

Optimum Synthesis of Lipase-catalyzed Biodiesel Using a Continuous Packed-bed Bioreactor

吳宗達、謝淳仁

E-mail: 9607699@mail.dyu.edu.tw

ABSTRACT

Biodiesel (fatty acid alkyl esters) is synthesized by transesterification of triglycerol with short-alcohol and have recently attracted attention due to its environmental benefits and renewable resource. Most of them are industrially produced by chemical method but it has many drawbacks such as high temperature, difficulty in recovery glycerol, the removal requirement of salt residues, and high energy cost. To overcome these drawbacks, the utilization of biocatalysts (enzymatic) to synthesize biodiesel by transesterification under mild conditions has attracted considerable attention in recent years. A useful method for enzymatic synthesis biodiesel catalyzed by immobilized lipase from *Candida antarctica* (NovozymR 435) in a continuous process was investigated. NovozymR 435 was packed in a packed-bed reactor to catalyze transesterification of methanol (or isopropanol) and soybean oil for biodiesel synthesis in tert-butanol (or solvent-free) system will be discussed in this study. Response surface methodology (RSM) and 3-factor-3-level Box-Behnken design were employed to evaluate the effects of synthesis parameters, such as flow rate (0.1 – 0.5 mL/min), temperature (40 – 50 °C), and substrate molar ratio of methanol (or isopropanol) to soybean oil (1:3 – 1:5) on percentage molar conversion of biodiesel by transesterification. The result shows that temperature and flow rate were significant effects on the percent molar conversion in the two systems (methanol and isopropanol). Based on ridge max analysis, (1) in the conversion of methyl esters, the optimum conditions for synthesis were: temperature 52.09 °C, flow rate 0.10 mL/min, and substrate molar ratio 1:4. The predicted value was $83.31 \pm 2.07\%$ and actual experimental value was $82.81 \pm 0.98\%$ molar conversion. (2) In the conversion of isopropyl esters, the optimum conditions for synthesis were: temperature 51.5 °C, flow rate 0.10 mL/min, and substrate molar ratio 1:4.14. The predicted value was $76.62 \pm 1.52\%$ and actual experimental value was $75.62 \pm 0.81\%$ molar conversion. Moreover, synthesis methyl and isopropyl esters with continuous process did not show any appreciable decrease in the percent molar conversion for over 30 d and 7 d, respectively. It demonstrates that synthesis of lipase-catalyzed biodiesel was produced by effective in scale-up of industrialization.

Keywords : Biodiesel ; Isopropanol ; Lipase ; Transesterification ; Packed-bed reactor ; Response surface methodology

Table of Contents

封面內頁 簽名頁 授權書.....	iii	中文摘要.....	iv	英文摘要.....	ix
要.....	vi	誌謝.....	viii	目錄.....	ix
表目錄.....	xii	圖目錄.....	xiii	1. 緒言.....	1
1.2. 文獻探討.....	5	2.1. 生質柴油.....	5	2.1.1 石油的枯竭與環境的影響.....	5
2.1.2 生質柴油的簡介.....	6	2.1.3 生質柴油之特性.....	7	2.1.4 全球生質柴油發展近況.....	9
2.1.5 生質柴油在臺灣之發展.....	12	2.1.6 生質柴油的合成方法.....	12	2.1.7 轉酯化反應.....	13
2.2 酵素.....	15	2.2.1 酵素之優點.....	15	2.2.2 酵素固定化之優點.....	16
2.2.3 脂解酵素.....	17	2.2.4 脂解酵素之應用.....	17	2.2.5 NovozymR 435之介紹.....	18
2.3 生物反應器.....	18	2.3.1 生物反應器種類.....	19	2.4 相關文獻.....	21
2.4.1 基質之相關探討.....	21	2.4.2 批次式生產生質柴油.....	23	2.4.3 連續式生產生質柴油.....	27
3. 材料與方法.....	29	3.1 材料與方法.....	29	3.1.1 藥品.....	29
3.1.2 儀器設備.....	29	3.2 實驗設計.....	30	3.2.1 反應變數範圍之選定.....	30
3.2.2 酵素之選擇.....	31	3.2.3 合成方法.....	34	3.2.4 測量酵素水含量.....	36
3.2.5 酵素活性分析.....	36	3.2.6 分析方法.....	39	3.2.7 產率計算.....	39
4. 結果與討論.....	40	4.1 以連續式反應酵素催化大豆油與甲醇合成生質柴油.....	40	4.1.1 溫度對連續式合成脂肪酸甲酯之莫耳轉換率影響.....	40
4.1.2 流速對連續式合成脂肪酸甲酯之莫耳轉換率影響.....	44	4.1.3 基質莫耳比對連續式合成脂肪酸甲酯之莫耳轉換率影響.....	44	4.1.4 脂肪酸甲酯之數據分析.....	44
4.1.5 脂肪酸甲酯之最優化合成探討.....	47	4.1.6 重複使用性之探討(脂肪酸甲酯).....	56	4.2 以連續式反應酵素催化大豆油與異丙醇.....	

