

以基因重組 *Candida rugosa* 脂肪同功酵素探討左旋薄荷醇衍生物之最優化合成條件

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摘要

左旋薄荷醇為一種芳香化合物，被廣泛應用於食品、香水、化妝品、醫藥等行業。為了提高其在水中的溶解度及更持久的清涼性和熱穩定性於各種工業應用，本研究預計使用具不同生化特性之基因重組*Candida rugosa* lipase (CRL)同功酵素 (LIP1~LIP 4) 作為生物觸媒，進行外消旋薄荷醇及乙酸乙酯之酯化反應。我們的目標是篩選出具有高光學專一性的重組同功酵素，並估計各種反應參數對的影響，如反應溫度30~40 °C、反應時間2~11天、酵素添加量0.05~0.5 U、基質莫耳比1:1~15:1 (乙酸乙酯:外消旋薄荷醇)、基質階梯式添加 1 (莫耳/天)、共溶液比3:1 (正己烷:甲苯)對高光學純度左旋乙酸薄荷酯的生產。從結果可知，基因重組LIP 4在合成左旋乙酸薄荷酯的產率比較時，其光學專一性高於其他同功酵素。而其最優化條件為：溫度35 °C、時間3天、基質莫耳比為8:1 (乙酸乙酯:外消旋薄荷醇；莫耳/莫耳)、酵素活性0.4 U、基質階梯式添加為1(莫耳/天)、共溶液比為3:1 (正己烷:甲苯)。以此條件的實際實驗值為52.62 ± 0.4%、鏡像過剩比值為100%，此產率約為商業化lipase AY的1.5倍。

關鍵詞：脂肪酵素、立體專一性、薄荷醇、同功酵素

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參考文獻

- 王永澤。2006。離子液體中脂肪?催化酯交換合成和拆分的研究:64 – 65。浙江大學生物化工博士學位論文。浙江，中國。
- 王惠珠、李菁菁、林苑暉、林旺熠、周薰修、康藏文、張永鍾、曹欽玉、邱文貴、黃培安、游銅錫、郭嘉信、劉建功、葉彥宏、陳榮輝、陳陸宏、謝喻文。2005。第534- 538頁。食品添加物。華格那出版。台中，台灣。
- 向天成、司紅岩、趙艷茹。2010。大孔樹脂固載Ce4+催化合成薄荷酯的研究。安徽農業科學38:6531 – 6533。
- 何智慧、羅嘉、練文柳。2006。薄荷捲菸研究進展。菸草科技9:38 – 42。
- 李立群。2001。假絲酵母(LIP2)重組基因之蛋白質表現生化特性及應用:47-56。國立海洋大學水產生物技術研究所碩士論文。基隆，台灣。
- 念保?，徐剛，楊立?。2006。I – 薄荷醇的合成及手性拆分研究進展。化工進展25:401 – 405。
- 范存?，楊忠寶。2002。左旋薄荷醇合成技術進展。香精香料化妝品4:28 – 30。
- 凌關庭。食品添加劑手冊第三版。第25 – 33頁。化學工業出版社。北京，中國。
- 張淑微。2001。以反應曲面法研究酵素合成己醇酯類之最優化:8。大葉大學食品工程研究所碩士論文。彰化，台灣。
- 張曉莉及黃世佑。1997。生物轉換法 – 有機溶劑中維持酵素活性之研究。化工進展44:71 – 84。
- 曹文正。2005。有機化學。第106頁。新文京開發出版股份有限公司。台北，台灣。
- 許祥純、傅慧音、甜欽仁、洪連樑、楊明華、蔣宗哲、賴盈璋、江文德、謝淳仁、江伯源、巢佳莉等。2006。食品生物化學。第449 – 451頁。華格那出版。台中，台灣。
- 陳旭敏、黃山、陸濤、趙小龍、姜標。2009。左旋薄荷醇的合成現狀及進展。有機化學29:884 – 890。
- 陳金珠，王宗德，宋湛?，姜志寬，韓招久，陳超。2006。薄荷醇酯類衍生物的合成及其驅蚊活性的初步研究。江西農業大學學報28:766 – 768。
- 陳國誠。1989。酵素工程學。第172 – 175頁。藝軒圖書出版社。台北，台灣。
- 彭志英。2004。食品酵素學。第285 – 287頁。九州圖書文物有限公司。台北，台灣。
- 鄢志仲。2004。利用directedevolution置換假絲酵

