0273-2289/1559–0291 (Online)/07/136–140/793–804/$30.00

Study on the Production of Biodiesel by Magnetic Cell Biocatalyst Based on Lipase-Producing *Bacillus subtilis*

Ming Ying[1–3] and Guanyi Chen*[,1,3]

[1]Section of Bioenergy and Environment, Faculty of Environmental Science and Engineering Tianjin University, Weijin Road 92, 300072, Tianjin, China; [2]School of Chemical Engineering and Technology, Tianjin University; and [3]State Kay Lab of Internal Combustion Engine, Tianjin University, E-mail: chen@tju.edu.cn

Abstract

Production of biodiesel from waste cooking oils by a magnetic cell biocatalyst (MCB) immobilized in hydrophobic magnetic polymicrosphere is studied here. The cells of lipase-producing *Bacillus subtilis* were encapsulated within the net of hydrophobic carrier with magnetic particles (Fe_3O_4), and the secreted lipase can be conjugated with carboxyl at the magnetic polymicrosphere surface. Environmental scanning electron microscope, transmission electron microscope, and vibrating magnetometer, and so on were used to characterize the MCB. The MCB was proved to be superparamagnetic; and could be recovered by magnetic separation; moreover it could be regenerated under 48 h of cultivation. When methanolysis is carried out using MCB with waste cooking oils under stepwise additions of methanol, the methyl esters in the reaction mixture reaches about 90% after 72 h reaction in a solvent-free system. The process presented here is environmentally friendly and simple without purification and immobilized process required by the current lipase-catalyzed process. Therefore, the process is very promising for development of biodiesel fuel industry.

Index Entries: *Bacillus subtilis*; biodiesel; magnetic cell biocatalyst; magnetic polymicrosphere; waste cooking oils.

Introduction

Up to now, biodiesel is usually produced through chemical-catalyzed process. Alkali or acid process always lead to large quantities of waste stream, which has to be treated before disposal. So lipase-catalyzed production of biodiesel has recently generated increasing interest because of its waste-free process *(1–3)*. However, the use of extracellar lipase as catalyst requires complicated recovery, purification, and immobilization process *(4)*. As part of a research program aimed at simplifying the process of

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

lipase-catalyzed biodiesel production from waste cooking oils, a magnetic cell biocatalyst (MCB) prepared with cells of *Bacillus subtilis* 1.198 were investigated. In this study, the *B. subtilis* cells were encapsulated in divinyl benzene magnetic polymicrosphere, which have superparamagnetic property. This kind of MCB can be better dispersed when it catalyzes the methanolysis reaction. It can also be more easily separated from reaction system, and stabilized in a magnetofluidized bed reactor by applying an external magnetic field. The use of magnetic particles can also reduce the capital and operational costs *(5)*. For these reasons, the process using MCB to produce biodiesel appears more promising for development of biodiesel industry.

In our previous work, we have reported the use of *Rhizopus oryzae* lipases efficiently for catalyzing the transesterification of waste cooking oils in a solvent-free medium. The cells of *R. oryzae* immobilized in polyurethane foam particles were used as whole cell biocatalyst in Kobe University (Japan). When methanolysis was carried out with stepwise additions of methanol, the level of methyl ester (ME) conversion was same as that using the extracellular lipase *(6–8)*. This forms the starting point of our research using cell-catalyzed biodiesel product. The lipase from *B. subtilis* used in this study is different from that of *R. oryzae* or *Candida antarctica* in term of the biochemical properties and three-dimensional structures *(9)*. First, lipolytic enzymes produced and secreted by *B. subtilis* include a lipase LipA (EC 3.1.1.3) and an esterase LipB (EC 3.1.1.1) *(10)*. *B. subtilis* 1.198 used in this study is a LipA-lipase overexpression strain. Second, LipA is a small protein with a molecular mass of 19.3 kDa *(11)*. The structures of LipA-lipase is clearly different from *R. oryzae* lipase studied early, which suggested no lid domain and interfacial activation *(12,13)*. This point will result in easy interaction with substrates in transesterification reaction system. Finally, LipA-lipase exhibits higher specific activities toward substrates with longer chain triacylglycerol, such as tricaprylin (C10 : 0), trilaurin (C12 : 0), and triolein (C18 : 1) Eggert et al. *(14)*.

