

# 以褐藻酸鈉為擔體進行假絲酵母脂肪?固定化及其生化特性分析之研究

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## 摘要

固定化酵素不僅可以保持酵素的催化特性，也可以克服游離酵素之不足之處，使其具有一般化學催化劑所包含的特性並且能夠回收以及重複使用的優點，另外在工業上能夠進行連續化與自動化。本研究初期先將Candida rugosa lipase固定在褐藻酸鈉與Bentonite兩種擔體上並比較其活性差異。研究結果顯示以褐藻酸鈉固定酵素所獲得之酵素活性及比活性較佳，以Bentonite作為擔體而言高出兩倍。明顯看出褐藻酸鈉固定C. rugosa脂肪?之效果優於Bentonite。因此本研究繼以褐藻酸鈉為載體，探討最適固定化條件及不同反應條件對固定化酵素之影響。結果顯示，褐藻酸鈉的濃度為6%時可以最優化固定化酵素的活性，氯化鈣濃度對固定化酵素之活性影響不大。固定化酵素之最適作用溫度與pH值分別為50 °C 與pH 7。酵素於40 °C 下具有最佳穩定性。在pH值為5時，酵素之穩定性最高。固定化酵素之儲存穩定性極佳，於4 °C 放置30天以後，依然保有90%以上之相對活性，而於30 °C 放置30天後也保有80%以上之活性。研究中亦利用掃描式電子顯微鏡對固定化顆粒表面進行拍攝。結果顯示，表面結構會根據濃度的不同而有所改變，濃度越高表面結構越緊密，反之亦越鬆弛。

關鍵詞：假絲酵母脂肪?、褐藻酸鈉、皂土、包埋法、固定化、最優化

## 目錄

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## 參考文獻

- 1.方彥程。2007。柴油降解菌應用於土耕法及結合細胞固定化技術降解水中柴油之研究：第28-32頁。國立成功大學環境工程學系碩士論文。台南
- 2.方菘信。1997。纖維水解酵素之固定化與其特性研究。大葉大學。彰化。
- 3.沈宗禮。1980。制放技術與微粒包埋。高立出版，第127-134頁。
- 4.何宜芳。2004。新型重組耐熱性Archaeoglobus Fulgidus 脂肪?之生化特性分析。國立台灣海洋大學碩士論文。基隆。
- 5.吳宗達。2007。以連續式填充床生物反應器探討脂解酵素催化生質柴油之最優化合成。大葉大學。彰化。
- 6.吳昭燕。2003年10月。科學發展。370期。第40-47頁。
- 7.李冠群。1999。Candida rugosa 脂肪?之基因調控與蛋白質工程。國立陽明大學博士論文。
- 8.陳信宏。2008。以反應曲面法探討酵素合成辛酸十六酯及異辛酸十六酯之最優化條件。大葉大學。彰化。
- 9.陳國誠。2000。生物固定化技術與

