JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Optimization of Culture Conditions for Production of a Novel Cold-Active Lipase from *Pichia lynferdii* NRRL Y-7723

Sun-Young Park,[†] Ji-Yeon Kim,[†] Jae-Han Bae,[†] Ching T. Hou,[§] and Hak-Ryul Kim*[,†]

[†]School of Food Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea

[§]Microbial Genomic and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultrual Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604, United States

ABSTRACT: Lipases with abnormal properties such as thermostability, alkalinity, acidity, and cold activity receive industrial attention because of their usability under restricted reaction conditions. Most microbial cold-active lipases originate from psychrotrophic and psychrophilic microorganisms found in Antarctic regions, which has led to difficulties in the practical production of cold-active lipase. Recently, a mesophilic yeast, *Pichia lynferdii* NRRL Y-7723, was reported to produce a novel cold-active lipase. This study focused on optimization of environmental factors, while giving particular attention to the relationships between given factors and incubation time, to maximize the production of a novel cold-active lipase from *P. lynferdii* NRRL Y-7723. Maximum lipase production was highly dependent on the incubation time at a given environmental factor. Lipase production varied with incubation time at a given temperature, and 20 °C was selected as the optimum temperature for lipase production. Fructose was selected as the best carbon source, and maximum lipase production was obtained when it was present at 0.7% (w/v). Yeast extract was an efficient organic nitrogen source, with maximum lipase production occurring at 0.9% (w/v). Specifically, at the optimum yeast extract level the lipase production was >10 times higher than the productivity under standard conditions. All natural oils tested showed lipase production, but their maximum productivities varied according to incubation time and oil species.

KEYWORDS: *lipase, cold-active, Pichia lynferdii, optimization, yeast*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3.) comprise one of the most important groups of industrial enzymes because they can catalyze numerous different reactions that are applicable for the production of industrial products in the food, chemical, pharmaceutical, and detergent industries.[1] However, industrial application of lipase is highly dependent on both the catalytic properties of the lipase and the environmental conditions of application. Hence, abnormal reaction conditions require special lipases with abnormal properties. Among these abnormal lipases, cold-active lipases are of great interest because they are attractive owing to their stability and cost-effectiveness when used in industrial applications such as cold-water washing detergent formulation, fine chemistry catalysis, and food processing.[2,3] Most microbial lipases exhibit high activity at between 30 and 50 °C, with the maximum activity being observed around 40 °C. At low temperature, their catalytic activities are severely reduced, decreasing to almost zero at 0 °C. However, cold-active enzymes show relatively high specific activity in the range of 0−20 °C, with the optimum activity occurring at 30−40 °C.[2−5]

Several microbial cold-active lipases were recently identified, but most of these originated from psychrotrophic and psychrophilic microorganisms found in Arctic and Antarctic seawaters.[6−10] The Antarctic strains are commonly cultivated at around 5 °C under laboratory conditions, and these organisms have long generation times of 2−10 h if there is no cellular stress. Although higher temperatures (>20 °C) shorten the generation time, they induce cellular stress, which leads to low density of cell growth and low extracellular enzyme production. These abnormal cultural conditions have caused limitations to commercial production of cold-active lipases in terms of cost effectiveness, leading to a new search for cold-active lipases from mesophilic microorganisms. In addition, high productivity of lipase is of great importance for industrial production of cold-active lipase. Production of lipase can be influenced by several factors including temperature, pH, medium composition, and the presence of inducers that can affect cell growth and lipase production. Specifically, the optimum incubation time for maximum production of a lipase can be altered according to the status of nutritional factors because microorganisms are supposed to produce lipase to hydrolyze available oil substrates into fatty acids, rendering them useful as energy sources. Therefore, the status of nutritional conditions is influenced by cell growth; accordingly, incubation time can be important for lipase production. During the past decade, extensive investigations of environmental factors have been carried out using different fermentation techniques to increase the productivity of lipases from different microbial sources.[11−14] However, such studies have not been thoroughly applied to the identification of cold-active lipases to date.

In our previous study, we report a novel cold-active lipase produced from the mesophilic yeast strain *Pichia lynferdii* NRRL Y-7723.[15] Here, we conducted an optimization study of environmental factors while focusing on the relationship

Received: November 19, 2012
Revised: January 9, 2013
Accepted: January 10, 2013
Published: January 10, 2013

dx.doi.org/10.1021/jf304919y | *J. Agric. Food Chem.* 2013, 61, 882−886

between nutritional factors and incubation time to maximize production of a cold-active lipase from *P. lynferdii* NRRL Y-7723.