合成生質柴油.....	58	4.2.1 溫度對連續式合成脂肪酸異丙酯之莫耳轉換率影響.....	58	4.2.2 流速對連續式合成脂肪酸異丙酯之莫耳轉換率影響.....	61
脂肪酸異丙酯之莫耳轉換率影響.....	58	4.2.3 基質莫耳比對連續式合成脂肪酸異丙酯之莫耳轉換率影響.....	61	4.2.4 脂肪酸異丙酯之數據分析.....	61
脂肪酸異丙酯之數據分析.....	61	4.2.5 脂肪酸異丙酯之最優化合成探討.....	64	4.2.6 重複使用性之探討(脂肪酸異丙酯).....	70
4.3 合成脂肪酸甲酯與脂肪酸異丙酯之結果比較.....	74	5. 結論.....	75	參考文獻.....	77
附錄.....	87				

REFERENCES

- 李晉嘉。2003。以反應曲面法研究生化柴油之最優化酵素合成。大葉大學碩士論文。彰化。
- 行政院經濟建設委員會。2006。中華民國96年國家建設計畫。下篇。第105 – 106頁。台灣。
- 陳國誠。2000。生物固定化技術與產業應用。第121 – 155頁。茂昌圖書有限公司。臺北。
- 經濟部能源局網站: <http://210.69.152.10/oil102/?group=g>
- Abigor, R. D., Uadia, P. O., Foglia, T. A., Haas, M. J., Jones, K. C., Okpefa, E., Obibuzor, J. U. and Bafor, M. E. 2000. Lipase-catalysed production of biodiesel fuel from some Nigerian lauric oils. *Biochemical Society Transactions* 28(6): 979 – 981.
- Ali, Y. and Hanna, M. A. 1994. Alternative diesel fuels from vegetable oils. *Bioresource Technology* 50(2): 153 – 163.
- Ali, Y., Hanna, M. A. and Leviticus, L. I. 1995. Emissions and power characteristics of diesel engines on methyl soyate and diesel fuel blends. *Bioresource Technology* 52(2): 185 – 195.
- Altin, R., C?etinkaya, S. and Yu?cesu, H. S. 2001. Potential of using vegetable oil fuels as fuel for diesel engines. *Energy Conversion and Management* 42(5): 529 – 538.
- Bai, S., Guo, Z., Liu, W. and Sun, Y. 2006. Resolution of (\pm)-menthol by immobilized *Candida rugosa* lipase on superparamagnetic nanoparticles. *Food Chemistry* 96(1):1 – 7.
- Balca?o, V. M. and Malcata, F. X. 1998. Lipase catalyzed modification of milkfat. *Biotechnology Advances* 16(2): 309 – 341.
- Balca?o, V. M., Paiva, A. L. and Malcata, F. X. 1996. Bioreactors with immobilized lipases: State of the art. *Enzyme and Microbial Technology* 18(6): 392 – 416.
- Ban, K., Kaieda, M., Matsumoto, T., Kondo, A. and Fukuda, H. 2001. Whole cell biocatalyst for biodiesel fuel production utilizing *Rhizopus oryzae* cells immobilized within biomass support particles. *Biochemical Engineering Journal* 8(1): 39 – 43.
- Ban, K., Hama, S., Nishizuka, K., Kaieda, M., Matsumoto, T., Kondo, A., Noda, H. and Fukuda, H. 2002. Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. *Journal of Molecular Catalysis B Enzymatic* 17(3 – 5): 157 – 165.
- Chang, H. M., Liao H. F., Lee C. C. and Shieh, C. J. 2005. Optimized synthesis of lipase-catalyzed biodiesel by Novozym 435. *Journal of Chemical Technology and Biotechnology* 80(3): 307 – 312.
- Chen, J. W. and Wu, W. T. 2003. Regeneration of immobilized *Candida antarctica* lipase for transesterification. *Journal of Bioscience and Bioengineering* 95(5): 466 – 469.
- Demirkol, S., Aksoy, H. W., Tu?ter, M., Ustun, G. and Sasmaz, D. A. 2006. Optimization of enzymatic methanolysis of soybean oil by response surface methodology. *Journal of the American Oil Chemists' Society* 83(11): 929 – 932.
- Derewenda, Z. S. and Sharp, A. M. 1993. News from the interface: The molecular structures of triacylglyceride lipases. *Trends in Biochemical Sciences* 18(1): 20 – 25.
- Dossat, V., Combes, D. and Marty, A. 1999. Continuous enzymatic transesterification of high oleic sunflower oil in a packed bed reactor: Influence of the glycerol production. *Enzyme and Microbial Technology* 25(3 – 5): 194 – 200.
