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Full Length Research Paper

Separation and identification of lipase - producing strains and immobilized transesterification efficiency

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12 strains of lipase - producing were isolated from 36 samples collected from oil - contaminated soil of machinery - repair plant, dining room, and vegetable market, respectively, of which strain (3 - 2) had the highest hydrolytic activity (17.1 U/ml). Immobilized cell of strain 3 - 2 onto sodium alginate and immobilized crude enzyme solution of strain (3 - 2) onto Sodium alginate were respectively used in two transesterification programs to transfer the Fatty acid methyl ester (FAME). Results showed that both immobilized crude enzyme solution and cell were of high transesterification efficiency for Transesterification Program I, respectively 32.72 and 26.84%. Cycle test done in this Program found that immobilized crude enzyme solution and cell still keep some transesterification efficiency after being recycled thrice at least. Meanwhile, hydrolytic activity of crude enzyme solution had some correlation with transesterification efficiency. However, hydrolytic activity of immobilized cell did not have a clear correlation with the latter. Strain (3 - 2) belonged to *Sphingobacterium multivorum.* Up to now, the transesterification research has not been reported.

Key words: Lipase, crude enzyme solution, bacterial cell, immobilized, transesterification.

INTRODUCTION

Biodiesel produced by lipase defines fatty acid monoester obtained by transesterification or esterification of triglyceride (or fatty acid) with short - chain alcohol (mostly methanol) under lipase catalysis. The main composition of biodiesel is Fatty Acid Methyl Esters (FAME). Relative to chemical method, Enzymatic method has simpler recycle process for byproduct (that is glycerin), without saponification and moderate reaction condition (Yang et al., 2003; Zhou et al., 1999; Song and Yinbo, 1999; Zhang et al., 2003; Ren and Yingkuo, 1996)

Biodiesel production by enzymatic method features extremely extensive potential and has been massively concerned with nowadays (Wang et al., 2005).

Lipase comes from microorganism, insoluble in solvent and easy agglomeration while reaction, so these greatly reduce lipase utilization. Immobilized lipase may improve its dispersion and thermodynamic stability in solvent and fit for recycle and continuous production (Kirsty et al., 2000; Qiao et al., 1998). Immobilization methods for lipase includes covalent method, cross-link method, entrapping method and adsorption method. Adsorption carrier relies on adhesion link with protein. This simple immobilized process is one of the most economic methods (Chun et al., 2008). Carriers frequently used in adsorption include diatomite and quartz sand etc. (Shi Qiaoqin, 1981; Chen et al., 2006).

We immobilized cell and crude enzyme solution of lipase - producing stain (3 - 2) (isolated from oil-contaminated soil), study on their transesterification and hydrolytic activity in two different transesterification system. This assay evaluates their transesterification efficiency and its application value in biodiesel production industry.

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Table 1. Immobilized transesterification program.

Key compositions for transesterification program	Program I (Chen et al., 2006)	Program II (Chun et al., 2008)
Olive oil (ml)	5	4.8
Methanol (ml)	0.8	0.09
Immobilized bacterial cell (crude enzyme) (g)	1.2	1.2
n-hexane (ml)	44.2	1
Water (ml)	0	0.18
Volume (ml)	50	7.3
Temperature (ºC)	32	32
Rotational speed (r/min)	200	200
Cultivation time (h)	24	24

MATERIALS AND METHODS

Materials

Lipase producing strain 3 - 2 isolated from Chengdu China, olive, diatomite, acetone, sodium alginate, n-hexane, and ethanol (95%). Enrichment medium, screening medium, fermentation medium and others, were all AR and supplied by Chengdu Kelong Reagent Co. Ltd, peptone were supplied by OXOID Reagent Co., Ltd (America) (AR).

Methods

Separation and identification of lipase - producing strain

36 samples were collected from oil-contaminated soil of machinery-repair plant, dining room and vegetable market in Chengdu and peripheral area. After enrichment cultivation, Strains with obvious transparent circle on initially screening medium (contain bromcresol purple) were screened out and then the strain with the highest hydrolytic activity (olive oil emulsion method Gao et al., 2002) were further screened out. BIOLOG MicrostationTM System MicroLog3 4.20 (BIOLOG Inc., Hayward, CA) was used to identify the classify-cation of that strain by metabolic modes of 95 kinds of substrate utilization on GN2 identification plate. At the same time, 16S rRNA gene sequence of this strain was analyzed (EL Herry et al., 2008). The strain was used in the following study.