MATERIALS AND METHODS

Materials. Vegetable oils (normal bleached refined oils) were purchased from a local market in Daegu, Korea, and used as inducers after filtration through a membrane filter (pore size = 0.45 μm) without further purification. All chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

Microorganism. *P. lynferdii* NRRL Y-7723 was obtained from the culture collection of the National Center for Agricultural Utilization Research (Peoria, IL, USA). The stock culture was maintained in a cryogenic vial containing 0.4 mL of glycerol and 0.6 mL of YM medium (1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone, w/v) at −70 °C prior to use.

Culture Conditions. Seed cultures were prepared by thawing the frozen stock culture in a water bath at 37 °C, after which they were used to inoculate 50 mL of YM medium in 100 mL Erlenmeyer flasks. Cultural incubation was carried out in a shaking incubator at 150 rpm and 25 °C. To produce lipase, 1% (v/v) of safflower oil was added to the YM medium at the beginning of cultivation as an inducer. The medium composition and culture conditions were modified from these standard conditions as described in the text for the optimization study. All experiments were carried out in triplicate, and data are presented as the average value with an error range within 10%, unless otherwise specified.

Determination of Cell Growth. One milliliter of culture from the flask was collected and centrifuged at 5000*g* for 10 min at room temperature. The cells were then washed once by resuspending the cell pellet in the same volume of distilled water, after which the harvested wet cells were resuspended in 1 mL of distilled water, and the absorbance at 600 nm was measured to determine the cell growth.

Determination of Lipase Activity. Lipase activity was determined by the spectrophotometric method using *p*-nitrophenyl-butyrate (*p*-NPB) as the substrate,[16] with slight modification. Briefly, 100 μL of enzyme solution was mixed with 900 μL of assay mixture containing 100 μL of substrate solution (10 mM *p*-NPB dissolved in 100% cold ethanol) and 800 μL of 50 mM sodium phosphate buffer (pH 7.0). The increase in absorbance at 410 nm caused by the release of *p*-nitrophenol from *p*-NPB by enzymatic hydrolysis at 15 °C was measured spectrophotometrically for a minimum of 2 min against an enzyme-free control. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 nmol of *p*-nitrophenol from *p*-NPB per minute. For preparation of the enzyme sample, 5 mL of yeast culture was collected and centrifuged at 10000*g* for 10 min at 4 °C. The cell-free supernatant was then collected and used for determination of the lipase activity.

RESULTS AND DISCUSSION

Effects of Incubation Time and Temperature on Lipase Production. In a previous study, lipase production by *P. lynferdii* NRRL Y-7723 was measured after 72 h of incubation under standard conditions.[15] However, the microbial growth rate is highly dependent on incubation time and temperature, which could affect lipase productivity. Microorganisms are supposed to produce lipase to hydrolyze triglycerides into fatty acids, rendering them useful as energy sources. As a result, determination of the status of nutritional conditions caused by the status of cell growth can be an important factor for lipase production. Therefore, we investigated the time course of lipase production at different incubation temperatures (15−35 °C). As shown in Figure 1A, lipase production was highly dependent on incubation time and temperature. Under standard conditions at 25 °C, the maximum lipase production (82 unit/ml) was obtained after

