Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 80 (1) 1–8 (2015)
JSCS–4691
UDC 582.282.123.4:547.26'11+577.152.3+543.24:665.327.3
Original scientific paper

Optimization of the fermentation conditions and substrate specifity of mycelium-bound ester hydrolases of *Aspergillus oryzae* Cs007

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(Received 1 April, revised 6 July, accepted 7 July 2014)

Abstract: In order to improve the activities of mycelium-bound ester hydrolases of *Aspergillus oryzae* Cs007, the main production conditions were investigated. The activities of the ester hydrolases were simultaneously determined by titration assay and spectrophotometric assay methods, using olive oil and *p*-nitrophenyl esters as substrates, respectively. The optimum carbon source and nitrogen source were olive oil and peptone, at concentrations of 1 and 2.2 %, respectively. The effects of the carbon source, the nitrogen source and their concentrations on the production of the enzymes were identical when the enzymes activities were assayed by the two methods. The mycelium-bound enzymes showed hydrolytic activity toward all the tested *p*-nitrophenyl esters, triglycerides and fatty acid ethyl esters, but it showed greater preference for long-chain triglycerides and short-chain *p*-nitrophenyl esters.

Keywords: ester hydrolases; lipases; carboxylesterases; *Aspergillus oryzae*; *p*-nitrophenyl esters; olive oil.

INTRODUCTION

Ester hydrolases represent a diverse group of enzymes that catalyze the cleavage and formation of ester bonds. Two typical and important classes of ester hydrolases are lipases (EC3.1.1.3, triacylglycerol hydrolases) and carboxylesterases (EC3.1.1.1, carboxyl ester hydrolases). Lipases can be distinguished from other ester hydrolases by the phenomenon of interfacial activation, which is a unique characteristic for lipases. Furthermore, lipases prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas carboxylesterases preferentially hydrolyze "simple" esters (*e.g.* ethyl acetate) and usually only triglycerides bearing fatty acids shorter than C6.[1] The most reported of the reactions realized by ester hydrolases, especially by lipases or carboxyl-

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doi: 10.2298/JSC140401071D

esterases, are hydrolysis, esterification, alcoholysis, acidolysis, transesterification and aminolysis. In addition, lipases or carboxylesterases show high regio- and stereospecificity. These make them attractive biocatalysts for use in detergent formulation,[2–5] food ingredients,[3–5] the pulp and paper industry,[3–5] the production of cosmetics,[4,5] biofuels production[4–6] and the synthesis of enantiopure compounds.[1,3,4,7–11] Ester hydrolases widely exist in animals, plants and microorganisms. However, the most frequently used ester hydrolases are of microbial origin, mainly bacteria[1,3,4,6,11–13] and fungi.[1,3–6] *Aspergillus oryzae* are one of the common fungal producers of ester hydrolases. In particular, they have the ability to produce extracellular and intracellular enzymes. A strain of *A. oryzae* WZ007 was isolated from soil that produced ester hydrolases showing high stereospecificity, which was used for the resolution of a biotin intermediate,[14] *α*-lipoic acid[15] and ethyl 2-(4-hydroxyphenoxy)propanoate.[16] In addition, it was a promising biocatalyst in the synthesis of flavour esters.[17] A mutant strain of *A. oryzae* Cs007 was obtained by ^{137}Cs gamma radiation of strain WZ007 that displayed high mycelium-bound ester hydrolases activity. To further improve production by the mycelium-bound ester hydrolases Cs007, the most important fermentation factors, including carbon source, nitrogen source and production time, were investigated in detail. Then the substrate specificities of the mycelium-bound enzymes for three typical esters, *i.e.*, *p*-nitrophenyl (*p*-NP) esters, triglycerides and straight-chain fatty acid ethyl esters, were investigated. To the best of our knowledge, most studies of the ester hydrolases from *A. oryzae* investigated systems with extracellular enzymes in the fermentation liquid. Mycelium-bound hydrolases were considered in only a few studies[14–17] and a mycelium-bound carboxylesterase, responsible for catalyzing ester synthesis in another.[18] Mycelium-bound enzymes could be conveniently used without additional immobilization. Therefore, it is very important to improve production by such bound enzymes.