As such, LipA-lipase of *B. subtilis* might be more suitable for biodiesel production. For the purpose of easily separating the catalyst from transesterification reaction system, and keeping stability in the fluidized-bed reactor by applying an external magnetic field, we encapsulated *B. subtilis* cells into the net of hydrophobic carrier with magnetic particles (Fe_3O_4) and prepared a MCB. Scanning electron microscope (SEM), transmission electron microscope (TEM), and vibrating magnetometer were used to characterize the MCB. The methanolysis activity was assayed at the same time.

Materials and Methods

Materials and Chemicals

The waste cooking oils were obtained from Yizhong restaurant. Olive oil (saponification value 175–195), vinyl acetate 2,2-azo-*bis*-isobutyronitrile (CR grade), and poly(vinyl alcohol) (Mr 72,000) were obtained from Tianjin

Kewei Reagent Company (Tianjin, China). Divinyl benzene (total isomers >80%) were obtained from Tianjin NanKai Chemical Factory (Tianjin, China), sodium dodecyl sulfonate, yeast extract, and tryptone were provided by Shanghai Sangon Biotechnology Co. Ltd (Shanghai, China). A neodymium permanent magnet (diameter 8 cm; magnetic field strength 2.4×10^5 A/m) from Institute of Metal Research Chinese Academy of science (Shenyang, China) was used for separation. All other chemicals were analytical grade and bought at local market.

Microorganism and Medium

All experiments were carried out using *B. subtilis* 1.198. The basal medium was LB containing: tryptone 10.0 g, yeast extract 5.0 g, and NaCl 10.0 g in 1 L tap water. For solid slant medium, 20.0 g/L agar was added into above liquid medium. The immobilized medium used ATCC573 *Bacillus* medium, which contains $(NH_4)_2SO_4$ 1.3 g, KH_2PO_4 0.37 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, $CaCl_2 \cdot 2H_2O$ 0.07 g, $FeCl_3$ 0.02 g, glucose 1.0 g, and yeast extract 1.0 g in 1.0 L distilled water and pH is adjusted to 4.0 with 10 *N* H_2SO_4. The multiplication medium of magnetic cells is GYE including 20.0 g tryptone, 10.0 g sucrose, 5.0 g olive oil, 1.0 g $(NH4)_2SO_4$, 1.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g KH_2PO_4, and 1.0 g Tween-80 in 1 L distilled water.

Preparation of MCB

Synthesis of Lipophilic Fe_3O_4 Nanocolloids

According to the method described previously *(15)*, $FeCl_3 \cdot 6H_2O$ 41.9 g and $FeCl_2 \cdot 4H_2O$ 20.9 g were added to a three-necked flask containing 350 mL of distilled water, which was heated by thermostat water bath and stirred by a mechanical agitator. When temperature of thermostat water bath increased to 60°C, 50 mL of 0.5 mol $NaHCO_3$ was poured into the flask under vigorous agitation. Five milliliters stearic acid was dropped into the mixture gradually, and incubated for 20 min at 60°C before the mixture was cooled to room temperature naturally.

Cultivation of *B. subtilis* cells for immobilization

Mycelia collected from LB agar slants were inoculated into 5 mL of LB liquid medium. The cultures were incubated for 20 h at 37°C on a rotary shaker at 225 rpm. And then the seed suspension (grown for 20 h on LB medium) was used to inoculate with 4.0×10^8 cells/mL to 250 mL ATCC573 *Bacillus* medium in a 500-mL shake-flask, which was incubated at 37°C and 225 rpm. The *B. subtilis* cells were collected into 10 mL oleic acid in the late logarithmic growth phase, and cultivated for 1 h at room temperature and 225 rpm.