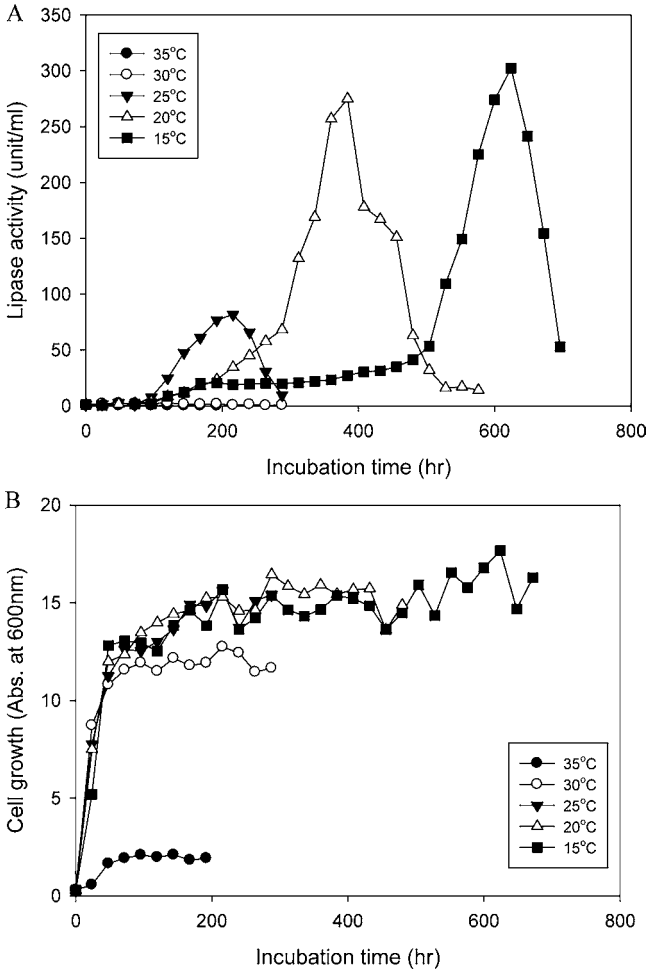

Figure 1. Effects of incubation time and temperature on lipase production by *P. lynferdii* NRRL Y-7723: (A) lipase production; (B) cell growth. Extracellular lipase activity was determined at 15 °C, and symbol designations are specified in the figure.

216 h of incubation. As temperature decreased, lipase production increased remarkably, reaching 275 and 302 units/mL at 20 and 15 °C, respectively. However, the incubation time required for maximum lipase production at a given temperature was greatly increased to 384 and 624 h at 20 and 15 °C, respectively. The lipase production at >30 °C was negligible. Unlike lipase production, cell growth at 15−25 °C showed similar efficiencies, reaching the stationary phase after 72 h of incubation (Figure 1B). Overall, the maximum cell growth at 30 °C corresponded to about 75% of that at 20 °C, whereas lipase production was negligible. At 35 °C, the maximum cell growth decreased to 13% of that at 20 °C. On the basis of these results and consideration of cost-effectiveness, 20 °C was selected as the optimum incubation temperature because the incubation time for maximum lipase production at 15 °C was 62.5% longer than that at 20 °C, whereas the maximum lipase production at 15 °C was 10% higher than that at 20 °C. There are no reports of the time course of production of the microbial cold-active lipase at different temperatures available; however, data from this study confirmed that induction of production of cold-active lipase by *P. lynferdii* NRRL Y-7723, and not cell growth, was greatly influenced by incubation temperature. These findings suggested that

dx.doi.org/10.1021/jf304919y | J. Agric. Food Chem. 2013, 61, 882−886

expression of the gene encoding cold-active lipase could be temperature-sensitive, although it should be confirmed further.

Effects of Carbon Source on Lipase Production. Although carbon sources are required for microbial growth and production of biochemical materials, their efficiencies for production of any target material can differ among microbial species and under different fermentation conditions. Therefore, the influences of different carbon sources on lipase production by *P. lynferdii* NRRL Y-7723 were tested. In our previous study, when lipase production was determined after 72 h of incubation at 25 °C, the effects of maltose and fructose on lipase production and cell growth were comparable to those of glucose (control), whereas sucrose induced the highest lipase production. In the present study, we determined the lipase production in response to different carbon sources after 192 h of incubation with 1% safflower oil as substrate at 25 °C (Figure 1). Among 11 different carbon sources tested, manitol, mannose, fructose, and glucose were efficient for both lipase production and cell growth (Table 1), and fructose showed the highest lipase production (170 units/mL). On the basis of these findings, fructose was selected as the best carbon source, and the optimum concentration of fructose was determined for lipase production. Glucose of YM medium was replaced with different concentrations of fructose (0.1−2.0%), and the time course of lipase production was determined (Figure 2). To determine the maximum lipase production, incubation was conducted at 20 °C because this was determined as the optimum incubation temperature for lipase production (Figure 1). As shown in the figure, lipase production changed greatly according to fructose concentration. The maximum lipase production and incubation time required for the maximum lipase production at a given concentration increased proportionally as the fructose concentration increased to 0.7%, reaching 1350 units/mL after 216 h of incubation. This productivity was almost 5 times higher than that observed under standard conditions at 20 °C. At 1.0%, the maximum lipase production decreased to 970 units/mL after 288 h of incubation. At >1.5%, lipase production decreased to <150 units/mL. There have been no specific reports of the effects of carbon sources on the production of cold-active lipases to date; however, most cases dealing with cold-active lipases used glucose as a carbon source.[17−19]