EXPERIMENTAL

Chemicals

p-Nitrophenyl acetate (*p*-NPA), *p*-nitrophenyl octanoate (*p*-NPO) and *p*-nitrophenyl palmitate (*p*-NPP) were purchased from Alfa (Ward Hill, MA). *p*-Nitrophenyl butyrate (*p*-NPB) was purchased from Sigma (St. Louis, MO). Corn oil and soybean oil were purchased from a local supermarket. All other chemicals were of the highest commercially available purity.

Microorganism, medium and growth conditions

A. oryzae Cs007, a mutant strain, was obtained by ^{137}Cs gamma radiation of *A. oryzae* WZ007, which was isolated from soil samples and stored in the China Center for Type Culture Collection with the accession number of CCTCC No. M206105.[14] The microorganisms were maintained on potato slant medium consisting of potato 200 g L^{-1}, sucrose 20 g L^{-1} and agar 20 g L^{-1}. Shake flask culturing was performed in a 250 mL Erlenmeyer flask containing 50 mL of the medium. The basal medium was composed of glucose 10 g L^{-1},

peptone 10 g L^{-1}, KH_2PO_4 1 g L^{-1}, $MgSO_4$ 0.5 g L^{-1}, NaCl 0.5 g L^{-1} and gum arabic 4 g L^{-1} with an initial pH of 5. The basal medium was supplemented with a range of carbon sources, mainly sucrose, maltose, starch, glycerol, olive oil, soybean oil and corn oil, which were screened to examine their capacities to support the growth of *A. oryzae* Cs007 and the production of enzymes. Each carbon source was added into the basal medium at a concentration of 10 g L^{-1} instead of glucose. Suspensions of spores of *A. oryzae* Cs007 with about 10^6 spores per millilitre were used as inoculums. The culture was incubated at 30 °C for 48 h on a rotatory shaker maintained at 200 rpm. The fermentation cultures were filtered through cotton gauze. The mycelia were washed with distilled water two times and used as the crude mycelium-bound enzymes sample.

Biomass measurement

The amount of biomass was determined by the dry weight of mycelia. The fermentation cultures were filtered through cotton gauze. The mycelia were washed two times with distilled water and then dried to a constant weight at 100 °C.

Ester hydrolases activity

Spectrophotometric assay (p-NPA assay). The ester hydrolases activity was assayed with spectrophotometric method using *p*-NPA as substrate, according to a modified version of the Moreau method.[19] The reaction mixture consisting of 0.1 mL of 30 mM *p*-NPA in acetonitrile, 2.8 mL of 50 mM Tris–HCl buffer (pH 7.5) and 20 mg wet-mycelia was incubated at 40 °C for 3 min. The released *p*-nitrophenol was measured at 405 nm in a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min under the assay conditions.

Titration assay (olive oil assay). The titration assay method was used according to the Watanabe method with some modifications.[20] An olive oil emulsion was prepared by mixing 50 mL of olive oil and 150 mL of 4 % poly(vinyl alcohol) solution (*Mw* = 1750±50, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The poly(vinyl alcohol) solution was prepared by dissolving poly(vinyl alcohol) in 50 mM phosphate buffer (pH 7.5). Prior to the assay, the olive oil emulsion was homogenized. The reaction mixture containing 5 mL of olive oil emulsion, 4 mL of 50 mM phosphate buffer (pH 7.5) and 50 mg wet-mycelia was incubated at 40 °C and 150 rpm for 10 min on a shaker. The enzyme reaction was terminated by adding 15 mL of ethanol to the reaction mixture. Then the liberated free fatty acids were titrated with 0.05 M NaOH. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of fatty acid per minute under the assay conditions.