Fermentation of lipase - producting strain

The activated strain was inoculated to 100 ml fermentation medium by 1 % (scale) and was shaking cultured for 48 h with speed of 160 r/min at 32ºC.

Immobilization of crude enzyme solution with diatomite

We added 8 g diatomite (dried under 300ºC before use) to 100 ml crude enzyme solution (supernatant of fermentation medium culture by centrifuge, 6000 r/min), and mixed them for 3 h at 30 - 35ºC, centrifuged to obtain precipitation, washed it 3 - 5 times by acetone to make particle dispersed, and storage it at 4ºC after freeze drying.

Immobilization of bacterial cell by sodium alginate

The fermented culture was centrifuged 10 min by 6000 r/min to collect bacterial cell. And then mixed 18.0 g of it with saline (the ration was 1 g: 1 ml) and with 4% sodium alginate (90 ml), then dropped into $CaCl_2$ solution (0.15 M) and mixed them all. The mixture was curinged at 4°C for 3 h to be formed into sodium alginate immobilized ball (2 - 2.5 mm), at last washed them with saline, filtered and dried out, then stored at 4ºC.

Transesterification conditions with immobilized crude enzyme solution and bacterial cell

Immobilized crude enzyme solution and immobilized bacterial cell was undergoing transesterification test according to the following two programs (Table 1).

Cycle transesterification measurement for immobilized crude enzyme solution and bacterial cell

After mentioned test above, we found a better transesterification program. Immobilized crude enzyme solution and Immobilized bacterial cell were tranesterified circularly in this system. The change of hydrolytic activity was measured after each cycle with olive oil emulsion method (Chun et al., 2008) GC-MS was used to analyze the content of methyl ester.

GC-MS analysis for transesterification product

The transesterification product was transported through chromato-graphic column Rtx-5si1MS (30 m × 0.25 mm × 0.25 m), with following conditions: helium acted as carrier gas, ion source under 200ºC and 1000 cm/s sample injection rate and no shunt. Sample injection volume was 1 l, and 50 min analyzing time. With area normalization method, the content of FAME in the product was analyzed.

RESULTS

Separation and identification for lipase - producing strain

12 strains of lipase - producing bacteria were obtained from 36 soil samples, and their hydrolytic activity were tested (Gao et al., 2002), shown in Table 2, including highest hydrolytic activity for strain (3 - 2) with 17.1U/ml. Strain (3 - 2) was used to study on transesterification efficiency in following.

Table 2. Comparison with 12 strians of lipase-producing bacteria.

Strain no.	Hydrolytic activity (U/ml)
4 - 1	0.5
4 - 3	0.6
4 - 4	10.2
4 - 5	0.7
4 - 6	0.3
5 - 2	3.3
5 - 3	1.1
5 - 4	1.2
3 - 1	3.7
(3 - 2)	17.1
N10-H	4.8
N1-H	0.5

16S rRNA sequence of strain (3 - 2) was obtained by 16S rDNA PCR. Through BLAST alignment, the similarity of strain (3 - 2), *Sphingobacterium* sp. GF2B, *Sphingobacterium* sp. QMT3-2 and *Sphingobacterium multivorum* was 97%. Construction of Evolutionary Tree of strain (3 - 2) with UPGMA (MEGA4.0 software) showed that strain (3 - 2) and *S. multivorum* were on the same evolutionary branch (Figure 1) at the same time; strain (3 - 2) was identified as *S. multivorum* by BIOLOG. On the basis of the characteristics of morphology, physiology and biochemistry, and analysis of 16S rRNA gene sequence, strain (3 - 2) was identified as *S. multivorum*.