Table 1. Effects of Carbon Source on Lipase Production

carbon source (1%)	cell growth (Abs at 600 nm)[a]	lipase activity (units/mL)
maltose	16.6 ± 1.2	4.9 ± 0.3
lactose	9.7 ± 1.1	6.7 ± 0.1
mannitol	14.9 ± 0.5	89.1 ± 0.6
mannose	15.8 ± 0.4	79.9 ± 3.1
galactose	13.1 ± 0.2	3.7 ± 0.1
xylose	6.4 ± 0.2	0.8 ± 0.2
fructose	17.3 ± 1.7	170.5 ± 2.1
starch	8.9 ± 1.5	2.5 ± 0.2
sucrose	13.9 ± 0.4	22.2 ± 1.3
glucose	15.8 ± 0.5	107.6 ± 5.5
glycerol	14.9 ± 0.4	35.2 ± 0.2

[a]Values were obtained from multiplication of analyzed data by the dilution factor.

Effects of Yeast Extract on Lipase Production. A previous study showed that yeast extract was the best nitrogen source for cold-active lipase production from *P. lynferdii* NRRL Y-7723 when tested as a single nitrogen source.[15] However, the effects of yeast extract concentration on lipase production could change with incubation time and temperature when the optimized carbon source was used. In this study, we tested the effects of varied concentrations of yeast extract on maximum lipase production. Different concentrations of yeast extract were added to YM medium with 0.7% fructose as a carbon source, and the time course of lipase production was determined at 20 °C. As shown in Figure 3, lipase production increased remarkably as the concentration of yeast extract increased to 0.9%, reaching 2775 units/mL after 336 h of incubation. This value was >10 times higher than the lipase production observed under standard conditions.

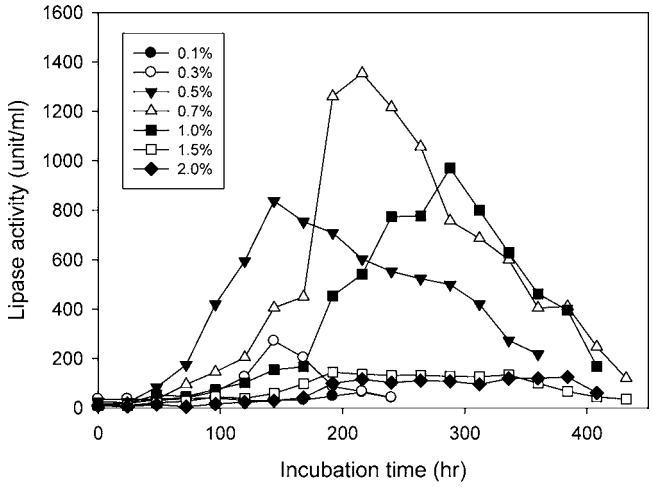

Figure 2. Effects of fructose concentration on lipase production by *P. lynferdii* NRRL Y-7723. Glucose of the standard control medium was replaced with different concentrations of fructose. Cultures were harvested at a given time, and extracellular lipase activity was determined at 15 °C under standard conditions.

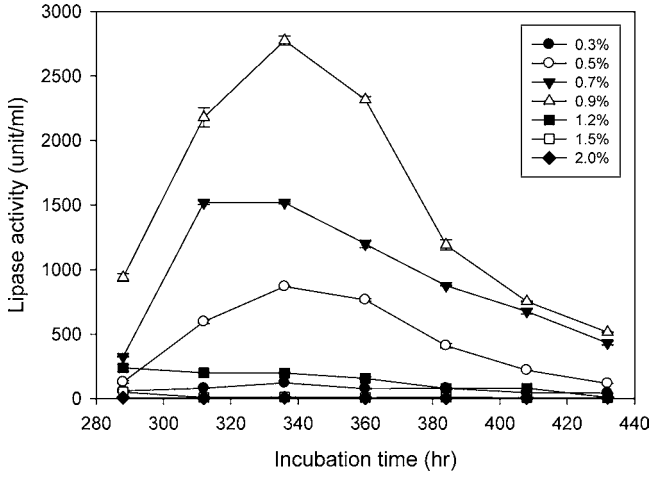

Figure 3. Effects of concentration of yeast extract on lipase production by *P. lynferdii* NRRL Y-7723. Various amounts of yeast extract were added to YM medium supplemented with 0.7% fructose, and the time course of lipase production was determined at 15 °C under standard conditions.

