Continuous Production of Ethyl Esters of Grease Using an Immobilized Lipase

An-Fei Hsu*, Kerby C. Jones, Thomas A. Foglia, and William N. Marmer

USDA, ARS, ERRC, Wyndmoor, Pennsylvania 19038

ABSTRACT: The continuous production of ethyl esters of grease using a phyllosilicate sol-gel immobilized lipase from *Burkholderias cepacia* (IM BS-30) as catalyst was investigated. Enzymatic transesterification was carried out in a recirculating packed-column reactor using IM BS-30 as the stationary phase and ethanol and restaurant grease as the substrates without solvent. The bioreactor was operated at various temperatures (40–60°C), flow rates (5–50 mL/min), and times (8–48 h) to optimize ester production. Under the optimal operating conditions (flow rate, 30 mL/min; temperature, 50°C; mole ratio of substrates, 4:1 ethanol/grease; reaction time, 48 h), the ester yields were >96%. The IM BS-30 could be reused in the reactor for continuous ester production. The conversion of grease to ester was monitored by HPLC and GC, whereas free and total glycerol content in the product was determined by GC. Either water-washing or silica column chromatography reduced the free and total glycerol content of the ethyl ester preparation to acceptable levels.

Paper no. J10788 in *JAOCS 81*, 749–752 (August 2004).

KEY WORDS: Alkyl esters, biodiesel, bioreactor, esterification, grease.

The alcoholysis of vegetable oils and animal fats with simple alcohols (methyl and ethyl) to produce alkyl esters for use as biodiesel has been studied extensively (1–3). The main advantages of these esters when used as diesel fuels, aside from being renewable fuels, are that they are biodegradable and impart improved engine emissions when used either neat or in blends with petroleum diesel. Currently, chemical methods to produce biodiesel have some difficulties, such as the need to use refined feedstocks, recovery of glycerol, removal of the alkaline catalyst, and the energy-intensive nature of the process (4). Chemical transesterification can be complicated further if the feedstock contains high FFA levels or water. Enzymatic approaches can overcome these problems since lipases can operate under a variety of conditions in the synthesis of alkyl esters (1,5,6). Owing to the high cost of the enzyme, however, the establishment of a continuous process of producing these simple esters using an immobilized enzyme is needed to decrease production costs (7).

Several studies have reported the enzymatic alcoholysis of vegetable oils in solvent or solvent-free systems using both primary and secondary alcohols (1,2,8,9). The use of a solvent-free system has the advantages of avoiding the problems associated with solvent separation, toxicity, and flammability. These reports, however, did not describe the continuous alcoholysis of oils and fats in solvent-free medium, which would be advantageous for the industrial production of esters as biodiesel. Recently, we reported a novel phyllosilicate sol-gel immobilization technique for lipases that produced a highly stable biocatalyst. The *Burkholderia cepacia* lipase immobilized (IM BS-30) in that study readily converted grease to simple alkyl esters in solvent-free medium. The purposes of the present study therefore were to investigate the continuous production of esters from grease using this immobilized lipase and to determine those factors affecting the overall ester yields. In addition, some biodiesel fuel properties of the esters such as viscosity, pour point, cloud point, acid number, and glycerol content were determined.

MATERIALS AND METHODS

Materials. Recycled restaurant grease, obtained from Kaluzny Bros., Inc. (Joliet, IL), contained 5.8% FFA, 91.3% triglycerides (TAG), 1.9% diglycerides (DAG), and 0.5% monoglycerides (MAG). The average M.W. calculated from its FA composition (FA, wt%: 14:0, 1.0; 16:0, 21.1; 16:1, 1.4; 18:0, 10.2; 18:1, 46.8; 18:2, 17.5; 18:3, 1.0) was 913 g/mol. Lipase from *B. cepacia* (BS-30) was obtained from Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). Phyllosilicate (montmorillonite Sy-1) was from Source Clay Minerals Repository (Columbia, MO). Cetyltrimethyl ammonium chloride (HDTMA) and tetramethylorthosilicate (TMOS) were obtained from Aldrich (Milwaukee, WI). Molecular sieves were obtained from J.T.Baker (Phillipsburg, NJ), silica gel (70–230 mesh) was obtained from Sigma (St. Louis, MO), and all other reagents used were of the highest purity available from commercial suppliers.