Transesterification efficiency for immobilized crude enzyme solution and bacterial cell

Sodium alginate immobilized bacterial cell were white small spherical, easy for separation from the reactant. Diatomite - immobilized crude enzyme had the main characters, as follows: white powder, insoluble in water and organic solvents. The same weight of immobilized enzyme and bacterial cell (each with 1.2 g) was added individually to the transesterification test (Chen et al., 2006; Chun et al., 2008). GC-MS was used to analyze transesterification efficiency through analyzing the content of FAMEs in products, and results showed that both immobilized crude enzyme solution and immobilized bacterial cell showed higher transesterification efficiency in transesterification program I (Table 3) compared to program II, respectively as 26.84 and 32.72%.

Analysis on transesterification product (GC-MC) in immobilized crude enzyme solution under program I, showed that (Figure 2), it has highest content of FAMEs, including Methyl Heptadecanoate, Methyl Linoleate, Methyl Oleate, Methyl Separate (arrow showed in Figure 2), totally 32.72% among products. Secondly, fatty acid (retention time: 42.928, 21.99% content), in addition, there also had fatty acid ethyl ester (retention time: 43.661, 7.52% content) and alkane (18.7% content).

Study on transesterification character of immobilized crude enzyme solution and bacterial cell under different cycle times

Change of hydrolytic activity for immobilized crude enzyme solution and bacterial cell under different cycle times

Optimal transesterification program I was used for transesterification test for cycle times with immobilized crude enzyme solution and bacterial cell as catalyst (shown in Table 4), and its results showed that, along with increased cycle times, immobilized crude enzyme solution may recycle, its hydrolytic activity gradually lowers with increasing cycle times. Immobilized bacterial cell has relatively less hydrolytic activity and much lower along increase in cycle times.

Change of transesterification efficiency under different cycle times

From Table 5, Program I with the immobilized crude enzyme solution and bacterial cell as catalyst separately show reduced transesterification efficiency with the increased cycle times. After three cycles, they also still had some transesterification efficiency.

DISCUSSION

Effects of immobilized material on transesterification

The gap between diatomite used to fix crude enzyme solution is small, it can prevent tiny particles to come in, free from chemical reaction with enzyme, free from affecting physical or chemical properties for enzyme, moreover, immobilized crude enzyme solution with diatomite recovered easy, and increase their interaction with cleaning up by acetone. Reuse of immobilized crude enzyme solution may reduce the cost of biodiesel production (Margolin et al., 1987). Long Zhangde et al. (2007) found that, *Serratia marcescens* lipase immobilized by diatomite and Eupergit C may increase its thermal stability and storage time. Lipase can dissolve in organic solvent, and need not covalent combination between immobilized lipase and, carrier, so diatomite adsorption method have been widely used (Sànchezetal, 2002; Kagaetal, 1994; Kharean and Nakajima, 2000) (Shuo-FenChang et al., 2007). This Article found that although the transesterification efficiency of immobilized bacterial cell was less than that of crude enzyme solution, its transesterification reducing rate is much lower than that of immobilized crude enzyme solution along with the increasing cycle. This may result from that immobilized