Synthesis of MCB

The MCB particles were synthesized by encapsulating the cells into the net of hydrophobic carrier with magnetic particles (Fe_3O_4) through divinyl

benzene radical suspension polymerization. In a basic preparation, 15 mL of the ferrofluid prepared as Synthesis of Lipophilic Fe_3O_4 Nanocolloids section, 10 mL of oleic acid *B. subtilis* cell suspension, and 1.8% 2,2-azo-*bis*-isobutyronitrile were dispersed in a mixture of 3.7 mL divinyl benzene and 16.8 mL vinyl acetate and sonicated for 10 min. The mixture was transferred into a 50-mL injector with a 60–70 μm of diameter pinhead. The mixture was injected drop by drop into a three-necked flask containing 300 mL aqueous solution of 10% poly(vinyl alcohol) plus 2.0 g/L sodium dodecyl sulfonate, which was put into a 30°C thermostat water bath and agitated at 300–500 rpm. Thereafter, the reaction mixture was maintained at 30°C stilly for 2 h. The magnetic cell microspheres were harvested by permanent magnet separation and washed three times with distilled water. Finally, the MCB was stored in 80% glycerol solution at 4°C before use.

Multiplication Culture of Magnetic Cells

Magnetic *B. subtilis* cells were separated from the glycerol solution by magnetic separation. After washed with routinely physiological saline solution, the magnetic cells were immerged in GYE multiplication medium and cultivated for 1–3 d at 225 rpm. And then the magnetic cells biocatalyst were separated from culture medium and dried under a vacuum for about 24 h after washed three times with sterile water. Magnetic cells biocatalyst with a water content of approx 5% were obtained and can be used as a methanolysis biocatalyst.

Methanolysis Reaction

The methanolysis were carried out at 40°C, 220 rpm and pH 6.5 in a 100-mL shake-flask incubating on a rotary shaker. The compositions of the reaction mixtures were as follows: waste cooking oils 68.85 g, 0.1 *M* phosphoric acid buffer (pH 6.5) 3.0 mL, and methanol 2.5 g with 3.0% MCB to the shake-flask. One molar equivalent of methanol was 2.5 g against 68.85 g waste cooking oil. To fully convert the oil to its corresponding MEs, when the ME content in the reaction mixture reached approx 30 and 60%, 2.5 g of methanol was added twice. In this case, the reaction mixture was incubated for 72 h. The composition of methanolysis products were analyzed by mass chromatography (MS) and the content of MEs were analyzed by capillary gas chromatography as described below.

Analysis

One unit enzyme activity of MCB was defined as the amount of lipase, which liberates 1 μmol fatty acids from olive oils/min under the assay condition. The released fatty acids were determined by titration with 5 m*M* NaOH solution. The initial enzyme activity of MCB is about 4800 U/g assayed by this method. The ME content of the reaction mixture was quantified using an Agilent 6890N Series GC–MS system (Agilent

Technologied Corp.), and HP6890 Chemstation software was used for data analysis. The GC was equipped with a HP9091s-413 capillary column (300 μm × 30 m). For GC analysis, 500 μL of sample supernatant and 500 μL hexane as diluent were mixed in a 1.5-mL bottle. One microliter aliquot of the dilute sample was injected into the gas chromatograph. A split injector was used with a split ratio of 20 : 1 and the temperatures of injector and detector were set at 300 and 260°C, respectively. The carrier gas was nitrogen with a flow rate of 20 mL/min. An FID detector was used and the oven was initially held at 50°C, then elevated to 130°C at 20°C/min holding for 5 min, and finally to 260°C at 2.5°C/min. The oven was held at this temperature for 10 min before returning to 50°C. Total run time for this method was about 70 min. Calibration of the GC method was carried out by analyzing standard solutions of methyl palmitate, linoleic acid methylester, methyl oleate, and stearic acid ME. The standards were diluted in hexane-like reaction samples. ME yield was expressed as the percentage of MEs produced relative to the theoretical maximum based on the amount of original oils. In this article, ME yield is sometimes expressed as ME content or conversion.