產業應用。茂昌圖書有限公司。台北。10.陳盛樂。2004。真菌Aspergillus terreus固定化技術之探討---包埋法：第23-37頁。朝陽科技大學應用化學系碩士論文。台中。11.曾政鴻。2003。固定化乳酸菌香蕉發酵的研究。國立中興大學食品科學系博士論文。台中 12.梁依婷。2008. 以脂解酵素LIP2合成左旋乙酸薄荷酯之最優化研究。大葉大學。彰化。13.蕭介夫。2002。數種脂肪?基因之表達與應用。知識創新22: 1-4。14.Araujo, M. L. G C., Oliveira, R. P., Giordano, R. C., and Hokka C. O. 1996. Comparative studies on cephalosporin C production process with free and immobilization cells of *Cephalosporium acremonium* ATCC 4827,Chem. Eng. Sci. 51: 2835-2840. 15.Aslani, P. and Kennedy, R. A. 1996. Studies on diffusion in alginate gels. I. Effect of cross-linking with calcium or zinc ions on diffusion of acetaminophen. J Control Release 42: 75-82. 16.Bai, S., Guo, Z., Liu, W. and Sun, Y. 2006. Resolution of (+)-menthol by immobilizeCandida rugosa lipaseon superparamagnetic nanoparticles. Food Chemistry 96(1):1-7. 17.Bhalchandra, K., and Vaidya, R. 2008. Use of insoluble yeast -glucan as a support for immobilization of Candida rugosa lipase. Colloids and Surfaces B: Biointerfaces 61: 101-105. 18.Bhalchandra, K., Vaidya., Ganesh, C., Ingavle., Ponrathnam, S. and Kulkarni, B. D. 2008. Immobilization of Candida rugosa lipase on poly(allyl glycidylether-co-ethylene glycol dimethacrylate) macroporous polymer particles. Bioresource Technology 99: 3623-3629. 19.Bickerstaff, G. F. 1997. Immobilization of enzymes and cells. In Methods in Biotechnology, vol. 1. : Immobilization of enzymes and cells. Bickerstaff GF (ed.), Humana Press, Totowa New Jersey, pp. 1-11. 20.Blanco RM., Terreros P., Perez M., Otero C. and Gonzalez GD. 2004. Functionalization of mesoporous silica for lipase immobilization: characterization of the support and the catalysts. J. Mol Catal B Enzyme(30):83-93. 21.Box, G. E. P. and Wilsoon, K. B. 1951. On the experimental attainment optimum conditions. J. Roy. Statist. Soc. 13: 1-45. 22.Carriere, F., Renou, C., Lopez, V., Caro, J.D., Ferrato, F., Lengsfeld, H., Caro, A.D., Laugier, R., and Verger, R. 2000. The Specific Activities of Human Digestive LipaseMeasured From the In Vivo and In Vitro Lipolysis of Test Meals. Gastroenterology 119: 949-960. 23.Carriere, F., Renou, C., Lopez, V., Caro, J.D., Ferrato, F., Lengsfeld, H., Caro, A.D., Laugier, R., and Verger, R. 2000. The Specific Activities of Human Digestive LipaseMeasured From the In Vivo and In Vitro Lipolysis of Test Meals. Gastroenterology 119: 949-960. 24.Cheetham, P. S. J, Blunk, K. W, Bucke, C. 1979. Physical studies on cell immobilization using calcium alginate gels. Biotechnol Bioeng 21: 2155-2168. 25.Chih-Jen, L., Chi-Mei, L and Chiou-Zong, H. 1996. Biodegradation of Chlorophenols by Immobilized Pure-Culture Microorganisms. Wear Science and Technology 34: 67-72. 26.Chibata, I. 1978. Immobilization Enzyme, Research and Development. Kodansha, Tokyo. 27.Cui, J. H. Goh, J. S. Kim, P. H. Choi, S. H. Lee, B. J. 2000. Survival and stability of bifidobacteria loaded in alginate poly- -lysine microparticles. Int J Pharm 210: 51-59. 28.D ' Souza, S. F. 1999. Immobilized enzymes in bioprocess. Current Science 77: 69-79Derewenda, Z.S., Derewenda U., and Dodson, G.G. 1992. The crystal and molecular structure of the Rhizomucor miehei triacylglyceride lipase at 1.9 Å resolution. J Mol Biol 227: 818-839. 29.Derewenda, Z.S., and Sharp, A.M. 1993. News from the interface: the molecular structures of triacylglyceride lipases. Trans Biochem Sci 18: 20-25. 30.Fundueanu, G. Nastruzzi, C. Carpov, A. Desbrieres, J. and Rinaudo M. 1999. Physico-chemical characterization of Ca-alginate microparticles produced with different methods. Biomaterials 20: 1427-1435. 