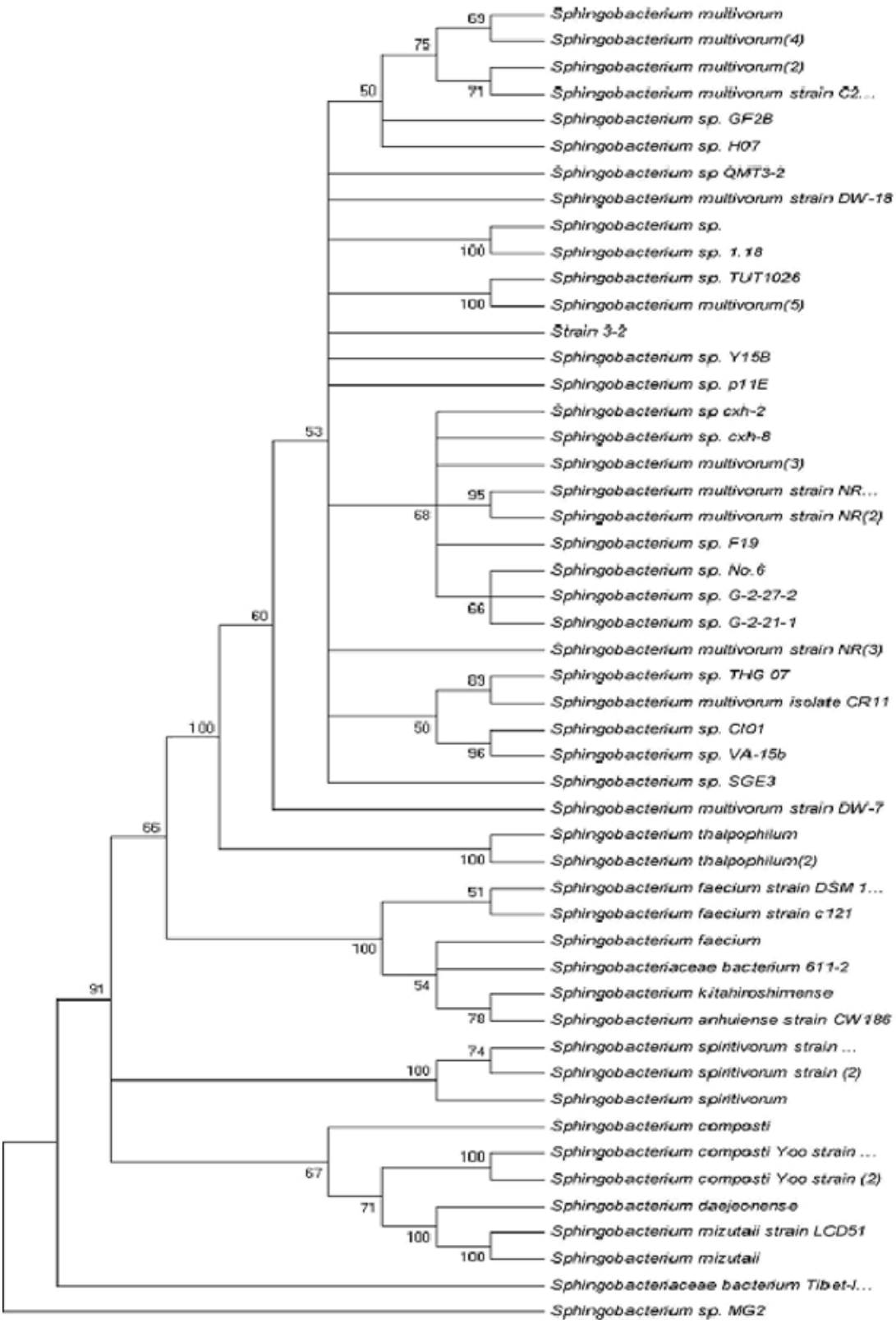

Figure 1. Construction of evolutionary tree of strain (3 - 2) with UPGMA (MEGA4.0 software).

bacterial cell continuously produce lipase through trans- esterification process, this is one of the benefits of immo-

Table 3. Transesterification efficiency in two programs for immobilized crude enzyme solution and Immobilized bacterial cell (unit: %).

Catalyst	Program I	Program II
Immobilized crude enzyme solution	32.72	10.85
Immobilized bacterial cell	26.84	10.91