Charaterization Analysis of MCB

The cross-section morphology of MCB particles was observed with a JEM-100CX II TEM system (JEOL, Japan). The paramagnetic property of the dried MCB particles was analyzed with an LDJ 9600-1 vibrating sample magnetometer ([VSM], LDJ Electronics, MI). The particle size distribution and density of the magnetic microspheres was measured with a Mastersizer 2000 particle size analyzer (Malvern Instruments, UK). Scanning electron micrographs were taken by an JSM-6700F field emission SEM (JEOL, Japan). The crystal construction of MCB was determined by X-ray detector X'pert Pro (Panalytical, Dutch).

Results and Discussion

Micromorphology of MCB

The magnetic cells polymicrospheres were prepared according to the method described in Preparation of MCB section, and the morphology of the particle observed by SEM was shown in Fig. 1A. Some micropores on rough microglobules surface can be found; which is a typical copolymer beads. The cross-section of magnetic cells biocatalyst particles was observed by TEM after 48 h cultivation in GYE multiplication medium, and the photographs was shown in Fig. 1B. It can be seen that the cells of *B. subtilis* (on the cycles) were encapsulated into the internal gaps of copolymer beads. Some cells have already became dormant, but the movement of other cells can be observed under the lane of TEM.

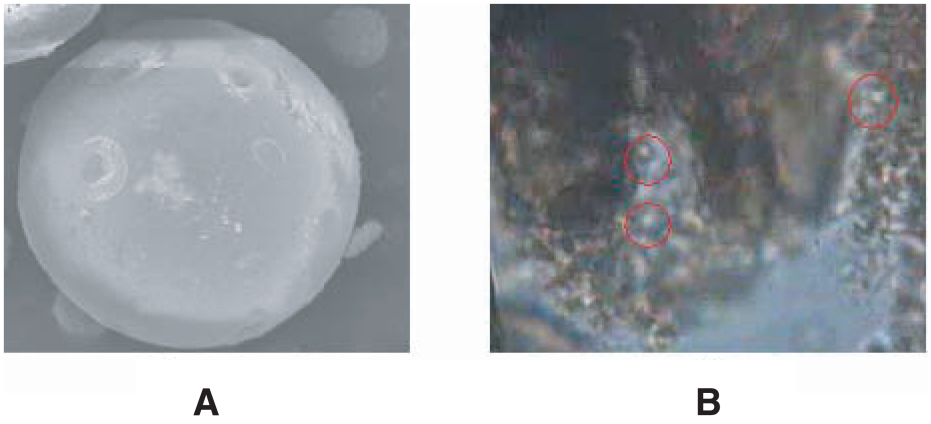

Fig. 1. (A) Surface features of the MCB particle observed with SEM. **(B)** Cross-section of the MCB particle observed with TEM.

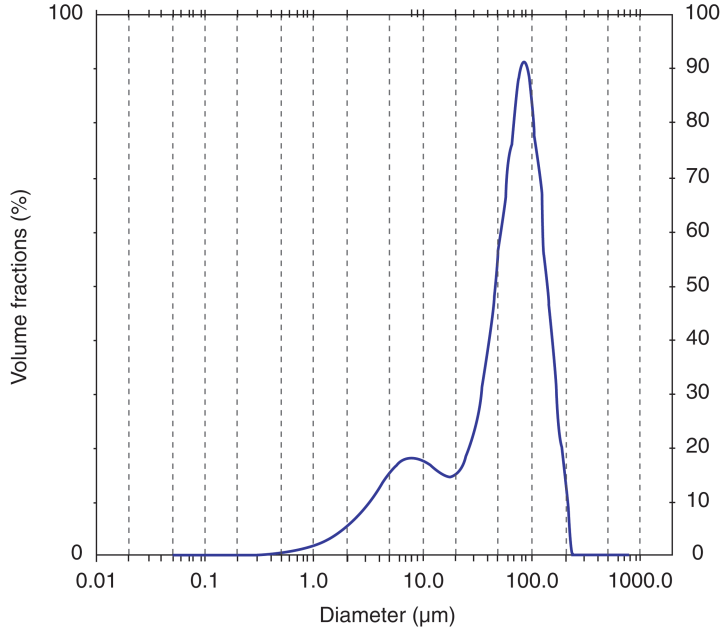

Fig. 2. Particle size distributions of MCB.