Substrate specificity

Hydrolysis of p-NP esters. Substrate specificities of the ester hydrolases of *A. oryzae* Cs007 for *p*-NP esters were determined by a spectrophotometric method[19,21] using the following *p*-NP esters with different chain length of fatty acids: *p*-NPA (C2), *p*-NPB (C4), *p*-NPO (C8) and *p*-NPP (C16). The solutions of *p*-NPA and *p*-NPB were prepared by adding 0.1 mL of 10 mM *p*-NP esters in acetonitrile into 2.8 mL of 50 mM Tris–HCl buffer (pH 7.5). The solutions of *p*-NPO and *p*-NPP were prepared by adding 0.1 mL of solution A (10 mM *p*-NP esters in acetonitrile) into 2.8 mL of solution B (2.22 g of Triton X-100 in 500 mL of 50 mM Tris–HCl buffer, pH 7.5). The reaction mixtures consisting of 2.9 mL of *p*-NP esters solution and 20 mg wet-mycelia were incubated at 40 °C for the appropriate time.

Hydrolysis of triglycerides and fatty acid ethyl esters. Substrate specificities of the ester hydrolase of *A. oryzae* Cs007 for triglycerides and fatty acid ethyl esters were determined by a previously described titration method.[20] Triglycerides, including triacetin and triolein, and natural olive oil were selected as substrates. Ethyl esters of different carboxylic acid chain lengths (C_1, C_2, C_6, C_8 and C_{12}) were chosen as the substrates.

RESULTS AND DISCUSSION

Effect of carbon source

As indicated in Table I, a better growth of the strain *A. oryzae* Cs007 on oils and glycerol than on carbohydrates was observed. Olive oil gave the best enzyme production. Similarly, oils as inducers or carbon sources enhanced lipase production.[22–24] The activities of mycelium-bound ester hydrolases achieved 160.2 and 472.6 U g^{-1} biomass according to the *p*-NPA assay and olive oil assay, respectively.

TABLE I. Effect of different carbon sources on cell growth and production of mycelium-bound ester hydrolases of *A. oryzae* Cs007

Carbon source	Biomass, g L^{-1}	Enzymes activity, U g^{-1} biomass	
		p-NPA assay	Olive oil assay
Glucose	5.0	18.5	0
Sucrose	7.2	20.6	0
Maltose	5.6	20.2	0
Starch	6.4	28.5	0
Glycerol	8.4	110.0	100.2
Olive oil	10.2	160.2	472.6
Soybean oil	9.8	134.3	356.3
Corn oil	10.3	146.7	282.7

Then the optimal concentrations of olive oil for ester hydrolases production and cell growth were determined. The results are given in Table II, which indicates that the biomass increased with increasing concentrations of olive oil. The mycelium-bound ester hydrolases activities increased with increasing concentration of olive oil up to 1 % after which the enzyme activities decreased with increasing concentration of olive oil. Therefore, 1 % olive oil was used in the subsequent experiments. Significantly, the optimum carbon source and its concentration for the production of enzymes were identical when the ester hydrolases activities were assayed by two methods.

TABLE II. Effect of different concentrations of olive oil on the cell growth and production of mycelium-bound ester hydrolases by *A. oryzae* Cs007

Concentration of olive oil, %	Biomass, g L^{-1}	Enzymes activity, U g^{-1} biomass	
		p-NPA assay	Olive oil assay
0.6	5.6	50.6	134.2
0.8	8.4	104.8	378.3
1.0	10.3	160.2	472.6
1.2	11.9	138.4	400.5
1.4	14.2	121.2	245.2
1.6	15.8	70.9	233.6

Effect of nitrogen source

Using 1 % olive oil as the carbon source, various nitrogen sources including: peptone, yeast extract, beef extract, soybean powder, corn powder, ammonium sulphate and sodium nitrate were selected to investigate growth of *A. oryzae* Cs007 and the production of enzymes. Each nitrogen source was added into the basal medium at a concentration of 10 g L^{-1} instead of peptone. As indicated in Table III, the organic mixed nitrogen sources, especially peptone, yeast extract and soybean powder, resulted in excellent cell growth. However, the inorganic nitrogen sources, such as ammonium sulphate and sodium nitrate, could not be utilized for cell growth. Peptone was overwhelmingly superior to the other nitrogen sources for the production of enzymes. In particular, soybean powder was a good nitrogen source for cell growth, but not a good one for the production of enzymes, with the mycelium-bound enzymes exhibiting low activity.