31.Gaskin, D.J., Romojaro, A., Turner, N.A., Jenkins, J., and Vulson, E.N. 2001. Alteration of lipase chain length specificity in the hydrolysis of esters by random mutagenesis. Biotechnol Bioeng 73: 433-441. 32.Gerbsch, N. Buchholz, R. 1995. New processes and actual trends in biotechnology. FEMS Microbiol Rev 16: 259-269. 33.Grant, GT. Morris, E. R. Rees, D. A. Smith, P. J, and Thom D. 1973. Biological interactions between polysaccharides and divalent cations. Egg-box model. FEBS Lett 32: 195. 34.Gupta, R., Rathi, P., Gupta, N., and Bradoo, S. 2003. Lipase assays for conventional and molecular screening: an overview. Biotechnol Appl Biochem37: 63-71. 35.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. 36.Hadzir, N. M., Rahman, M. B.A., Razak, C. N. A., Rahman, R. N. Z. A., and Salleh, A. B. 2001. Enzymatic alcoholysis of triolein to produce wax ester. Journal of chemical Technology and Biotechnology 76:511-515. 37.Hari Krishna, S., Manohar, B., Divakar, S., Prapulla, S. G. and Karanth, N. G. 2000. Optimization of isoamyl acetate production by immobilized lipase from Mucor miehei by response surface methodology. Enzyme and Microbial Technology 26(2-4): 131-136. 38.Himmelblau, D. M. 1970. Process analysis by statistical methods. p.230-293. John Wiley and Sons, New York. 39.Jaeger, K.-E., Ransac, S., Dijkstra, B.W., Colson, C., Heuvel, M.V., and Misset, O. 1994. Bacterial lipases. FFMS Microbiology Reviews 15:29-63. 40.Jaeger, K.E., Dijkstra, B.W., and Reetz, M.T. 1999. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipase. Annu Rev Microbiol 53: 315-351. 41.Kikuchi, A. Kawabuchi, M. Sugihara, M. and Sakurai Y. 1997. Pulsed dextran releasfrom calcium-alginate gel beads. J Control Release 47: 21-29. 42.Kim, E.L., Jang, W.H., Ko, J.H., Kang, J.S., Noh, M.J., and Yoo, O.J. 2001. Lipase and its modulator from *Pseudomoans* sp. Strain KFCC 10818: proline-to-glutamine substitution at position 112 induces formation of enzymatically active lipase in the absence of the modulato. J Bacteriol 183: 5937-5941. 43.Kiran, K. R., Manohar, B. and Divakar S. 2001. A central composite rotatable design analysis of lipase catalyzed synthesis of lauroyl lactic acid at bench-scale level. Enzyme and microbial technology 29:122-128. 44.Kouker, G., and Jaegar, K.E. 1987. Specific and sensitive plate assay for bacterial lipase. Appl Environ Microbiol 53: 211-213. 45.Lang, D., Hofmann, B., Haalck, L., Hecht, H.J., Spener, F., Schmid, R.D., and Schomburg, D. 1996. Crystal structure of a bacterial lipase from *Chromobacterium viscosum* ATCC 6918 refined at 1.6 angstroms resolution. J Mol Biol 259: 704-717. 46.Lee, B. J. and Min, G. H. 1996. Oral controlled release of melatonin using polymer-reinforced and coated alginate beads. Int J Pharm 144: 37-46. 47.Liodakis, A., Drew, J., Chen, R.Y.S., and Sawyer, W.H. 1991. Spectorfluormetric determination of lipase activity. Biochemistry international 23: 825-834. 48.Longhi, S., Fusetti, F., Grandori, R., Lotti, M., Vanoni, M., and Alberghina, L. 1992. Cloning and nucleotide sequence of two Lip genes from *Candida cylindracea*. Biochim. Biophys. Acta. 1131:227-232. 49.Lotti, M., Grandori, R., Fusetti, F., Longhi, S., Tramontano, A., and Akberghina, L. 1993. Molecular cloning and analysis of *Candida cylindracea* lipase sequences. Gene 124:45-55. 50.Macrae, A. R. 1983. Lipase-catalyzed interesterification of oil and fats. J. Am. Oil Chem. Soc.