Crystal Construction of MCB

The size distributions of MCB particles were analyzed by a Mastersizer 2000 particle size analyzer. The results are described in Fig. 2, which indicates the mean diameter is 64.7 μm, the density is 1.00 g/cm^3, and specific surface area is 0.5092 m^2/g. The magnetic particles for the preparation of the polymicrospheres were analyzed by X-ray diffraction, Fig. 3 shows the result. The spectrum of magnetic particles consisting with Fe_3O_4 spectrum of database indicates the core of polymicrospheres is Fe_3O_4 indeed. According to Scherrer

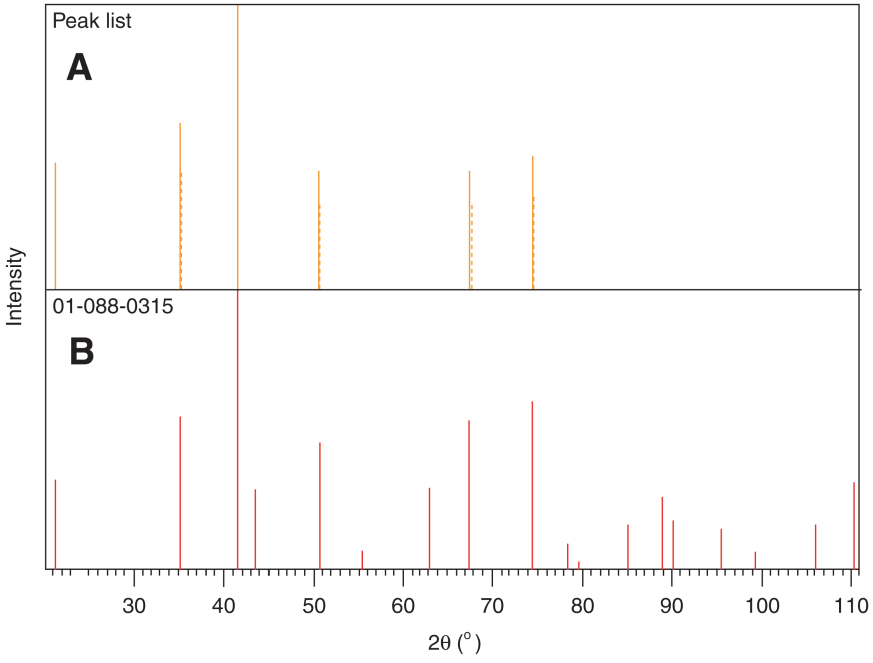

Fig. 3. XRD pattern of MCB comparing with standard pattern of Fe_3O_4, **(A)** XRD pattern of MBC. **(B)** Standard pattern of Fe_3O_4 in database.

equation $D_{XRD} = k\lambda/\beta\cos\theta$, the size of Fe_3O_4 is about 5.5–17.8 nm with a mean diameter of about 11.5 nm. For ultrafine magnetic particles, there exists a critical size, 25 nm, below which the microspheres can obtain single magnetic domains even in zero magnetic fields (*13*). According to this magnetic theory, the MCBs prepared in this study are superparamagnetic.

Superparamagnetic Property of MCB

Drawing the magnetization curve of MCB with a VSM is used to further analyze the magnetic property of the MCB particles at room temperature (300 K). The magnetization curve shown in Fig. 4 can be fitted as Langevin Eq. 1, which describes the superparamagnetic behavior well *(16)*. This provides a strong support to the superparamagnetic property of MCB

$$B = \varepsilon_m B_s \int_0^\infty \left[\coth\left(\frac{kT}{\mu_0 B_s VH} \right) - \frac{kT}{\mu_0 B_s VH} \right] f(V)\mathrm{d}V \qquad (1)$$

where B is the magnetization (emu/g), B_s is the saturation magnetization of magnetite colloids (emu/g), H is the applied magnetic field strength (Oe), V is the volume of magnetite colloids (cm^3), μ_0 is the permeability in vacuum, T is the absolute temperature (K), and k is the Boltzmann's constant. The values of saturation magnetization B_s, the remanence B_r, coercivity H_c, and maximum field strength H_{max} measured by VSM are listed in Table 1 *(17)*.