母(LIP4)之受質結合位置以改變受質選擇性性之研究:47 – 56。國立嘉義大學農業生物技術研究所碩士論文。嘉義，台灣。18.劉薇。2004。非水相中固定化脂肪酸拆分(±) – 薄荷醇的研究:15 – 18。天津大學博士論文。天津，中國。19.鄭世雄。1999。有機化學(上冊)。第211 – 212頁。藝軒圖書出版社。台北，台灣。20.謝維？，余光前，馮章明，聶暉，蔣佑清。2009。I – 乳酸薄荷酯的合成及其在個人護理用品中的應用研究。日用化學工業39:253 – 256。21.韓亞明。2005。乳酸分子蒸餾純化與乳酸薄荷醋合成:8 – 9。廣東中山大學碩士論文。廣東，中國。22.竇宏濤、馮武煥。2007。薄荷優質高產栽培與加工。第10 – 15頁。中國農業出版社。北京，中國。23.Akoh, C.C., Lee, G.C., Shaw, J.F. 2004. Protein engineering and applications of *Candida rugosa* lipase isoforms. *Lipids* 39: 513 – 26. 24.Athawale, V., Manjrekar, N., Athawale, M. 2001. Enzymatic synthesis of chiral menthyl methacrylate monomer by *pseudomonas cepacia* lipase catalysed resolution of (±) – menthol. *Journal of Molecular Catalysis B: Enzymatic* 16 169 – 173. 25.Babali, B., Aksoy, H.A., Tuter, M., & Ustun, G. 2001. Enzymatic esterification of (–)-menthol with lauric acids in isoctane by sorbitan monostearate-coated Lipase from *Candida rugosa*. *Journal of the American Oil Chemists Society*, 78, 53 – 56, 173 – 175. 26.Bai, S., Guo, Z., Liu, W. 2006. Resolution of (±)-Menthol by Immobilized *Candida rugosa* Lipase on Superparamagnetic Nanoparticles. *Food Chemistry*, 96 : 1 – 7. 27.Bellot, J.C., Choisnard, L., Castillo, E., Marty A. 2001. Combining solvent engineering and thermodynamic modeling to enhance selectivity during monoglyceride synthesis by Lipase-catalyzed esterification. *Enzyme and Microbial Technology*, 28, 362 – 369. 28.Benjamin, S., Pandey, A. 1998. *Candida rugosa* lipases: molecular biology and versatility in biotechnology. *Yeast*, 14, 69 – 87. 29.Burgess, K., Henderson, I., and Ho, K.K. 1992. Biocatalytic resolution of sulfinylakanoates: a facile route to optically active sulfoxides. *Journal of Organic Chemistry*, 57, 1290 – 1295. 30.Cantacuzene, D., Pascal, F., and Guerreiro, C. 1987. Synthesis of amino acid esters by papain. *Tetrahedron*, 43, 1823 – 1826. 31.Castillo, E., Pezzotti, F., Navarro, A., pez-Mungu'a A. Lo. 2003. Lipase-catalyzed synthesis of xylitol monoesters: solvent engineering approach. *Journal of Biotechnology*, 102, 251 – 259. 32.Chen, H.C., Liang, Y.T., Chen, J.H. 2009. Optimization of Immobilized *Candida rugosa* Lipase LIP2-catalyzed resolution to produce l-menthyl acetate. *Biocatalyzed and Biotransformation*, 27, 296 – 302. 33.Chen, H.Z., Jin, S.Y. 2006. Effect of ethanol and yeast on cellulase activity and hydrolysis of crystalline cellulose. *Enzyme and Microbial Technology*, 39, 1430 – 1432. 34.Cygler, M., Schrag, JD. 1999. Structure and conformational flexibility of *Candida rugosa* lipase. *Biochimica Biophysica Acta*, 1441, 205 – 214. 35.Dastoli, F.R., Musto, N.A., and Price, S. 1966. Reactivity of active sites of chymotrypsin suspended in an organic medium. *Archives of Biochemistry and Biophysics*, 115, 44 – 47. 36.Dixon, M., Webb, E.C., Thorne, C.S.R., and Tipton, K.F. 1979. *Enzymes*, 3rd edn. Longman, London. 12 – 25 37.Docherty, A., Bodmer, M.W., Anyal, S., Verger, R., Riviere, C., Lowe P. 1985. Rat lingual Lipase. *Nucleic Acids Research*, 13, 1891 – 1903. 38.Eccles, R. 1994. Menthol and related cooling compounds . *Journal of Pharmacy and Pharmacology*, 46, 618 – 630. 39.Ferrer, P., Montesinos, J.L., Valero, F., Sola, C. 2001. Production of native and recombinant lipases by *Candida rugosa*: a review. *Applied Biochemistry Biotechnoogy*, 95, 221 – 255. 40.Kamiya, N., and Goto, M. 1997. How is enzymatic selection of menthol esterification catalyzed by surfactant-coated Lipase determined in organic media *Biotechnology Progress*, 13, 488 – 492. 41.Kirchner, G., Scollar, M.P., and Klipanov, A.M. 1985. Resolution of racemic mixtures via Lipase catalysis in organic solvents. *Journal of American Chemical Society*, 107, 7072 – 7076. 