. Continuous cell growth can be achieved in a tank fermentor operating in continuous or fed-batch mode. The principal defect of continuous tank fermentation is a low efficiency of subtrate utilization, which has resulted inherently from the residence time distribution of the substrate. In contrast to having a limited industrial application with continuous culture, fed-batch culture has been widely used since antiquity.

This study, therefore, was intended to investigate the use of fed-batch culture for alkaline lipase production by *Acinetobacter radioresistens.* The operation of the fed-batch culture was in a fill-and-draw mode (sometimes referred to as semicontinuous culture), in order to keep a constant liquid volume, and thus to simplify the analysis. In addition, fill-and-draw has been reported to facilitate the determination of kinetic parameters, because various steady-state conditions can be achieved *(8).* The yield and productivity of lipase were compared between batch and the fill-and-draw cultures.

MATERIALS AND METHODS

A. radioresistens was originally isolated from sludge of waste water, and was provided by Ming-Cheung Chang, Department of Biochemistry, National Cheng Kung University, Taiwan. Preliminary experiments showed that the lipase produced was extracellular, and had an optimal temperature of 37°C and an optimal pH of 10.0. However, the optimal temperature and pH for lipase production were 30°C and pH 7.0, respectively.

This strain was maintained at 4°C on Luria-Bertani (LB) slopes containing (per liter) 10 g tryptone (Difco, Detroit, MI), 5 g yeast extract (Difco), 10 g NaCl, and 15 g agar. Prior to the preparation of precultures, the cells were transferred to a LB agar plate and incubated at 30°C for 10 h. Precultures were prepared by inoculating one loopful of cells from an agar plate in a 500-mL Erlenmeyer flask containing 120 mL of LB broth, and were incubated for 12 h in a rotary shaker operating at 120 rpm and 30°C. Tank fermentations were carried out in a 2.5-L fermentor (M-100,

Tokyo Rikakikai, Japan), with a working volume of 1.2 L. The medium contained (per liter): 10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 g NH_4Cl, 20 mL *n*-hexadecane, 1 mL olive oil (Sigma, St. Louis, MO), and 1 g gum arabic (Sigma). The addition of NH_4Cl was to repress protease activity *(6)*. The use of *n*-hexadecane as the carbon (C) source for lipase production was according to the work of Kok et al. *(9)* for *A. calcoaceticus*, and was found to greatly improve the productivity of the present strain. Olive oil was designated as an inducer for lipase production, as well as a supplemental C source for cell growth. The cells were cultivated at 30°C with a 10% (v/v) inoculum. The pH was maintained at 7.0 by the use of 1 *N* NaOH/1 *N* HCl. The agitation speed was 400 rpm, and aeration rate was 1 vvm.

Cell density was determined by turbidity at 600 nm in a spectrophotometer (UV-1201, Shimadzu, Tokyo, Japan), and was correlated with dry cell w. Lipase activity was determined by the pH-stat method, using a Mettler-Toledo DL25 titrator (Hightstown, NJ). The substrate was prepared by stirring 20 mL olive oil and 10 g gum arabic in 200 mL deionized water for more than 2 h, to ensure a homogeneous emulsion. For the enzyme assay, 1 mL of fermentation broth was added, with stirring, to 10 mL of the substrate solution. The titration was achieved by using 0.02–0.1 N NaOH. The concentration of NaOH was adjusted according to the lipase activity in the samples. One lipase unit was defined as the amount of enzyme required to liberate 1 μmol/min fatty acid at 37°C and pH 10.0. Protease activity was measured by proteolysis of azocasein (Sigma), according to Leighton et al. *(10)*. One unit of protease was defined as the amount of enzyme required for an increase of 0.01 in absorbance at 440 nm/min.

RESULTS AND DISCUSSION

A batch fermentation with *A. radioresistens* is shown in Fig. 1. Cell growth reached the stationary phase at 16 h; however, a nonobvious secondary growth was observed during prolonged cultivation. Because the biphasic growth was not observed if *n*-hexadecane was excluded from the medium (data not shown), the slight increase in cell density during prolonged cultivation could be caused by a later growth on *n*-hexadecane. Obviously, this growth required an adaptation time. The maximum lipase activity (17 U/mL) was obtained at the beginning of the stationary phase (16 h), and the formation of lipase mostly followed a growth-associated pattern. Protease activity was detected since the end of the exponential phase, and was considered to be responsible for the decrease of lipase activity *(6,7)*. However, the sharp decrease of lipase activity might also be the result of excessive foaming observed at the early stationary phase; the occurrence of excessive foaming could have resulted in the adsorption of