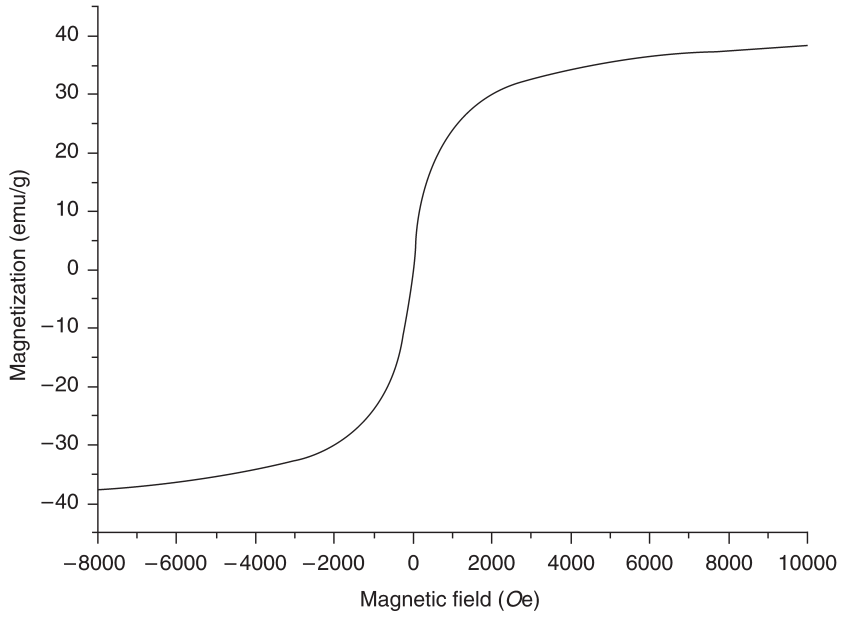

Fig. 4. Magnetization curves of MCB particle microspheres at 300 K. The solid line is calculated from the data measured by VSM.

Table 1
Some Magnetic Parameters of MCB Measured by VSM

Maximum field strength (H_{max} [Oe])	9960
Coercivity (H_c [Oe])	−0.1094
Remanence (B_r [emu/g])	0.01004
Saturation magnetization (B_s [emu/g])	3.867

From the results of VSM, it can be seen that remanence (0.01004 emu/g) and coercivity (–0.1094 emu/g) were so small that hysteresis could hardly be observed *(18)*. This feature is also typical of superparamagnetism *(19)*. So the MCB particles could be easily settled within 22 s under the permanent magnet field. When the external magnet was removed, the MCB particles can be well dispersed by gentle shaking. The above conclusion indicates the advantage of the MCB, i.e., easy for recovery and recycling *(20)*.

Methanolysis Activity of MCB

Figure 6 shows the composition of ME, which is converted by MCB from waste cooking oil. The reaction is under the conditions: temperature 40°C, pH 6.5, loading of MCB 3.0%, adding methanol in two stepwise and reacting for 72 h. The analytic result of Fig. 5 is listed in Table 2. It can be seen that all the components are ME and basically belong to longer carbon