- Du, D., Sato, M., Mori, M. and Park, E. Y. 2006. Repeated production of fatty acid methyl ester with activated bleaching earth in solvent-free system. *Process Biochemistry* 41(8): 1849 – 1853.
- Du, W., Wang, L. and Liu, D. 2007. Improved methanol tolerance during Novozym435-mediated methanolysis of SODD for biodiesel production. *Green Chemistry* 9(2): 173 – 176.
- Du, W., Xu, Y. and Liu, D. 2003. Lipase-catalysed transesterification of soya bean oil for biodiesel production during continuous batch operation. *Biotechnology and Applied Biochemistry* 38(2): 103 – 106.
- Du, W., Xu, Y. Y., Liu, D. H. and Li, Z. B. 2005. Study on acyl migration in immobilized lipozyme TL-catalyzed transesterification of soybean oil for biodiesel production. *Journal of Molecular Catalysis B: Enzymatic* 37(1 – 6): 68 – 71.
- Du, W., Xu, Y., Liu, D. and Zeng, J. 2004. Comparative study on lipase-catalyzed transformation of soybean oil for biodiesel production with different acyl acceptors. *Journal of Molecular Catalysis B: Enzymatic* 30(3 – 4): 125 – 129.
- Dunn, R. O., Shockley, M. W. and Bagby, M. O. 1996. Improving the low-temperature properties of alternative diesel fuels: Vegetable oil-derived methyl esters. *Journal of the American Oil Chemists' Society* 73(12): 1719 – 1728.
- Fernando, S., Karra, P., Hernandez, R. and Jha, S. K. 2007. Effect of incompletely converted soybean oil on biodiesel quality. *Energy* 32(5): 844 – 851.
- Foidl, N., Foidl, G., Sanchez, M., Mittelbach, M. and Hackel, S. 1996. *Jatropha curcas* L. as a source for the production of biofuel in Nicaragua. *Bioresource Technology* 58(1): 77 – 82.
- Fomuso, L. B. and Akoh, C. C. 2002. Lipase-catalyzed acidolysis of olive oil and caprylic acid in a bench-scale packed bed bioreactor. *Food Research International* 35(1): 15 – 21.
- Freedman, B., Pryde, E. H. and Mounts, T. L. 1984. Variables affecting the yields of fatty esters from transesterified vegetable oils. *Journal of the American Oil Chemists' Society* 61(10): 1638 – 1643.
- Guthalugu, N. K., Balaraman, M. and Kadimi, U. S. 2006. Optimization of enzymatic hydrolysis of triglycerides in soy deodorized distillate with supercritical carbon dioxide. *Biochemical Engineering Journal* 29(3): 220 – 226.
- Hacking, M. A. P. J., Akkus, H., Van Rantwijk, F. and Sheldon, R. A. 2000. Lipase and esterase-catalyzed acylation of hetero-substituted nitrogen nucleophiles in water and organic solvents. *Biotechnology and Bioengineering* 68(1): 84 – 91.
- Hama, S., Yamaji, H., Fukumizu, T., Numata, T., Tamalampudi, S., Kondo, A., Noda, H. and Fukuda, H. 2007. Biodiesel-fuel production in a packed-bed reactor using lipase-producing *Rhizopus oryzae* cells immobilized within biomass support particles. *Biochemical Engineering Journal* 34(3): 273 – 278.
- Hari Krishna, S., Manohar, B., Divakar, S., Prapulla, S. G. and Karanth, N. G. 2000. Optimization of isoamyl acetate production by using immobilized lipase from *Mucor miehei* by response surface methodology. *Enzyme and Microbial Technology* 26(2-4): 131 – 136.
- Hsu, A. F. Jones, K. C. Foglia, T. A. and Marmer, W. N. 2004. Continuous production of ethyl esters of grease using an immobilized lipase. *Journal of the American Oil*