TABLE III. Effect of different nitrogen sources on cell growth and production of mycelium-bound ester hydrolases by *A. oryzae* Cs007

Nitrogen source	Biomass, g L^{-1}	Enzyme activity, U g^{-1} biomass	
		p-NPA assay	Olive oil assay
Peptone	10.2	160.2	472.5
Yeast extract	9.2	45.6	150.1
Beef extract	6.2	38.8	120.6
Soybean powder	9.3	16.0	109.7
Corn powder	6.4	15.0	99.7
Ammonium sulphate	0	0	0
Sodium nitrate	0	0	0

To find the optimal concentration of peptone for cell growth and enzyme production, various concentrations of peptone from 10 to 30 g L^{-1} were investigated. The obtained results are presented in Table IV. The biomass increased with increasing the concentration of peptone to 18 g L^{-1}, reaching a constant value of about 11.6 g L^{-1} even though higher concentrations of peptone were used. Similarly, the mycelium-bound ester hydrolases activities increased with

TABLE IV. Effect of different concentrations of peptone on the cell growth and production of mycelium-bound ester hydrolases by *A. oryzae* Cs007

Concentration of peptone, g L^{-1}	Biomass, g L^{-1}	Enzymes activity, U g^{-1} biomass	
		p-NPA assay	Olive oil assay
10	10.2	160.2	472.5
14	11.0	188.5	623.3
18	11.6	363.8	1100.1
22	11.4	365.5	1150.5
26	11.8	359.7	1018.3
30	11.5	361.7	970.2

increasing the concentration of peptone to 22 g L^{-1}, achieving a maximum value of 365.5 U g^{-1} biomass by *p*-NPA assay and 1150.5 U g^{-1} biomass by olive oil assay. Thus, 22 g L^{-1} peptone was selected as the optimal concentration for the production of ester hydrolases of *A. oryzae* Cs007. The effects of the nitrogen source and its concentration on the production of the mycelium-bound enzymes were also identical when the enzymes activities were assayed by the two methods.

Time course of cell growth and the production of ester hydrolases of A. oryzae *Cs007*

The time courses of cell growth and production of ester hydrolases of *A. oryzae* Cs007 using the optimal medium are depicted in Fig. 1. The maximum biomass was obtained at 36 h. The activity of the mycelium-bound ester hydrolases showed a sharp increase from 24 to 36 h. The activity of enzymes reached maximum values at 36 h using the *p*-NPA assay but attained maximum values at 42 h using the olive oil assay. A further increase in the incubation time did not lead to increases in the activity of the enzymes and a slow decrease was evidenced up to 48 h.

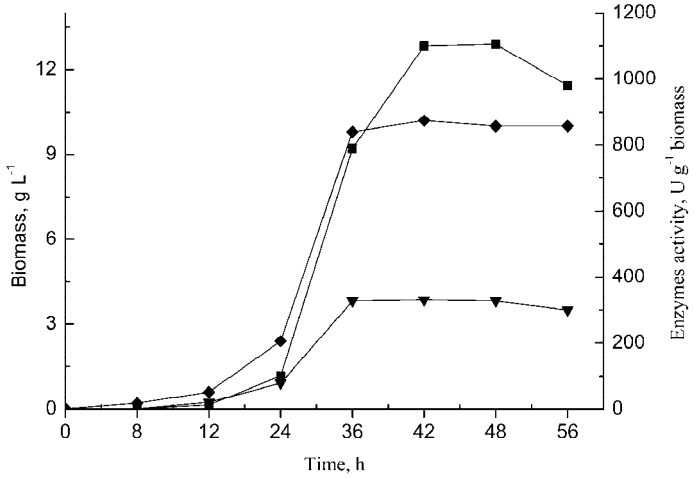

Fig. 1. Time courses of cell growth (◆), mycelium-bound ester hydrolases activity (▼) by the *p*-NPA assay and (■) by the olive oil assay of *A. oryzae* Cs007.