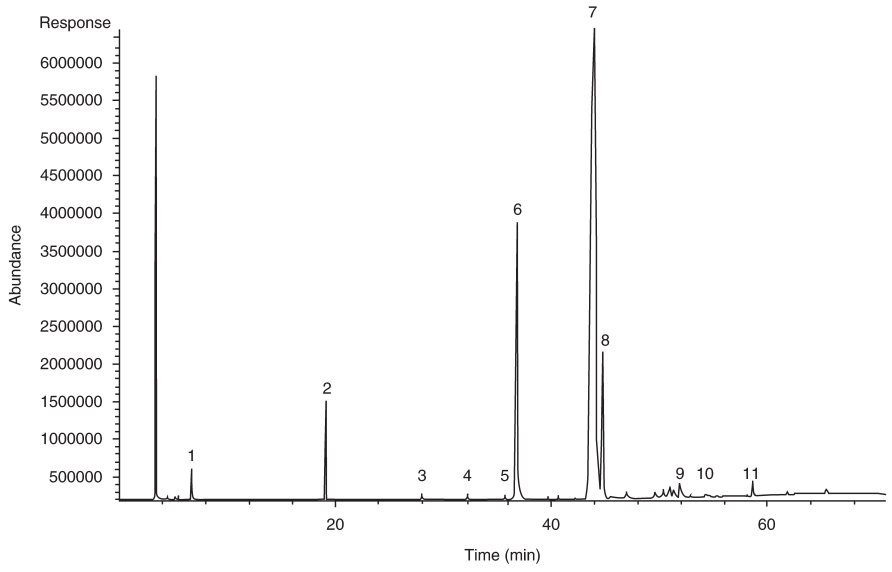

Fig. 5. The GC–MS spectrum of ME converted by MCB from waste cooking oil.

Table 2
Result of GC–MS Analysis on Chemical Composition of Methanolysis Production

No.	Compositions name	Retention time (min)
1	Decanis acid and ME	8.206
2	Methyl tetradecanoate	19.094
3	9-Hexadecenoic acid and ME	28.495
4	Hexadecanoic acid and ME	33.758
5	Pentadecanoic acid and 14-methyl ME	37.725
6	8,11-Octadecadienoic acid and ME	38.538
7	9-Octadecenoic acid and ME	44.034
8	Eicosanoic acid and ME	46.396
9	Docosanoic acid and ME	53.046
10	Tricosanoic acid and ME	56.126
11	Etracosanoic acid and ME	59.217

chain (>C_{12}). The main components are octadecadienoic acid ME (C18 : 1; C18 : 2), which is involved in the range of biodiesel fuel *(21)*. The ME content analyzed by capillary gas chromatography is about 90%. All of the peaks are methyl ester except for the first one which is the diluent.

Regeneration Property of MCB

The MCB particles can be repeated for use many times; however, the methanolysis activity will decrease gradually like immobilized lipase. The method of MCB regeneration was investigated in this study. When

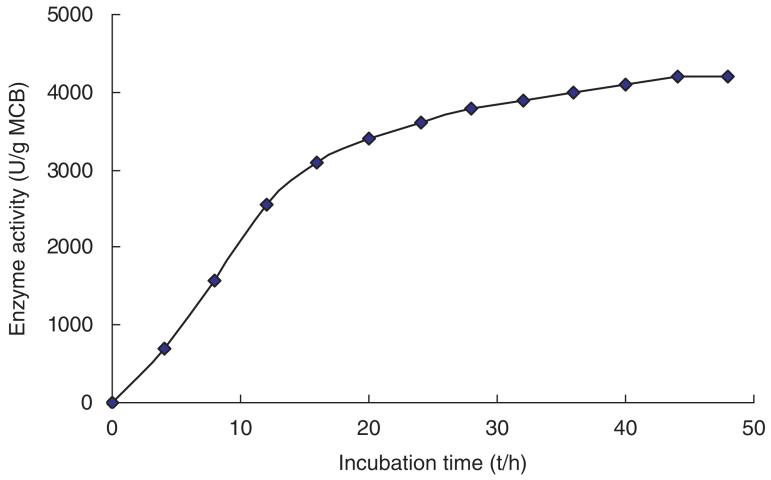

Fig. 6. Curve of enzyme activity change of MBC incubated in GYE medium.

enzyme activity of MCB decreases, the MCB particles separated from reactive mixture with a permanent magnet were washed with acetone and physiological saline solution before added into the GYE medium. The enzyme activity of MCB was assayed as described in Preparation of MCB section at interval of 4 h during 48 h cultivation. Figure 6 shows the curve of enzyme activity change when MCB particles regenerate. The results indicate *B. subtilis* cells encapsulated within polymicrosphere can propagate in the rich GYE medium, at the same time the enzyme activity of MCB increase gradually. All the results demonstrate the advantage of MCB in terms of easy regeneration, like cultivation of microorganisms.