Lipase immobilization. The procedure used to entrap lipase BS-30 within a phyllosilicate sol-gel matrix was as described by Hsu *et al*. (8) with slight modification. In the standard preparation, crude lipase BS-30 powder was suspended in water (150 mg/mL). The mixture was vortexed for 10 min and then centrifuged at 2,000 × *g* to pelletize the insoluble material. Protein content (1–1.5 mg/mL) in the supernatant was determined by a modified Lowry assay using serum albumin as the standard (10). To a suspension of clay in water (5.4 mL, 3.3% wt/vol) was added 600 μL of HDTMA, and to this mixture were added 3.5 mL of the enzyme supernatant, 1 mL of 1 M sodium fluoride, and TMOS (1.05 mL). The mixture was mixed vigorously, placed in ice for 4 h, then set overnight at

*To whom correspondence should be addressed at USDA, ARS, ERRC, 600 E. Mermaid Lane, Wyndmoor, PA 19038. E-mail: ahsu@errc.ars.usda.gov

room temperature to complete TMOS polymerization. The mixture was washed with distilled water (2 × 25 mL) to remove unretained protein and dried at room temperature to a water activity of ~0.5. The dried residue served as the IM BS-30 biocatalyst. For larger-scale preparations of IM BS-30 (20–25 g), the amounts of the components were increased proportionately.

Reactor design. A schematic of the apparatus used in this work for the continuous transesterification of grease is shown in Figure 1. The unit consists of an enzyme reactor (13.5 cm × 2.5 cm i.d., 67 mL), substrate reservoir (375 mL), and a product detector unit (ELSD–HPLC). Both the enzyme reactor and substrate reservoir unit were jacketed, through which was circulated thermostated water at various temperatures by a recirculating water bath (Model RTE-110; NESLAB Inc., Portsmouth, NH).

Grease (125 mL, 0.13 mol) and 30 mL of absolute alcohol (0.52 mol) were mixed together prior to placement into the substrate reservoir. The enzyme reactor was packed with 10 packets of IM-BS-30 [commercial paper coffee filters (20 mm diameter)], each of which contained 1 g of IM BS-30. The enzyme packets were slightly compressed by the movable reactor end rods. Substrates were circulated through the reactor by means of a circulating pump at various flow rates (from 10 to 50 mL per min). The standard reaction was conducted at 50°C for 48 h at a flow rate of 30 mL/min. After the substrate exited the enzyme reactor, it was passed through a valve that enabled the mixture to be sampled. Approximately 1 mL of the solution was isolated for HPLC analysis to measure ethyl ester (EE) production, while the remainder of the reaction mixture was returned to the substrate reservoir and recirculated to the enzyme reactor for further transesterification.

EE purification. EE obtained from the reactor were purified by column chromatography or water extraction to remove the glycerol co-product in the crude esters. For column purification, 100 mL of crude ester was passed through a column (14 cm × 2 cm i.d.) loaded with silica (30 g). After sample loading, the column was eluted by nitrogen pressure and the eluant analyzed by HPLC and GC for its lipid composition. For water extraction, 1 mL of crude ester was mixed vigorously with 1 mL of NaCl (0.9%); then the mixture was extracted with hexane/ether (1:1) (3 × 3 mL). The organic phases were combined, dried over anhydrous Na_2SO_4, and filtered. The solvent was removed under a stream of nitrogen and the washed ester analyzed by HPLC and GC. Results are the average of two trials with duplicate determinations of each data point. The relative SD was <5% calculated using HP-Chemstation software (Agilent Technologies, Wilmington, DE).

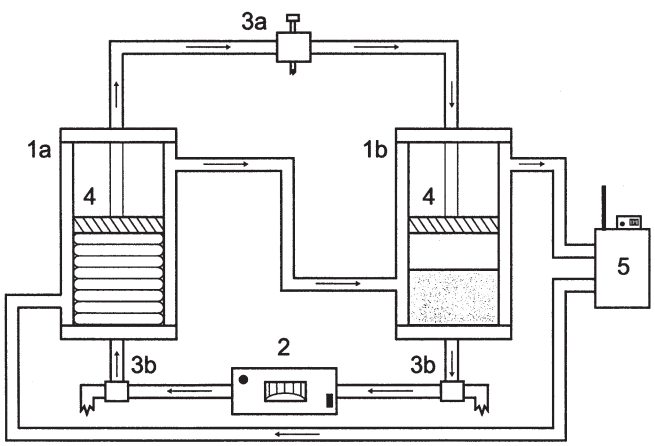

FIG. 1. Schematic for immobilized lipase BS-30 bioreactor. Bioreactor consists of (1a) water-jacketed column, (1b) substrate reservoir, (2) recirculating pump (5 to 100 mL/min), (3) three-way valves [(3a) sampling valve, (3b) drain valves], (4) movable plungers, and (5) recirculating water bath.