60:291-294. 51.Manco, G., Giosue, E., D ' Auria, S., Herman, P., Carrea, G., and Rossi, M. 2000. Cloning, overexpression, and properties of a new thermophilic and thermostable esterase with sequence similarity to hormone-sensitive lipase subfamily from the archaeon *Archaeoglobus fulgidus*. *Arch Biochem Biophys* 373: 182-192. 52.Martinsen A, Skja\*k-Br? k G.and Smidsr?d O. 1989. Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. *Biotechnol Bioeng* 33: 79-89. 53.Maurich, V., Zacchigna, M., and Pitotti, A. 1991. p-nitrophenyllaurate: a substrate for the high-performance liquid chromatographic determination of lipase activity. *J Chromatogr* 566: 453-459. 54.Nielson, J. M. and Giffin, E. G. 1916. Adsorption of invertase. *J. Am.Chem. Soc*38: 1109-1115. 55.Noble, M.E., Cleasby, A., Johnson, L.N., Egmond, M.R., and Frenken, L.G. 1993. The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate. *FEBS Lett* 331: 123-128. 56.Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolov, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., and Schrag, J. 1992. The alpha/beta hydrolase fold. *Protein Eng* 5: 197-211. 57.Ostberg, T, Lund, E. M. and Graffner C. 1994. Calcium alginate matrices for oral multiple unit administration: IV. Release characteristics in different media. *Int J Pharm* 112: 241-248. 58.Palocci, C., Falconi, M., Chronopoulou L. and Cernia,E. 2008. Lipase-catalyzed regioselective acylation of tritylglycosides in supercritical carbon dioxide. *Journal of Supercritical Fluids*45: 48-93. 59.Posorske, L. H. 1984. Industrial-scale application of enzymes to the fats and oil industry. *Journal of the American Oil Chemists ' s Society* 61(11): 1758-1760. 60.Pouderoyen, G.V., Eggert, T., Jaeger, K.-E., and Dijkstra, B.W. 2001. The crystal structure of *Bacillus subtilis* lipase: a minimal alpha/beta hydrolase fold enzyme. *J Mol Biol* 309: 215-226. 61.Radzi, S. M., Basri M., Salleh, A.B., Ariff, A., Mohammad, R., Abd. Rahman, M. B., and Abd. Rahman, R. N.Z.R. 2005. High performance enzymatic synthesis of oleyl oleate using immobilized lipase from *Candida antartica*. *Electronic Journal of Biotechnology* 8: 292-298. 62.Rajendran, A. and Thangavelu, V. 2007. Sequential optimization of culture medium composition for extracellular lipase production by *Bacillus sphaericus* using statistical methods. *Journal of Chemical Technology and Biotechnology* 82:460-470. 63.Sabra, W. Zeng, A. P. Deckwer, W. D. 2001. Bacterial akginate: physiology, product quality and process aspects. *Appl Microbiol Biotrchnol* 56: 315-325. 64.Serkan, E., and Mustafa, Y. 2009. Synthesis of calyx [n]arene-based silica polymers for lipase immobilization. *Journal of Molecular Catalysis Catalysis B: Enzymatic* 58: 29-35. 65.Shao-Hua C., Wen-Teng W. 2004. Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. *Biomaterials*(25):197-204. 66.Shaw, J. F. Chang, R. C., Wang, F. F., Wang Y. J. 1990. Lipolytic activitiea of lipase immobilized on six support materials. *Biotechnol. Bioeng.* 35:132-137. 67.Shiel, 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. 68.Smidsr?d O, Skja\*k-Braek G. 1990. Alginate as immobilization matrix for cells. *Trend Biotechnol* 8:71-78. 69.Thomosn, D. 1982. Response surface experimentation. *J. Food Process. Pres.* 6: 155-188. 70.Tien-Chieh, H., Giridhar, R., Shao-Hua, C. and Wen-Teng, W. 2003. Binary immobilization of *Candida rugosa* lipase on chitosan. *Journal of Molecular Catalysis B: Enzymatic* 26: 69-78. 71.Torres, C. and Otero, C. 1999. Part I. Enzymeatic synthesis of lactate and glycolate esters of fatty alcohols. *Enzyme and Microbial Technology* 25:745-752. 72.Uppenberg, J., Hansen, M.T., Patkar, S., and Jones, T.A. 1994. The sequence,crystal structure determination and refinement of two crystal forms of lipase B from *Candida antarctica*. *Structure* 2: 293-308. 73.Verger, R., and Haas, G,H,D. 1976. Interfacial enzyme kinetics of lipolysis. *Annu Rev Biophys Bioeng* 5: 77-117. 74.Walde, P., and Luisi, P.L. 1989. A Continuous Assay for Lipases in Reverse Micelles Based on Fourier Transform Infrared Spectroscopy *Biochemistry* 28: 3353. 75.Winfried, H. 1988. Methods of Immobilization. *Immobilized Biocatalysts* 6: 22-50. 76.Winkler, F.K., D ' Arcy, A., and Hunziker, W. 1990. Structure of human pancreatic lipase. *Nature* 343: 771-774. 77.Woodward, J. 1985. Immobilised cells and enzymes : a practical approach., Oxford, England., Washington, DC : IRL Press. 78.Yesim. and Yesiloglu. 2005. Utilization of bentonite as a support material for immobilization of *Candida rugosa* lipase. *Process Biochemistry* 40:2155-2159. 79.Yotsuyanagi, T. Ohkubo, T. Ohhadhi, T. and Ikeda, K. 1987. Calcium-induced gelation of alginic acid and pH-sensitive reswelling of dried gels. *Chem Pharm Bull* 35:1555-1563. 80.Zandonella, G., Haalck, L., Spener, F., and Hermetter, A. 1997. New fluorescent glycerolipids for a dual wavelength assay of lipase activity and stereoselectivity. *Journal of Molecular Catalysis B: Enzymatic* 3: 127-130. 81.Zheg-Cang, W., Jian-Qin, W., Zhi-Kang, X. 2006. Immobilization of lipase from *Candida rugosa* on electrospun polysulfone nanofibrous membranes by adsorption. *Journal of Molecular Catalysis B: Enzymatic*(42):45-51