Conclusions

A novel method of immobilizing cells within magnetic material has been developed for production of biodiesel from waste cooking oils. This new type of biocatalyst was characterized by various techniques, and the results demonstrate the MCB particles have superparamagnetic property and have the advantages of easy recovery, recycling, and regeneration. The conclusions of our work indicate MCB can avoid the disadvantage of extracellular lipase-catalyzed reaction, such as complicated recovery, purification, and immobilization process required. Moreover, the methanolysis of waste cooking oils with MCB shows high conversion up to 90%. Further work should be concentrated on optimizing the various reaction parameters in order to shorten the reaction time and also on investigating kinetic behavior of methanolysis reactions, which may be included in the nonaqueous media.

Acknowledgments

This work was financially supported by Ministry of Science and Technology (China) through National Key Fundamental Research Programme (Contract No. 200556617).

References

1. Du, W., Xu, Y. Y., Liu, D. H., and Zeng, J. (2004), *J. Mol. Catalysis B: Enzymatic* **30,** 125–129.
2. Köse, Ö., Tüter, M., and Aksoy, A. H. (2002), *Bioresour. Technol*. **83,** 125–129.
3. Iso, M., Chen, B. X., Eguchi, M., Kudo, T., and Shrestha, S. (2001), *J. Mol. Catalysis B: Enzymatic* **16,** 53–58.
4. Oda, M., Kaieda, M., Hama, S., et al. (2005), *Biochem. Eng. J.* **23,** 45–51.
5. Bahar, T. and Celebi, S. (2000), *Enzyme Microb. Technol*. **26,** 28–37.
6. Kondo, A., Liu, Y., Furuta, M., Fujita, Y., Matsumoto, T., and Fukuda, H., (2000), *Enzyme Microbial. Technol.* **27,** 806–811.
7. Ban, K., Hama, S., Nishizuka, K., et al. (2002), *J. Mol. Catalysis B: Enzymatic* **17,** 157–165.
8. Ban, K., Kaieda, M., Matsumotoc, T., Kondo, A., and Fukuda, H. (2001), *Biochem. Eng. J.* **8,** 39–43.
9. Ruiz, C., Blanco, A., Pastor, F. I. J., and Diaz, P. (2002), *FEMS Microbiol. Lett*. **217,** 263–267.
10. Eggert, T., Brockmeier, U., Dröge, M. J., Quax, W. J., and Jaeger, K. E. (2003), *FEMS Microbiol. Lett*. **225,** 319–324.
11. Ruiz, C., Pastor, F. I. J., and Diaz, P. (2003), *Lett. Appl. Microbiol*. **37,** 354–359.
12. Eggert, T., Pouderoyen, G. V., Pencreac'h, G., et al. (2002), *Colloids Surf. B: Biointerfaces*. **26,** 37–46.
13. Pouderoyen, G. V., Eggert, T., Jaeger, K. E., and Dijkstral, B. W. (2001), *J. Mol. Biol*. **309,** 215–226.
14. Eggert, T., Pouderoyen, G. V., Dijkstrab, B. W., and Jaeger, K. E. (2001), *FEBS Lett*. **502,** 89–92.
15. M. Z. Y., Guan, Y. P., Liu, X. Q., and Liu, H. Z. (2005), *Chin. J. Chem. Eng*. **13,** 239–243.
16. Bean, C. P. and Livingston, J. D. (1959), *J. Appl. Phys*. **30,** 120–129.
17. Xue, B. and Sun, Y. (2002), *J. Chromatogr. A*. **947,** 185–193.
18. Chantrell, R. W. and Popplewell, J. (1978), *IEEE Trans. Mag*. **14,** 975–980.
19. Zhang, M. L. and Sun, Y. (2001), *J. Chromatogr. A*. **912,** 31–38.
20. Kobayashi, H. and Matsunaga, T. (1991), *J. Colloid Interface Sci*. **141,** 505–511.
21. Kröbitz, W. (1999), *Renewasble Energy* **16,** 1078–1083.