Substrate specificity

As shown in Table V, the mycelium-bound ester hydrolases of *A. oryzae* Cs007 showed a broad range of substrate specificities, with hydrolytic activity toward all tested *p*-NP esters, triglycerides and fatty acid ethyl esters. Of the *p*-NP esters, the enzymes showed greater preference for those with short-chain fatty acids. The highest hydrolytic activity was found toward *p*-NPA, with a specific activity of 365.5 U g^{-1} biomass. The enzymes showed the lowest hydrolysis of

medium- and long-chain *p*-NP esters, such as *p*-NPO (C8) and *p*-NPP (C16). Similarly, lipases from *A. fumigatus*[25] also showed preference for *p*-NP esters with short-chain fatty acids, especially toward *p*-NPA. For triglycerides and fatty acid ethyl esters, the highest hydrolytic activity was found towards triolein with specific activity of 1975.4 U g^{-1} biomass. Moderate enzymes activities were observed towards the short-chain triglyceride (triacetin) and all the tested fatty acid ethyl esters. Of the tested fatty acid ethyl esters, ethyl caproate afforded a relatively high activity with a specific activity of 659.2 U g^{-1} biomass. The enzymes showed greater preference for long-chain triglycerides (triolein) and natural oil (olive oil). The preference of ester hydrolases of *A. oryzae* Cs007 for long-chain triglycerides may due to the hydrophobic nature of the long-chain fatty acid, which promotes helix lid opening, making the catalytic residues accessible to the substrates and exposing the hydrophobic surface of the enzyme. Thus, the enzymatic activity increased dramatically.[26]

TABLE V. Substrate specificities of mycelium-bound ester hydrolases of *A. oryzae* Cs007

Substrate	Enzymes activity, U g^{-1} biomass
p-NPA	365.5
p-NPB	238.7
p-NPO	32.9
p-NPP	44.6
Ethyl formate	394.6
Ethyl acetate	491.3
Ethyl caproate	659.2
Ethyl caprylate	559.1
Ethyl laurate	461.4
Olive oil	1150.5
Triolein	1975.4
Triacetin	395.8

CONCLUSIONS

This work showed the main production conditions and substrate specificities of mycelium-bound enzymes from *A. oryzae* Cs007. The main production conditions, including the optimum carbon and nitrogen sources, the optimal concentration of the carbon and nitrogen source and the production time, were optimized as follows. Olive oil and peptone were the optimum carbon and nitrogen source, and the optimal concentrations of the carbon and nitrogen source were 1 and 2.2 %, respectively. The activity of the enzymes reached maximum values at 36 h using the *p*-NPA assay and 42 h using the olive oil assay. The mycelium-bound enzymes showed hydrolytic activity toward all the tested *p*-NP esters, triglycerides and fatty acid ethyl esters. However, it was interesting that the enzymes preferred short-chain *p*-NP esters and long-chain triglycerides.

Acknowledgment. The authors are grateful for financial support from Zhejiang Provincial Education Department (Project No. Y201121810).

И З В О Д

ОПТИМИЗАЦИЈА УСЛОВА ФЕРМЕНТАЦИЈЕ И СУПСТРАТНА СПЕЦИФИЧНОСТ ЕСТАРСКИХ ХИДРОЛАЗА ИЗ *Aspergillus oryzae* CS007 ВЕЗАНИХ ЗА МИЦЕЛИЈУМ

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Испитивани су производни услови у циљу побољшања активности естарских хидролаза из *Aspergillus oryzae* Cs007 везаних за мицелијум. Активности естарских хидролаза су истовремено одређиване методом титрације и спектрофотометријски, користећи маслиново уље и *p*-нитрофенил естре као супстрате. Оптимални извори угљеника и азота су били маслиново уље и пептон, у концентрацијама 1, односно 2,2 %. Исти резултати су добијени применом обе методе праћења активности ензима. Ензими везани за мицелијум су испољили хидролитичку активност спрам свих тестираних *p*-нитрофенил естара, триглицерида и етил-естара масних киселина. Највећа активност је испољена спрам дуголанчаних триглицерида и кратколанчаних *p*-нитрофенил естара.