Chemists' Society 81(8): 749 – 752. 34.Iso, M., Chen, B., Eguchi, M., Kudo, T. and Shrestha, S. 2001. Production of biodiesel fuel from triglycerides and alcohol using immobilized lipase. *Journal of Molecular Catalysis B: Enzymatic* 16(1): 53 – 58. 35.Joshi, R. M. and Pegg, M. J. 2007. Flow properties of biodiesel fuel blends at low temperatures. *Fuel* 86(1-2): 143 – 151. 36.Kaieda, M., Samukawa, T., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H., Nomoto, F., Ohtsuka, K., Izumoto, E. and Fukuda, H. 1999. Biodiesel fuel production from plant oil catalyzed by *Rhizopus oryzae* lipase in a water-containing system without an organic solvent. *Journal of Bioscience and Bioengineering* 88(6): 627 – 631. 37.Knapp, T. and Mookerjee, R. 1996. Population growth and global CO₂ emissions: A secular perspective. *Energy Policy* 24(1): 31 – 37. 38.Knothe, G. 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Processing Technology* 86(10): 1059 – 1070. 39.Knothe, G., Matheaus, A. C. and Ryan III, T. W. 2003. Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester. *Fuel* 82(8): 971 – 975. 40.Kojima, S., Du, D., Sato, M. and Park, E. Y. 2004. Efficient production of fatty acid methyl ester from waste activated bleaching earth using diesel oil as organic solvent. *Journal of Bioscience and Bioengineering* 98(6): 420 – 424. 41.Kořbitz, W. 1999. Biodiesel production in Europe and North America, an encouraging prospect. *Renewable Energy* 16(1-4): 1078 – 1083. 42.Kořse, O., Tuřter, M., and Aksoy, H. Ays?e. 2002. Immobilized *Candida antarctica* lipase-catalyzed alcoholysis of cotton seed oil in a solvent-free medium. *Bioresource Technology* 83(2): 125 – 129. 43.Krisnangkura, K. 1986. Simple method for estimation of cetane index of vegetable oil methyl esters. *Journal of the American Oil Chemists' Society* 63(4): 552 – 663. 44.Kuo, T. M. and Gardner, H. W. 2002. *Lipid biotechnology*. p. 387 – 398. Marcel Dekker. New York. USA. 45.Ladommatos, N., Parsi, M. and Knowles, A. 1996. The effect of fuel cetane improver on diesel pollutant emissions. *Fuel* 75(1): 8 – 14. 46.Lai, C. C., Zullaikah, S., Vali, S. R. and Ju, Y. H. 2005. Lipase-catalyzed production of biodiesel from rice bran oil. *Journal of Chemical Technology and Biotechnology* 80(3): 331 – 337. 47.Lang, X., Dalai, A. K., Bakhshi, N. N., Reaney, M. J. and Hertz, P. B. 2001. Preparation and characterization of bio-diesels from various bio-oils. *Bioresource Technology* 80(1): 53 – 62. 48.Lara Pizzarro, P. V. and Park, E. Y. 2004. Potential application of waste activated bleaching earth on the production of fatty acid alkyl esters using *Candida cylindracea* lipase in organic solvent system. *Enzyme and Microbial Technology* 34(3-4): 270 – 277. 49.Li, W., Du, W. and Liu, D. 2007. Optimization of whole cell-catalyzed methanolysis of soybean oil for biodiesel production using response surface methodology. *Journal of Molecular Catalysis B: Enzymatic* 45(3 – 4): 122 – 127. 50.Li, L., Du, W., Liu, D., Wang, L. and Li, Z. 2006. Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *Journal of Molecular Catalysis B: Enzymatic* 43(1 – 4): 58 – 62. 51.Lin, C. Y. and Lin, H. A. 2006. Diesel engine performance and emission characteristics of biodiesel produced by the peroxidation process. *Fuel* 85(3): 298 – 305. 52.Linko, Y. Y., Lařmsař, M., Wu, X., Uosukainen, E., Seppařlař, J. and Linko, P. 1998. Biodegradable products by lipase biocatalysis. *Journal of Biotechnology* 66(1): 41 – 50. 53.Ma, F., Clements, L. D. and Hanna, M. A. 1998. The effects of catalyst, free fatty acids, and water on transesterification of beef tallow. *Transactions of the American Society of Agricultural Engineers* 41(5): 1261 – 1264. 