Monitoring of ester production. The percentages of EE, FFA, MAG, DAG, and TAG in reaction mixtures were determined by HPLC using a method developed in our laboratory (11). HPLC analyses were performed with a Hewlett-Packard HP1050 series liquid chromatograph equipped with helium degasser, autosampler, and quaternary pump module. Lipid class separations were obtained on a Phenomenex (Torrance, CA) cyanopropyl column (250 × 4.6 mm i.d., 5 μm) with guard column (30 × 4.6 mm i.d., 5 μm) of the same phase using a binary solvent mixture of *n*-hexane (solvent A) and methyl *t*-butyl ether (solvent B), each fortified with 0.4% acetic acid. The modified binary solvent profile used was as follows: After an initial isocratic period for 5 min at 100% A, the solvent composition was changed to 20% A/80% B over 10 min, held for 2 min, then returned to 100% A over 1 min. The column was equilibrated for 10 min before the next injection, for a total run time of 30 min. A Varex N_2 (Burtonsville, MD) MK III ELSD operated at 30°C with a N_2 flow of 1.51 standard liters per min was used for detection. Ester content was determined from standard curves of the ELSD detector response vs. amount for each neutral lipid class (11). Sample injection volume was 20 μL. Acquisition and processing of data were done using HP-Chemstation software.

Free and bound (acylglycerol) glycerol content in the EE mixtures was measured by GC according to ASTM Method D 6751-02 (12). Sample analyses were carried out on a DB-1ht fused-silica capillary column (15 m × 0.32 mm i.d., 0.1 μm film thickness; J&W Scientific, Folsom, CA). Samples (1 μL) were injected on-column by an HP 7673 autosampler injector at an oven temperature of 50°C. After an isothermal period of 1 min, the GC oven was heated at 15°C/min to 180°C, then at 7°C/min to 230°C and at 30°C/min to 370°C (hold for 10 min), for a total run time of 31.5 min. Helium was used as carrier gas at a linear velocity of 62 cm/s measured at 50°C. The detector temperatures were 380°C, and helium served as the detector makeup gas (26.4 mL/min). Acquisition and processing of data were carried out using HP-Chemstation software.

RESULTS AND DISCUSSION

Effect of reactor flow rate on EE production. The experimental design of the bioreactor is shown in Figure 1. To avoid clogging of the reactor by deposition of the immobilized lipase onto the reactor end seals, the IM BS-30 was packaged in filter paper, which did not affect the flow rate (data not shown). Additionally, to increase the substrate contact with the enzyme, the amount of IM BS-30 loaded into the reactor was divided into

10 equal portions, each of which was individually packaged. The volume of the reactor was approximately 100 mL after enzyme loading. Ethanolysis was initiated by circulation of the mixed grease/alcohol reactants from the reactor substrate reservoir through the enzyme reactor at 50°C. From our previous studies (8) on batch lipase-catalyzed alcoholysis using IM BS-30 lipase, the grease-to-alcohol mole ratio of 1:4 was used throughout this study. Figure 2 shows ester production curves as a function of flow rate (5, 15, 30, and 50 mL/min). At various time points (2 to 24 h), samples were withdrawn from the system through the sampling valve and conversion of the grease to EE (percentage of total lipid to EE) was measured by HPLC. The data show that ester production was a direct function of flow rate and residence time in the reactor. For each time point, the ester production was maximal at a flow rate of approximately 30 mL/min. Accordingly, the standard flow rate for the bioreactor in subsequent experiments was set at 30 mL/min.