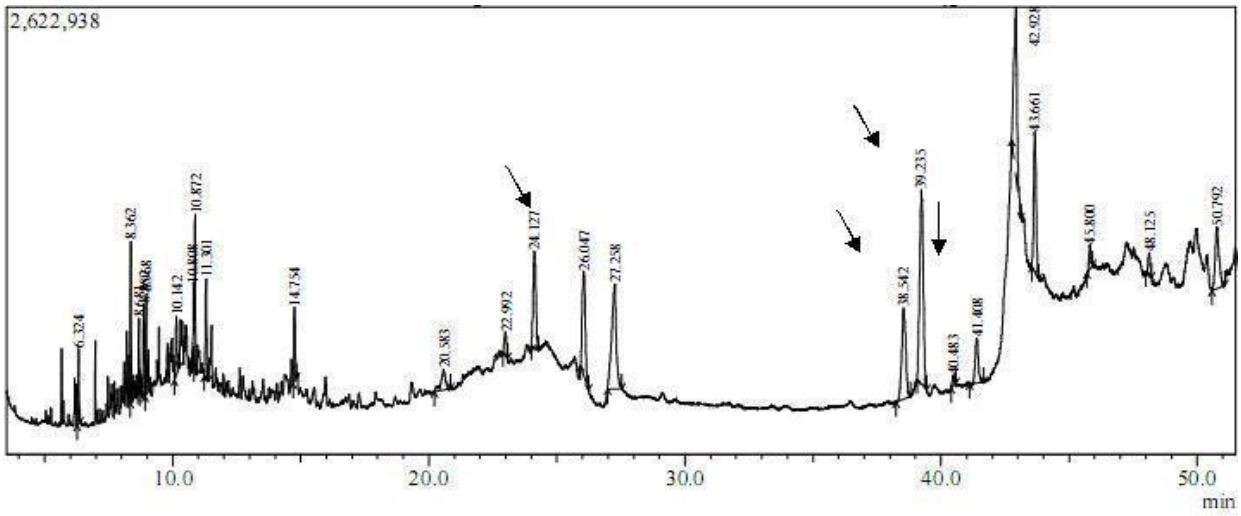

Figure 2. Product (GC-MS) analysis of immobilized crude enzyme solution in transesterification Program I.
Note: a, Methyl Heptadecanoate, b. Methyl Linoleate, c, Methyl Oleate, d. Methyl Separate;

Table 4. Hydrolytic activity change for different cycle times for immobilized crude enzyme solution and bacterial cell (Unit: U/ml).

Cycle times	Immobilized crude enzyme solution	Immobilized bacterial cell
1	16.8	9.3
2	10.2	5.7
3	2.5	0

Table 5. GC analysis result for transesterification product from immobilized crude enzyme solution and bacterial cell under different cycle times (Unit: %).

Cycle times	Immobilized crude enzyme solution	Immobilized bacterial cell
1	32.72	26.84
2	18.38	16.13
3	12.24	10.51

bilized bacterial cell in actual production.

Relationship between transesterification efficiency and hydrolytic activity

Goujard et al. (2009) reported there may no correlation between hydrolytic activity and transesterification efficiency of lipase. Xiao Yan Wu et al. (1996) utilized nine kinds of commercial lipases to research on their hydrolytic activity, esterification and transesterification activity, and found that there was some correlation between esterification and transesterification activity only for *Black Rhizomuco* lipase, but there was no correlation with hydrolytic activity. Hydrolytic and esterification activity for lipase from other source (including *purple bacteria*) has no correlation at all; even lipase with high hydrolytic activity has no transesterification activity. Toshiyuki et al.

(1995) found that, in 50% dioxide solution, transesterification activity for lipase might be estimated by hydrolytic activity by Colormetric Testing only when they use p-Nitrophenyl esters as substrate. Our results showed that, hydrolytic and transesterification activity for strain (3 - 2) crude enzyme and bacteria cell were in turn lowers with increasing cycle times. It showed that there was certain correlation between the two activities. While the immobilized bacterial cell had lower hydrolytic activity, this might result from exoenzyme secrete time, the test only had 10 min reaction time while measuring. If there was not full time to excrete enzyme, this may affect test result. So immobilized bacterial cell hydrolytic activity and transesterification efficiency has no obvious correlation; immobilized bacterial cell still has 10.51% transesterification efficiency even if hydrolytic activity measurement value is zero.

Transesterification reaction analysis for strain (3 - 2)

Two transesterification programs was used in the study, both refer to commercial lipase transesterification method, immobilized crude enzyme solution and immobilized bacterial cell both had higher efficient for transesterification under program I than program II, which may be resulted from that alcohol/oil molar ratio of program I is higher than that of program II, namely 0.17:1, and program I reaction volume reaches 50 ml in total, which made immobilized crude enzyme solution or immobilized bacterial cell fully contact with reactant; but program II has less volume, worse reaction contact. Even if alcohol/oil molar ratio of the two programs are not high, this may find that strain (3 - 2) also has some potential for study of production of FAME in biodiesel industry, fatty acid ethyl ester and alkane of esterified products also valuable in diesel compound. After identification, strain (3 - 2) are *s. multivorum* , such a microbial stain is -poly-L-lysine tolerant strain, which has excellent solubility and biological degradation, edibility and nontoxicity. It has extensive prosperity in food preservation, biodegradable fiber, drug carrier (Ing- LungShih et al., 2006). Most important, such microbial strain has not been reported yet with respect to study of lipase and applied to transesterification.