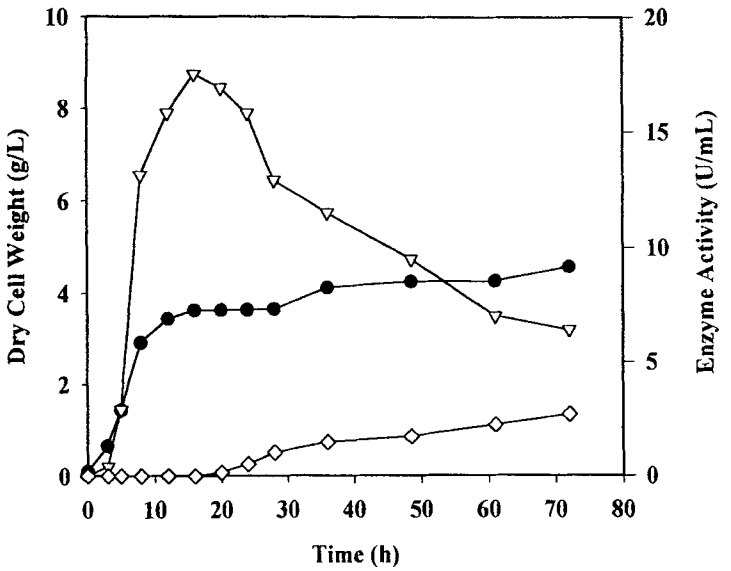

Fig. 1. Batch culture of *A. radioresistens*. ●, dry cell wt; ∇, lipase activity; ◇, protease activity.

a significant amount of lipase on the air–water interface, and thus biased the sampling. In a word, the production of *A. radioresistens* lipase encountered one problem: Protease formation and excessive foaming occurred during the interruption of cell growth, which led to a restriction on lipase yield. Because the lipase production follows the growth-associated pattern, this problem could be overcome if the cultivation is controlled, so that cell growth will not be interrupted.

In the operation of the fill-and-draw culture, the amount of medium and the time interval of the exchange are two critical factors. According to Fig. 1, lipase was produced most rapidly at the middle of the exponential phase. With consideration of maintaining an adequate cell density, therefore, the authors' first test involved exchanging one-third of the medium (400 mL). The exchange is equivalent to letting the culture go back to the 7-h status. As to the interval of exchange, 4 h seems a compromise between recovery of cell density and rate of lipase production. The result of the fill-and-draw operation, starting at 16 h, is shown in Fig. 2. As one can see, cell density was not fully recovered; it decreased to a somewhat lower level (from 3.5 to 2.8 g/L). This is not surprising, if one considers that 4 h is obviously not sufficient for the culture to go back to the 16-h status. The lipase yield also decreased to a lower level (from 17 to 13 U/mL) as a result of the overdilution. Formation of protease and occurrence of excessive foaming, as expected, were not observed throughout the fermentation, because the culture never reached the stationary phase. In addition to the

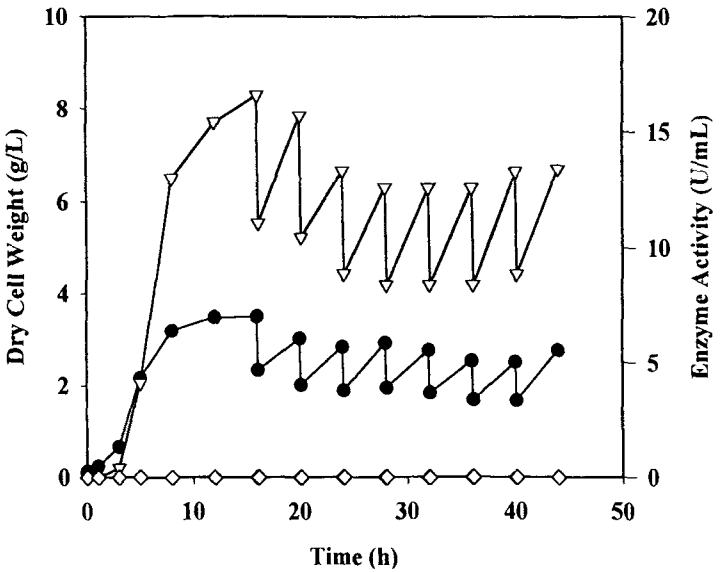

Fig. 2. Fill-and-draw culture of *A. radioresistens* with an exchange of 400 mL medium in a period of 4 h. ●, dry cell wt; ∇, lipase activity; ◇, protease activity.

yield, the volumetric productivity should also be examined to evaluate a fermentation process. The lipase productivity of batch culture, defined as the slope of the straight line connecting the origin and the lipase activity at 16 h, was 1060 U/L/h. On the other hand, the lipase productivity of the fill-and-draw culture, defined as the total activity obtained in one replacement divided by the total working volume and the time interval, was 1080 U/L/h, which was roughly the same as that of its batch part.