Effect of temperature on EE production. The continuous enzymatic transesterification of grease was examined in the temperature range of 40 to 60°C at a flow rate of 30 mL/min (Fig. 3). At short reaction times (<10 h), the highest ester yields were obtained at 60°C, but at the longer reaction times (>30 h) there was a minimal difference in ester yields at 40, 50, or 60°C. The effect of temperature on ester yields was further demonstrated when ester yields at 50°C were normalized to 100% and compared with the ester yields obtained at 40 or 60°C. Figure 4 indicates that at reactor reaction times <20 h ester production was slightly favored at 60°C, whereas for reaction times >20 h ester production was slightly favored at 40°C. Based on these data, 50°C was used for the routine operation of the bioreactor.

Reusability of lipase IM BS-30 in the reactor. The reusability of IM BS-30 lipase for continuous ester production was investigated. In this series of experiments the ethanolysis of grease was carried out at 50°C for 18 h with continuous circulation of the substrates through the reactor at 30 mL/min after which the substrates were removed from the reactor and ester production was

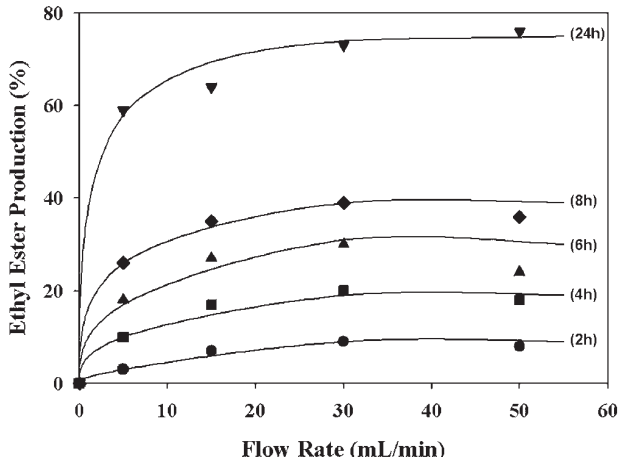

FIG. 2. Effect of reactor flow rate on ethyl ester production at 50°C. IM BS-30 was packed in the reactor with the grease and alcohol substrates circulated through the reactor at 5, 15, 30, and 50 mL per min. At selected times (2, 4, 6, 8, and 24 h), 1 mL of sample was taken from the splitting valve and EE production was determined by HPLC.

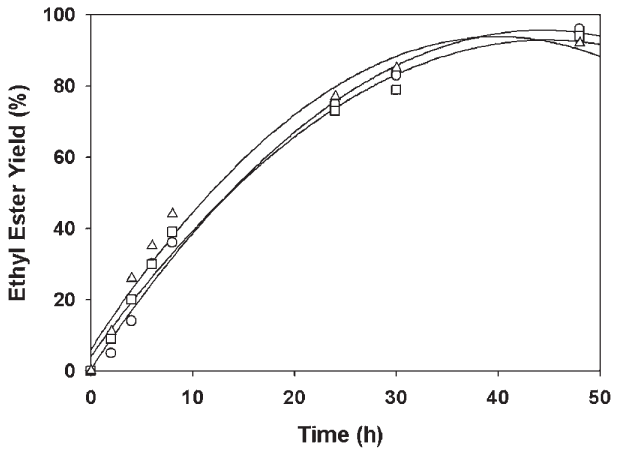

FIG. 3. Effect of temperature on EE production at a 30-mL/min flow rate. IM BS-30 was loaded into the reactor and the grease and ethyl alcohol substrates were circulated through the reactor at 40, 50, and 60°C for 48 h. At each time interval, a 1-mL sample was taken from the splitting valve and ethyl ester production was determined by HPLC (40°C, ○; 50°C, □; 60°C, △). See Figure 2 for abbreviation.