54.Ma, F. and Hanna M. A. 1999. Biodiesel production: a review. *Bioresource technology* 70(1): 1 – 15. 55.Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill Jr., C. G. and Amundson, C. H. 1990. Immobilized lipase reactors for modification of fats and oils. A review. *Journal of the American Oil Chemists' Society* 67(12): 890 – 910. 56.Modi, M. K., Reddy, J. R. C., Rao, B. V. S. K. and Prasad, R. B. N. 2007. Lipase-mediated conversion of vegetable oils into biodiesel using ethyl acetate as acyl acceptor. *Bioresource Technology* 98(6): 1260 – 1264. 57.Nelson, L. A., Foglia, T. A. and Marmer, W. N. 1996. Lipase-catalyzed production of biodiesel. *Journal of the American Oil Chemists' Society* 73(9): 1191 – 1195. 58.Nie, K., Xie, F., Wang, F. and Tan, T. 2006. Lipase catalyzed methanolysis to produce biodiesel: Optimization of the biodiesel production. *Journal of Molecular Catalysis B: Enzymatic* 43(1 – 4): 142 – 147. 59.Nielsen, N. S., Yang, T., Xu, X. and Jacobsen, C. 2006. Production and oxidative stability of a human milk fat substitute produced from lard by enzyme technology in a pilot packed-bed reactor. *Food Chemistry* 94(1): 53 – 60. 60.Noureddini, H., Gao, X. and Philkana, R. S. 2005. Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. *Bioresource Technology* 96(7): 769 – 777. 61.Pahl, G. 2005. *Biodiesel: growing a new energy economy*. p. 1 – 120. Chelsea green publishing company. USA. 62.Park, E. Y. and Mori, M. 2005. Kinetic study of esterification of rapeseed oil contained in waste activated bleaching earth using *Candida rugosa* lipase in organic solvent system. *Journal of Molecular Catalysis B: Enzymatic* 37(1 – 6): 95 – 100. 63.Park, E.Y., Sato, M. and Kojima, S. 2006. Fatty acid methyl ester production using lipase-immobilizing silica particles with different particle sizes and different specific surface areas. *Enzyme and Microbial Technology* 39(4): 889 – 896. 64.Pizarro, A. V. L. and Park, E. Y. 2003. Lipase-catalyzed production of biodiesel fuel from vegetable oils contained in waste activated bleaching earth. *Process Biochemistry* 38(7): 1077 – 1082. 65.Posorske, L. H. 1984. Industrial-scale application of enzymes to the fats and oil industry. *Journal of the American Oil Chemists' Society* 61(11): 1758 – 1760. 66.Pramanik, K. 2003. Properties and use of *Jatropha curcas* oil and diesel fuel blends in compression ignition engine. *Renewable Energy* 28: 239 – 248. 67.Pryde, E. H. 1983. Vegetable oils as diesel fuels: overview. *Journal of the American Oil Chemists' Society* 60(8): 1577 – 1588. 68.Royon, D., Daz, M., Ellenrieder, G. and Locatelli, S. 2007. Enzymatic production of biodiesel from cotton seed oil using t-butanol as a solvent. *Bioresource Technology* 98(3): 648 – 653. 69.Salis, A., Solinas, V. and Monduzzi, M. Wax esters synthesis from heavy fraction of sheep milk fat and cetyl alcohol by immobilised lipases. *Journal of Molecular Catalysis B: Enzymatic* 21(4-6): 167 – 174. 70.Salis, A., Pinna, M., Monduzzi, M. and Solinas, V. 2005. Biodiesel production from triolein and short chain alcohols through biocatalysis. *Journal of Biotechnology* 119(3): 191 – 299. 71.Samukawa, T., Kaieda, M., Matsumoto, T., Ban, K., Kondo, A., Shimada, Y., Noda, H. and Fukuda, H. 2000. Pretreatment of immobilized *Candida antarctica* lipase for biodiesel fuel production from plant oil. *Journal of Bioscience and Bioengineering* 90(2): 180 – 183. 72.Schumacher, L. G., Borgelt, S. C., Fosseen, D., Goetz, W. and Hires, W. G. 1996. Heavy-duty engine exhaust emission tests using methyl ester soybean oil/diesel fuel blends. *Bioresource Technology* 57(1): 31 – 36. 73.Shieh, C. J., Akoh, C. C. and Yee, L. N. 1996. Optimized enzymatic synthesis of geranyl butyrate with lipase AY from *Candida rugosa*.