The lipase yield, however, can be improved by decreasing the rate of displacement, that is, by decreasing the amount of medium exchanged, or increasing the time interval. The result of the fill-and-draw culture, with an exchange of 200 mL medium in an interval of 4 h, is shown in Fig. 3. Under this exchange rate, cell density and lipase activity could be recovered. In the fill-and-draw period, the lipase yield and productivity were 18 U/mL and 750 U/L/h, respectively. Formation of protease and occurrence of excessive foaming were also not observed throughout the processs. Compared with the result obtained from an exchange rate of 400 mL/4 h (Fig. 2), it is apparent that the increase in lipase yield was at the expense of productivity. This rationale was further confirmed in a similar experiment with an exchange rate of 100 mL/4 h, which resulted in a lipase yield of 19 U/mL and productivity of 395 U/L/h (Fig. 4).

Although a decrease in productivity that accompanies an increase in yield is expected in the fill-and-draw culture, it is worth examining various exchange intervals. Figure 5 shows the time-course of the fill-and-

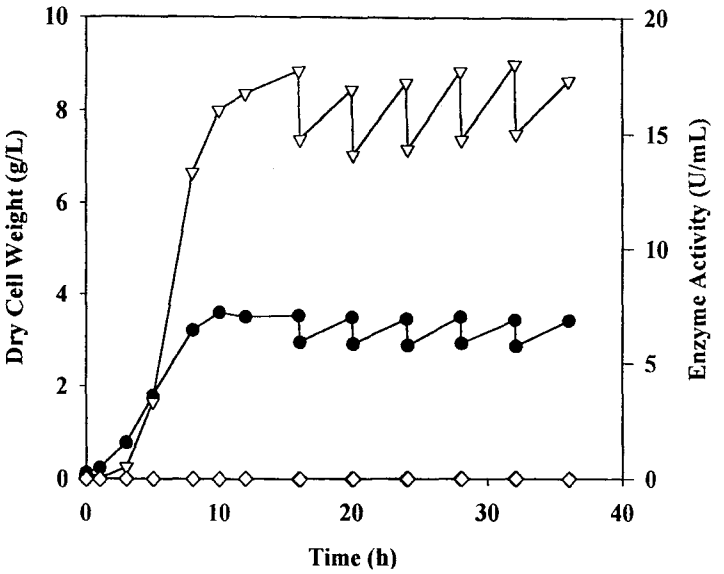

Fig. 3. Fill-and-draw culture of *A. radioresistens* with an exchange of 200 mL medium in a period of 4 h. ●, dry cell wt; ∇, lipase activity; ◇, protease activity.

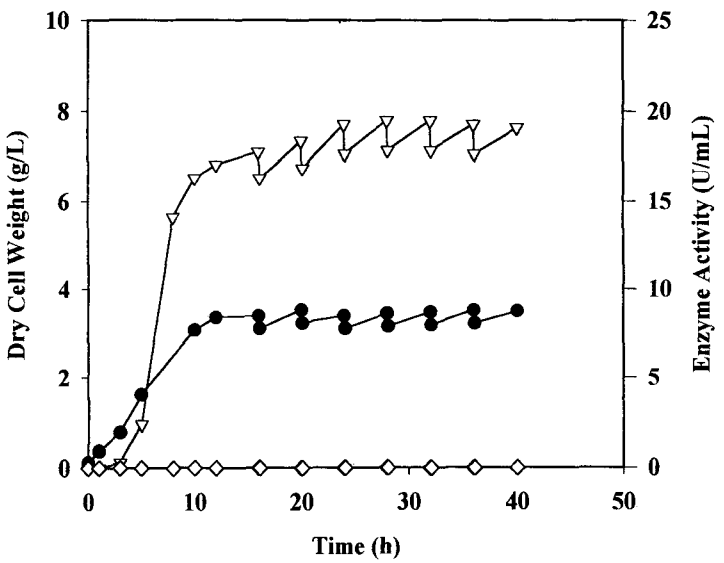

Fig. 4. Fill-and-draw culture of *A. radioresistens* with an exchange of 100 mL medium in a period of 4 h. ●, dry cell wt; ∇, lipase activity; ◇, protease activity.