42.Klipanov, A.M. 1983. Basic biology of new developments in biotechnology. Plenum, New York. 497 – 517. 43.Klipanov, A.M. 1989. Enzymatic catalysis in anhydrous organic solvent. *Trends in Biochemical Sciences*, 14, 141 – 144. 44.Klipanov, A.M., Samokhin, G.P., Martinek, K., and Berezin, I.V. 1977. A new approach to preparative enzymatic synthesis. *Biotechnology Bioengineering*, 19, 1351 – 1361. 45.Kobayashi, T., Nagao, T., Watanabe, Y., Shimada, Y. 2007. Analysis of equilibrium state for synthesis of oleic acid l-menthyl ester in an oil – aqueous biphasic system with *Candida rugosa* lipase. *Enzyme and Microbial Technology*, 40, 1300 – 1304. 46.Laane, C., Boeren, S., Vos, K., and Veeger, C. 1987. Rules for optimization of biocatalysis in organic solvents. *Biotechnology Bioengineering*, 30, 81 – 87. 47.Lee, G.C., Lee, L.C., SAVA, V., and Shaw, J.F. 2002. Multiple mutagenesis of non-universal serine codons of the *Candida rugosa* LIP2 Lipase overexpressed in *Pichia pastoris*. *Biochemical Society*, 366, 603 – 611. 48.Li, Z., Wang, Y., Li, J., Wang, P., Wei, W., Gao, Y., Fu, C., Dong, W. 2009. Dual response surface-optimized synthesis of l-menthyl conjugated linoleate in solvent-free system by *Candida rugosa* lipase. *Bioresource Technology*, 101, 1305 – 1309. 49.Lopez, N., Pernas, MA., Pastrana, LM., Sanchez, A., Valero, F., Rua, ML. 2004. Reactivity of pure candida rugosa lipase isoenzymes (Lip1, Lip2, and Lip3) in aqueous and organic media. *Biotechnology Progress*, 20, 65 – 73. 50.Lotti, M., Alberghina, L., 1996. In: Malcata, F.X. (Ed.), Enginnering of/with Lipases, 1st edn. Kluwer, Dordrect, 115 – 124. 51.Mancheno J.M., Pernas M.A., Martinez M.J., Ochoa, B., Rua, M.L., Hermoso, J.A. 2003. Structural insights into the lipase/esterase behaviour in the *Candida rugosa* lipases family: crystal structure of the lipase 2 isoenzyme at 1.97 Å ° resolution. *Journal of Molecular Biology*, 332, 1059 – 1069. 52.Maria, P.D., Sanchez-Montero, J.M., Sinisterra, J.V., Alcantara, A.R. 2006. Understanding *Candida rugosa* lipases: An overview. *Biotechnology Advances*, 24, 180 – 196. 53.Ognntimein, G B., Anderson, W A. , Young, M. M. 1995. Synthesis of geraniol esters in a solvent-free system catalyzed by *Candida antarctica* lipase. *Biotechnology Letters*, 17, 77 – 82. 54.Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolov, F., Franken, S.M., Harel, M., Remington, S.j., Silman, I., Schrag J. 1992. The alpha/beta hydrolase fold. *Protein Engineering*, 5, 197 – 221. 55.Othman, S.S., Basri, M., Hussein, M.Z., Rahman, M.B., Rahman, R.N.Z., Salleh, A.B. Jasmani, H. 2008. Production of highly enantioselective (?)-menthyl butyrate using *Candida rugosa* lipase immobilized on epoxy-activated supports. *Food Chemistry*, 106, 437 – 443. 56.Pernas, M.A., Lopez, C., Pastrana, L., Rua, M.L. 2000. Purification and characterization of Lip2 and Lip3 isoenzymes from a *Candida rugosa* pilot-plant scale fed-batch fermentation. *Journal of Biotechnology*, 84, 163 – 174. 57.Ren, M.Y., Bai, S., Sun, Y. 2009. Resolution of (±)-Menthol in Ionic Liquid Catalyzed by Immobilized Lipase on Magnetic Microspheres. *Chinese Journal of Bioprocess Engineering*, 3, 1 – 5. 58.Ren, M.Y., Bai, S., Zhang, D.H., Sun, Y. 2008. pH Memory of Immobilized Lipase for (±)-Menthol Resolution in Ionic Liquid. *Journal Agricultural and Food Chemistry* 56, 2388 – 2391. 59.Secundo, F., Carrea, G., Tarabiono, C., Pietro, G.L., Brocca, S., Lotti, M., Jaeger, K.E., Puls, M., Eggert T. 2006. The lid is a structural and functional