Biotechnology and Bioengineering 51(3): 371 – 374. 74. Shieh, C. J., Liao, H. F. and Lee, C. C. 2003. Optimization of lipase-catalyzed biodiesel by response surface methodology. *Bioresource Technology* 88(2): 103 – 106. 75. Shimada, Y., Watanabe, Y., Samukawa, T., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. 1999. Conversion of vegetable oil to biodiesel using immobilized *Candida antarctica* lipase. *Journal of the American Oil Chemists' Society* 76(7): 789 – 793. 76. Shimada, Y., Watanabe, Y., Sugihara, A. and Tominaga, Y. 2002. Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *Journal of Molecular Catalysis B: Enzymatic* 17(3 – 5): 133 – 142. 77. Shah, S., Sharma, S., and Gupta, M. N. 2004. Biodiesel preparation by lipase-catalyzed transesterification of *Jatropha* oil. *Energy and Fuels* 18(1): 154 – 159. 78. Shah, S. and Gupta, M. N. 2007. Lipase catalyzed preparation of biodiesel from *Jatropha* oil in a solvent free system. *Process Biochemistry* 42(3): 409 – 414. 79. Soumanou, M. M. and Bornscheuer, U. T. 2003. Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. *Enzyme and Microbial Technology* 33(1): 97 – 103. 80. Soumanou, M. M. and Bornscheuer, U. T. 2003. Lipase-catalyzed alcoholysis of vegetable oils. *European Journal of Lipid Science and Technology* 105(11): 656 – 660. 81. Vicente, G., Coteron, A., Martinez, M. and Aracil, J. 1998. Application of the factorial design of experiments and response surface methodology optimize biodiesel production. *Industrial Crops and Products* 8(1): 29 – 35. 82. Wang, L., Du, W., Liu, D., Li, L. and Dai, N. 2006. Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with absorbent present in tert-butanol system. *Journal of Molecular Catalysis B: Enzymatic* 43(3 – 5): 29 – 32. 83. Watanabe, Y., Pinsirodom, P., Nagao, T., Yamauchi, A., Kobayashi, T., Nishida, Y., Takagi, Y. and Shimada, Y. 2007. Conversion of acid oil by-produced in vegetable oil refining to biodiesel fuel by immobilized *Candida antarctica* lipase. *Journal of Molecular Catalysis B: Enzymatic* 44(3 – 4): 99 – 105. 84. Watanabe, Y., Shimada, Y., Sugihara, A., Noda, H., Fukuda, H. and Tominaga, Y. 2000. Continuous production of biodiesel fuel from vegetable oil using immobilized *Candida antarctica* lipase. *Journal of the American Oil Chemists' Society* 77(4): 355 – 360. 85. Watanabe, Y., Shimada, Y., Sugihara, A. and Tominaga, Y. 2001. Enzymatic conversion of waste edible oil to biodiesel fuel in a fixed-bed bioreactor. *Journal of the American Oil Chemists' Society* 78(7): 703 – 707. 86. Watanabe, Y., Shimada, Y., Sugihara, A., Tominaga, Y. 2002. Conversion of degummed soybean oil to biodiesel fuel with Immobilized *Candida antarctica* lipase. *Journal of Molecular Catalysis B: Enzymatic* 17(3 – 5): 151 – 155. 87. Xi, W. W. and Xu, J. H. 2005. Preparation of enantiopure (S)-ketoprofen by immobilized *Candida rugosa* lipase in packed bed reactor. *Process Biochemistry* 40(6): 2161 – 2166. 88. European Biodiesel Brand. <http://www.ebb-eu.org/>