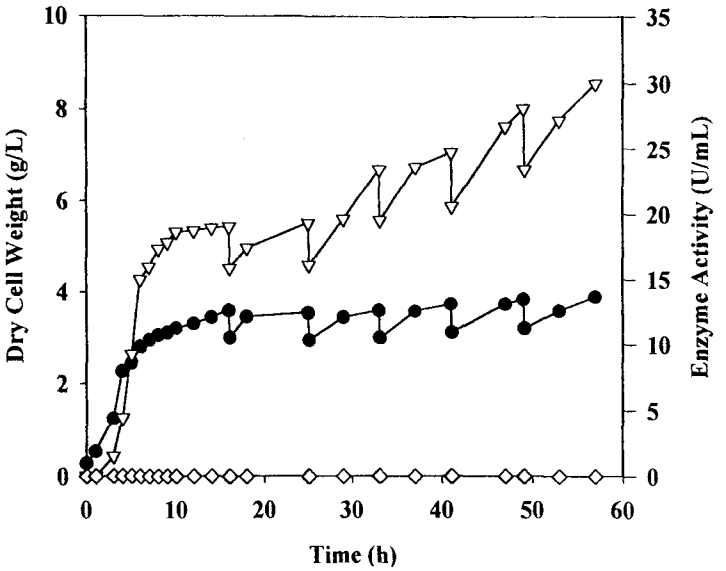

Fig. 5. Fill-and-draw culture of *A. radioresistens* with an exchange of 200 mL medium in a period of 8 h. ●, dry cell wt; ∇, lipase activity; ◇, protease activity.

draw culture under an exchange rate of 200 mL/8 h, which results in the same dilution rate as Fig. 4. As one can see, cell density increased very slightly, and the lipase yield increased gradually from 18 to 30 U/mL. Again, formation of protease and occurrence of excessive foaming were not observed during the entire process. As discussed above (see Fig. 1), the cells required an adaptation time to start a secondary growth. Apparently, this delay was so long that it allowed protease formation and excessive foaming to occur. In other words, the timing of this minor growth was too late to be helpful for lipase production. However, this situation can be improved by the fill-and-draw operation. It can be seen from Fig. 5 that cell density increased gradually during the repeated culture, indicating that the cells gradually adapted to the medium. In other words, during the prolonged cultivation, the cells entered the second growth phase on *n*-hexadecane, and thus lipase formation continued, and protease formation and excessive foaming did not occur. The lipase productivity calculated from a yield of 30 U/mL was 625 U/L/h. Compared with the batch culture (in which the average values were 18 U/mL and 1100 U/L/h for lipase yield and productivity, respectively), this exchange rate seems promising for the lipase production by the fill-and-draw culture.

Figure 6 shows the result of the lipase production by the fill-and-draw culture operated at a further decreased exchange rate of 200 mL/12 h. Cell density and lipase yield increased gradually, because of the adaptation of

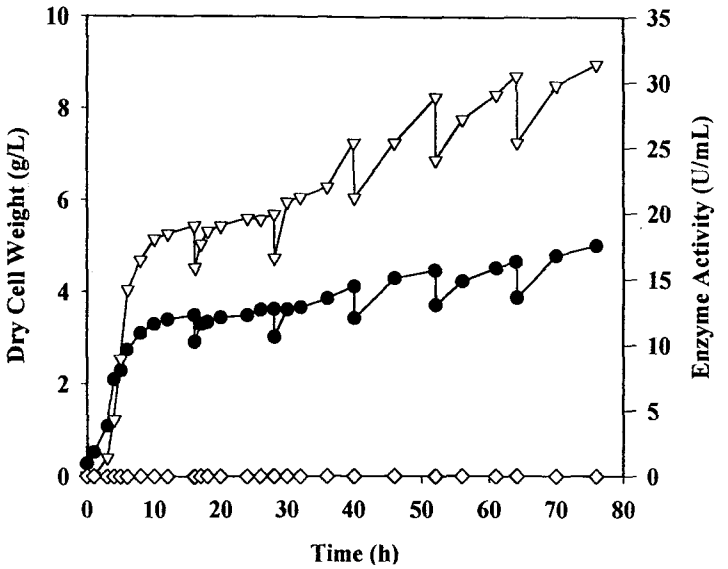

Fig. 6. Fill-and-draw culture of *A. radioresistens* with an exchange of 200 mL medium in a period of 12 h. ●, dry cell wt; ∇, lipase activity; ◇, protease activity.

the cells to the medium. No protease activity was detected; however, slight foaming was observed. The lipase yield was 31 U/mL, and the productivity was down to 430 U/L/h; the results, on the whole, were worse than that of Fig. 5. This is qualitatively realized, because the gain in yield has a limit (as can be seen in Figs. 2–4), but the decrease in productivity by the replacement is an event of proportion.