determinant of lipase activity and selectivity. *Journal of Molecular Catalysis B: Enzymatic*, 39, 166 – 170. 60.Serra, S., Brenna, E., Fuganti, C., et al. 2003. *Tetrahedron. Asymmetry*, 14, 3313 – 3319. 61.Shih, I.L., Hung, S.H., Chen, F.Y., Ju, H.Y., Shieh, C.J. 2007. Optimized synthesis of lipase-catalyzed L-mentyl butyrate by *Candida rugosa* lipase. *Food Chemistry*, 100, 1223 – 1228. 62.Shin, J.S., Luque, S., Klipanov, A.M. 2000. Improving lipase enantioselectivity in organic solvents by forming substrate salts with chiral agents. *Biotechnology and Bioengineering*, 69, 577 – 583. 63.Shu, Z.Y., Jiang, H., Lin, R.F., Jiang, Y.M., Lin, L., Huang, J.Z. 2010. Technical methods to improve yield, activity and stability in the development of microbial lipases. *Journal of Molecular Catalysis B: Enzymatic*, 62, 1 – 8. 64.Vidaluc, J.L., Baboulene, M., Speziale, V., Lattes, A., Monsan, P. 1983. Optimisation of the enzymatic synthesis of amino – acid esters. *Reaction in polyphasic medium*. *Tetrahedron*, 39, 269 – 274. 65.Villeneuve, P., and Foglia, T.A. 1997. Lipase specificities : potential application in lipid bioconversions. *Inform*, 8, 640 – 651. 66.Vorlova1, S., Bornscheuer, U.T., Gatfield, I., Hilmer, J.M., Bertram, H.J., Schmid, R. 2002. Enantioselective hydrolysis of D,L-mentyl benzoate to (R)-menthol by recombinant *Candida rugosa* lipase lip1. *Advanced Synthesis and Catalysis*, 344, 1152 – 1155. 67.Wang, D.L., Nag, A., Lee, G.C., Shaw, J.F. 2002. Factors affecting the resolution of DL-menthol by immobilized lipase-catalyzed esterification in organic solvent. *Journal of Food Chemistry*, 50, 262 – 265. 68.Wu, W.H., Akoh, C.C., Phillips, R.S. 1996. Lipase-catalyzed stereoselective esterification of DL-menthol in organic solvents using acid anhydrides as acylating agents. *Enzyme and Microbial Technology*, 18, 536 – 539. 69.Yuan, Y., Bai, S., Sun, Y. 2006. Comparison of lipase-catalyzed enantioselective esterification of (±)-menthol in ionic liquids and organic solvents. *Journal of Food Chemistry*, 97, 324 – 330. 70.Zaks, A., Klipanov, A.M. 1984. Enzymatic catalysis in organic media at 100 degrees c. *Science*, 224, 1249 – 1251. 71.Zhang, D.H., Bai, S., Ren, M.Y., Sun, Y. 2008. Optimization of Lipase-catalyzed enantioselective esterification of (±)-menthol in ionic liquid. *Journal of Food Chemistry*, 109, 72 – 80. 72.Zheng G W, Zhang J D, Xu J H. 2008. Enzymatic production of L-menthol by a high concentration substrate tolerable esterase from a newly isolated *Bacillus subtilis* ECU0554. *Journal of Biotechnology*, 136, 366 – 383. 73.Zheng, G.W., Pan, J., Yu, H.L., Ngo-Thi, M.T., Li, C. Xi., Xu, J.H. 2010. An efficient bioprocess for enzymatic production of L-menthol with high ratio of substrate to catalyst using whole cells of recombinant *E. coli*. *Journal of Biotechnology*, 150, 108 – 114.