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REFERENCES

Chen Z, Wu H, Zong M (2006). Lipase-catalyzed Production of Biodiesel from High Acid waste oil. Chin. J. Catal. 27(2): 146-150

Chun H, Xie W, Wang Y, Wang T (2008). Study on immobilized lipase catalysis tansesterification into biodiesel, Grain Oil Grease 8: 12-15.

EL Herry Soumeya, Nasri Hichem, Bouaicha Noureddine (2008). Morphological and phylogénétic analysis of colonies of Microcystis morphospecies isolated from the lebna dam, Tunisia. Afr. J. Microbiol. Res. l2(12): 340-348.

Gao Gui, Han Siping, Wang Zhi, Weng Liang, Wang Baijing, Feng Yan, Cao Shugui, Gao Shujuan (2002). Comparison of lipase activity detection methods [J]. Med. Biol. Technol. 9(5):281-284.

Goujard L, Villeneuve P, Barea B, Lecomte J, Pina M, Claude S, Le Petit J, Ferré E. (2009). A spectrophotometric transesterication-based assay for lipases in organic solvent. Anal. Biochem. 385: 161-167.

Ing-LungShih, Ming-HawShen, Yi-TsongVan (2006). Microbial synthesis of poly (-lysine) and its various applications, Bioresour. Technol. 97: 1148-1159.

Kirsty K, Maria CBP, Marcia MCM, William M, Ledingham JLLF, Maria MDM (2000). Immobilization of lipase from *Fusarium solani* FS, Brazilian J. Microbiol. 31: 220-222.

Long Zhangde, XU Jianhe, PAN Jiang (2007). Immobilization of *Serratia marcescens* Lipase and Catalytic Resolution of Trans-3-(4'-methoxyphenyl) glycidic. Chin. J. Catal. 28(2): 175-179

Margolin AL, Crenne JY, Klibanov AM (1987). Selection Oligomerization Catalyzed by Lipase in Organic Solvents. Biotechnol. Lett. 9: 1607-1610. property of alkaline lipase-producting strain. Ind. Microorganism. 29(4): 22-26.

Qiao Hongqun, Xu Hong, Fu Shanlei et al (1998). Research on screening and enzymatic propery of lipase-producting strain. J. Nanjing Chem. Uni. J. 20(l): 15-19.

Ren Lihong, Zhou Yingkuo (1996). Study on screening for lipase-producing strain and opimal lipase production condition of *Aspergillus*. Ind. Microorganism 26(1): 23-26.

Shi Qiaoqin (1981). Research on alkaline lipase. Microorganism Bull. 8(3): 108-110.

Shuo-FenChang, Shu-WeiChang, Yue-HorngYen, Chwen-Jen Shieh (2007). Optimum immobilization of *Candida rugosa* lipase on Celite by RSM. Appl. Clay Sci. 37: 67-73.

Song Xin, Qu Yinbo (1999). Research on breeding and enzymatic.

Toshiyuki urutani, Ronghui Su, Hiroshi Ooshima, Jyoji Kato (1995). Simple screening method for lipase for transesterification in organic solvent, Enzyme Microb. Technol. 17: 1067-1072.

Wang Zhong, Yuan Yinnan, Li Baolu, Mei Deqing, Sun Ping (2005). Study on emission test for biodiese. J. Agric. Ind. 21(7): 77-80.

Xiao Yan Wu, Sanna JEiskeliiinen, Yu-Yen Linko (1996). An investigation of crude lipases for hydrolytic, esterification, and transesterification, Enzyme. Microb. Technol. 19: 226-231.

Yang Yongmei, Han Wang, Wei Wu, Hong Fang, Liu Chengjun (2003).Study on selection of strain produced by alkaline lipase and enzyme-producting condition and enzymatic property. J. Sichuan Univ. Nat. Sci. 40(5): 935-938.

Zhang Liying, Wei Dongzhi, Tong Wangyu (2003). Study on optimal fatty acid production condition of *Candida rugosa*. Study Life Sci. 7(4): 320-323.

Zhou Xiaoyun, Huang Jianning, Wang Huizhong, Wang Rongwei (1999). Study on Streptomyces alkaline lipase. J. Appl. Environ. Biol. 5(5): 529-532.