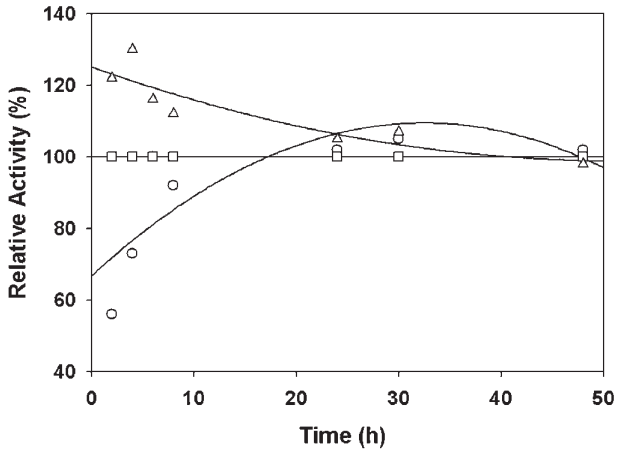

FIG. 4. Ethyl ester production in the reactor as a function of time. The conditions for carrying out ethyl ester synthesis are the same as in Figure 3. The data are normalized to ethyl ester yields obtained at 50°C. Ester yields at the same time points at 40 and 60°C are normalized to ester production as a relative percentage compared with the corresponding time point ester yield at 50°C (40°C, ○; 50°C, □; 60°C, △). See Figure 2 for abbreviation.

measured by HPLC. Between each cycle, *n*-hexane (3 × 120 mL) was passed through the reactor, enzyme bed, and substrate reservoir and the catalyst bed air-dried before reuse. The next alcoholysis cycle was carried out with fresh substrates using the recovered enzyme. The ester conversions obtained after five cycles of enzyme use were normalized, with the conversion for the first cycle being set at 100%. The conversion to esters for the second cycle decreased to ~90% and then remained constant for the next three cycles. The observed decrease in ester yields after the first cycle could have resulted from mechanical loss of enzyme during the washing process or from leakage of the enzyme in the first cycle. Continued reuse of this immobilized enzyme was not studied in detail, but other data (not shown) suggested that it could be reused further. The use of this immobilized lipase in

ester production could decrease the cost of biodiesel production from FFA-containing feedstocks, such as recycled restaurant grease.

Purification of EE as biodiesel. Residual glycerol, both free and total (sum of free and acylglycerol bound glycerol), must meet specified levels when fatty esters are used as biodiesel fuel (12). In addition, the recovery of glycerol from the reaction is necessary since its sale as a co-product reduces the cost of biodiesel production. Table 1 lists the free glycerol and acylglycerol (MAG, DAG, TAG) levels found in the crude and purified EE obtained from the reactor. For the crude esters, both free and total glycerol (Table 2) exceeded the ASTM specification. Free glycerol in the crude esters was reduced to undetectable levels after purification (either silica or water wash, Table 1), which also reduced acylglycerol levels and hence bound glycerol. In this manner, a biodiesel fuel was obtained that met both the free and total glycerol ASTM specification (Table 2). A recent report (13) indicated that short-chain alcohols also could be used to remove glycerol from lipase-catalyzed alcoholysis reaction products. Removal of glycerol from the biocatalyst in this manner also stabilized the lipase, which allowed for its reuse. In our study, the biocatalyst was washed with *n*-hexane before reuse, which allowed for retention of its catalytic activity.

TABLE 1
Analysis of Ethyl Esters Produced by Ethanolysis of Grease Catalyzed by IM BS-30

Sample	Glycerol[a]	MAG[a]	DAG[a]	TAG[a]	Ester[a]
Crude biodiesel[b]	0.9	0.7	1.4	0.1	97
Silica-treated[c]	ND[d]	0.1	0.2	0.1	98
Solvent-extracted[e]	ND	0.1	0.1	0.1	98

[a]Data expressed as wt% as determined by GC according to ASTM Method D 6584 (12).
[b]Crude esters obtained directly from the bioreactor after 48 h reaction at 50°C.
[c]Esters purified by passing through a silica column.
[d]ND = not detected by GC.
[e]Esters purified by washing with 0.9% NaCl and extracting with hexane/ether (1:1).

TABLE 2
Selected Properties for Esters Produced by Ethanolysis of Grease Catalyzed by IM BS-30

	Crude esters[a]	Purified esters[b]	ASTM specification	Units
Viscosity (40°C)[c]	5.9	5.1	1.9–6.0	mm^2/s
Pour point (PP)[c]	2.0	3.0	NS[d]	°C
CFPP[c]	–21	–23	NS[d]	°C
Cloud point (CP)[c]	8.0	2.0	–3 to –12[e]	°C
Acid number	1.53	0.72	0.80	mg KOH/g
Free glycerin[f]	0.09	ND	0.02	wt%
Total glycerin[f]	0.89	0.20	0.24	wt%
Density	0.8689	0.8651	NS[d]	g/cc

[a]Crude esters: esters obtained after ethanolysis of grease at 50°C for 48 h.
[b]Purified esters: crude esters passed through a silica column.
[c]Properties measured following ASTM Methods (12). Pour points (PP), cold filter plugging point (CFPP), and cloud point (CP) determined by Dr. Robert Dunn, USDA, ARS, NCAUR (Peoria, IL).
[d]No specification (NS) for PP, CFPP, and CP for biodiesel given in ASTM Method D6751-02.
[e]CP range listed is for biodiesel obtained from various refined oil feedstocks.
[f]Free glycerin and total glycerin determined by GC as specified in ASTM Method D 6751-02.