(Примљено 1. априла, ревидирано 6. јула, прихваћено 7. јула 2014)

REFERENCES

1. U. T. Bornscheuer, *FEMS Microbiol. Rev.* **26** (2002) 73
2. F. Hasan, A. A. Shah, S. Javed, A. Hameed, *Afr. J. Biotechnol.* **9** (2010) 4836
3. K.-E. Jaeger, M. T. Reetz, *Trends Biotechnol.* **16** (1998) 396
4. F. Hasan, A. A. Shah, A. Hameed, *Enzyme Microb. Technol.* **39** (2006) 235
5. A. K. Singh, M. Mukhopadhyay, *Appl. Biochem. Biotechnol.* **166** (2012) 486
6. A. Bajaj, P. Lohan, P. N. Jha, R. Mehrotra, *J. Mol. Catal., B* **62** (2010) 9
7. A. Ghanem, H. Y. Aboul-Enein, *Tetrahedron: Asymmetry* **15** (2004) 3331
8. A. Ghanem, H. Y. Aboul-Enein, *Chirality* **17** (2005) 1
9. H. Monti, G. Audran, *Mini-Rev. Org. Chem.* **2** (2005) 265
10. A. Ghanem, *Tetrahedron* **63** (2007) 1721
11. T. Panda, B. S. Gowrishankar, *Appl. Microbiol. Biotechnol.* **67** (2005) 160
12. J. L. Arpigny, K.-E. Jaeger, *Biochem. J.* **343** (1999) 177
13. R. Gupta, N. Gupta, P. Rathi, *Appl. Microbiol. Biotechnol.* **64** (2004) 763
14. J.-Y. Zheng, Z. Wang, Q. Zhu, Y.-J. Zhang, H.-D. Yan, *J. Mol. Catal., B* **56** (2009) 20
15. H.-D. Yan, Z. Wang, L.-J. Chen, *J. Ind. Microbiol. Biotechnol.* **36** (2009) 643
16. J.-Y. Zheng, J.-Y. Wu, Y.-J. Zhang, Z. Wang, *J. Mol. Catal., B* **97** (2013) 62
17. H.-D. Yan, Q. Zhang, Z. Wang, *Catal. Commun.* **45** (2014) 59
18. F. Molinari, R. Gandolfi, A. Converti, M. Zilli, *Enzyme Microb. Technol.* **27** (2000) 626
19. H. Moreau, A. Moulin, Y. Gargouri, J.-P. Noël, R. Verger, *Biochemistry* **30** (1991) 1037
20. N. Watanabe, Y. Ota, Y. Minoda, K. Yamada, *Agric. Biol. Chem.* **41** (1977) 1353
21. U. K. Winkler, M. Stuckmann, *J. Bacteriol.* **138** (1979) 663
22. N. Kulkarni, R. V. Gadre, *Biotechnol. Lett.* **21** (1999) 897
23. Y.-Q. He, T.-W. Tan, *J. Mol. Catal., B* **43** (2006) 9
24. R. Kaushik, S. Saran, J. Isar, R. K. Saxena, *J. Mol. Catal., B* **40** (2006) 121
25. J.-J. Shangguan, Y.-Q. Liu, F.-J. Wang, J. Zhao, L.-Q. Fan, S.-X. Li, J.-H. Xu, *Appl. Biochem. Biotechnol.* **165** (2011) 949
26. M. S. M. Ali, C. C. Yun, A. L. T. Chor, R. N. Z. R. A. Rahman, M. Basri, A. B. Salleh, *Protein J.* **31** (2012) 229.