As described by Schnapp et al. *(8)*, fill-and-draw provides a means of examining the data in terms of effective dilution rate D_{eff}:

$$D_{eff} = \frac{1}{\Delta t} \times \frac{V_r}{V_w}$$

where Δt is the replacement interval, V_r is the volume removed, and V_w is the working volume. The production of *A. radioresistens* lipase by fill-and-draw culture under various exchange rates is summarized in Table 1 and plotted in Fig. 7. The discontinuities in the yield, productivity, and specific production rate indicate that the cells experienced different growth phases, one on olive oil and the other on *n*-hexadecane, and the data, therefore, should be treated separately. From Table 1 and Fig. 7, the lipase production can be pictured: The lipase is chiefly a growth-associated product, as can be judged from the slope and intercept of the data of specific production rate; higher dilution rate results in lower yield, but higher vol-

Table 1
Comparison of Lipase Yield and Productivity Between Batch and Fill-and-Draw Cultures

Operating mode	Effective dilution rate (h^{-1})	Yield (U/mL)	Productivity (U/L/h)	Specific production rate (U/g cell/h)
Batch[a]	–	18	1100	–
Fill-and-draw:				
400 mL/4 h				
400 mL/4 h	0.083	13	1080	432
200 mL/4 h	0.042	18	750	221
100 mL/4 h	0.021	19	395	120
200 mL/8 h	0.021	30	625	179
200 mL/12 h	0.014	31	430	96

[a] Data was taken as the average of the five fill-and-draw runs at 16 h.

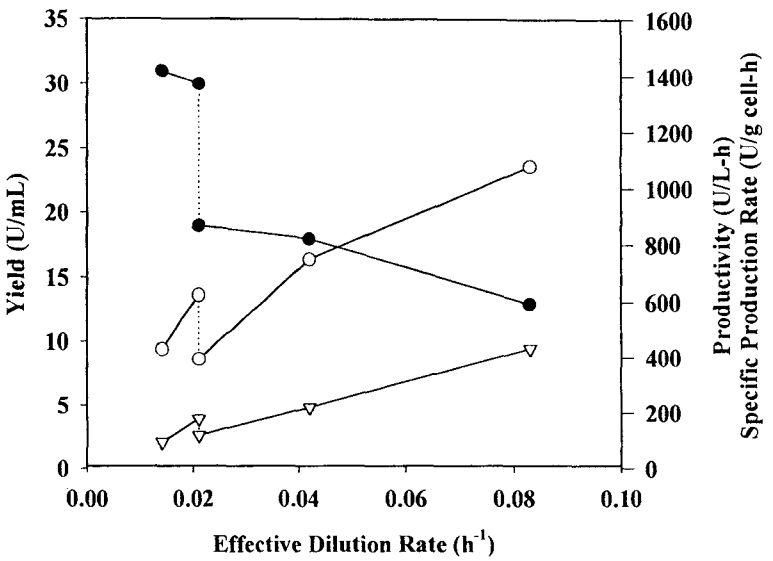

Fig. 7. Effect of effective dilution rate on yield (●), productivity (○), and specific production rate (∇) of *A. radioresistens* lipase.

umetric productivity; the higher productivity is the result of a more efficient protein synthesis (*see* the data on specific production rate); and the lipase yield is higher when the cells grow on *n*-hexadecane.

CONCLUSIONS

In a medium containing olive oil and *n*-hexadecane, the production of *A. radioresistens* lipase by batch culture reached a maximum yield of 18 U/mL and then fell off, because of protease formation during prolonged cultivation. It is suggested that the substrate consumed before the occurrence of the maximum yield is olive oil, and, to grow on *n*-hexadecane, the cells need an adaptation time. This limitation could be surpassed in the fill-and-draw culture, because of the adaptation of the cells to *n*-hexadecane during the repeated operation. As a result, a 66% increase in the lipase yield could be obtained. However, the improvement in lipase yield was accompanied by a decrease in productivity. Nevertheless, the fill-and-draw culture could be superior to batch culture for lipase production.

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