Selected properties of grease EE. Table 2 lists some additional fuel properties for both the crude and purified (water-washed) EE produced in the bioreactor. Cloud points obtained for the grease esters are higher than those reported for a variety of biodiesels prepared from refined vegetable oils, but more importantly, the cold filter plugging point value was acceptable. The purified biodiesel had free glycerin and total glycerin values within the ASTM specification, as was the acid number. Based on the above data, we conclude that EE production from grease using the continuous enzymatic process described herein can meet the required ASTM specifications for use as a biodiesel fuel. Further studies, however, need to address the cold-temperature properties of these esters. Other properties, such as oxidative stability and combustion properties, also need to be determined.

REFERENCES

1. Shimada, Y., Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda, and Y. Tominaga, Conversion of Vegetable Oil to Biodiesel Using Immobilized *Candida antarctica* Lipase, *J. Am. Oil Chem. Soc. 76*:789–793 (1999).
2. Selmi, B., and D. Thomas, Immobilized Lipase-Catalyzed Ethanolysis of Sunflower Oil in a Solvent-Free Medium, *Ibid. 75*:691–695 (1998).
3. Mittelbach, M., Lipase-Catalyzed Alcoholysis of Sunflower Oil, *Ibid. 67*:168–170 (1990).
4. Ali, Y., and M.A. Hanna, Alternative Diesel Fuels from Vegetable Oils, *Bioresource Technol. 50*:153–163 (1994).
5. Hsu, A.-F., K. Jones, T.A. Foglia, and W.N. Marmer, Immobilized Lipase-Catalyzed Production of Alkyl Esters of Restaurant Grease as Biodiesel, *Biotechnol. Appl. Biochem. 36*:181–186 (2002).
6. Nelson, L.A., T.A. Foglia, and W.N. Marmer, Lipase-Catalyzed Production of Biodiesel, *J. Am. Oil Chem. Soc. 73*:1191–1195 (1996).
7. Hsu, A.-F., T.A. Foglia, and S. Shen, Immobilization of *Pseudomonas cepacia* Lipase in a Phyllosilicate Sol-Gel Matrix: Effectiveness as a Biocatalyst, *Biotechnol. Appl. Biochem. 31*:179–183 (2000).
8. Hsu, A.-F., K. Jones, W.N. Marmer, and T.A. Foglia, Production of Alkyl Esters from Tallow and Grease Using Lipase Immobilized in a Phyllosilicate Sol-Gel, *J. Am. Oil Chem. Soc. 78*:585–588 (2001).
9. Noureddini, H., X. Gao, S. Joshi, and P.R. Wagner, Immobilization of *Pseudomonas cepacia* Lipase by Sol-Gel Entrapment and Its Application in the Hydrolysis of Soybean Oil, *Ibid. 79*:33–40 (2002).
10. Bensadoun, A., and D. Weinstein, Assay of Proteins in the Presence of Interfering Materials, *Anal. Biochem. 70*:241–250 (1976).
11. Foglia, T.A., and K.C. Jones, Quantitation of Neutral l Lipid Mixtures, Using High-Performance Liquid Chromatography with Light-Scattering Detection, *J. Liq. Chromatogr. Rel. Technol. 20*:1829–1838 (1997).
12. Standard Specification for Biodiesel Fuel (B100) Blend Stock for Distillate Fuels, Designation D 6751-02, American Society for Testing and Materials, West Conshohocken, PA, 2002.
13. Du, W., Y.-Y. Xu, and D.-H. Liu, Lipase-Catalyzed Transesterification of Soybean Oil for Biodiesel Production During Continuous Batch Operation, *Biotechnol. Appl. Biochem. 38:*103–106 (2003).

[Received January 6, 2004; accepted June 15, 2004]