Effects of Oil Substrate on Lipase Production. Lipase production is induced by the presence of oil substrate; hence, the concentration and species of oil substrate can be important

dx.doi.org/10.1021/jf304919y | J. Agric. Food Chem. 2013, 61, 882−886

factors for efficient lipase production. In this study, the effects of safflower oil concentration on lipase production by *P. lynferdii* NRRL Y-7723 were first determined because safflower oil was used as an oil substrate under standard conditions. Different concentrations of safflower oil (0.5−7%) were added to the culture in the YM medium with 0.7% fructose at the beginning of the incubation period, and the time course of lipase production was determined at 20 °C. The maximum lipase production increased remarkably as the oil content increased, reaching 2500 units/mL at 3% (Figure 4). At >5% oil

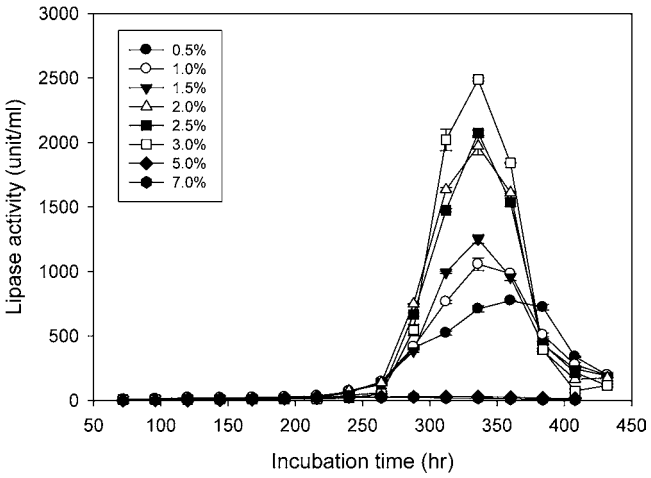

Figure 4. Effects of oil content on lipase production by *P. lynferdii* NRRL Y-7723. Corresponding amounts of safflower oil were added to YM medium supplemented with 0.7% fructose prior to microbial inoculation, and the time course of lipase production was determined at 15 °C under standard conditions.

content, lipase production decreased to the base level. These changing patterns were quite similar to those observed in a previous study in which soybean oil was used as oil substrate instead of safflower oil, although the level of lipase production with safflower oil was much higher than that with soybean oil.[15] These results suggested that species of oil could be another important factor for production of the cold-active lipase from *P. lynferdii* NRRL Y-7723. Therefore, we tested the effects of different vegetable oil species on lipase production by *P. lynferdii* NRRL Y-7723. For rapid screening of the best oil substrate, the incubation conditions were fixed at 264 h and 20 °C with 1% oil and 0.7% fructose. Among 20 natural vegetable oils tested, red pepper seed oil (935 units/mL), black seed oil (741 units/mL), hemp seed oil (726 units/mL), and palm oil (1446 units/mL) showed relatively high lipase production, although all oils tested produced lipase at >150 units/mL (Table 2). These results suggested that the fatty acid composition of oil could be an important factor for the production of cold-active lipase from *P. lynferdii* NRRL Y-7723, although the fatty acid compositions of oils tested were not clearly presented in this study.

As palm oil was determined as the best oil substrate for lipase production, we attempted to determine the optimum concentration of palm oil for lipase production. To accomplish this, different concentrations of palm oil (0.5−5.0%) were added to the culture, and the time course of lipase production was determined (Figure 5). Maximum lipase production increased as the oil content increased to 2.0%, reaching 2216 units/mL, after which it decreased. However, maximum lipase

Table 2. Effects of Natural Oil Substrate on Lipase Production

oil (1%)	lipase activity (units/mL)	oil (1%)	lipase activity (units/mL)
apricot seed	190.9 ± 7.3	avocado	415.7 ± 20.1
soybean	156.7 ± 8.1	grape seed	315.2 ± 14.2
castor	219.1 ± 12.6	almond	447.6 ± 12.5
sunflower	159.9 ± 13.1	emu	399.2 ± 24.0
olive	255.7 ± 12.1	jojoba	284.6 ± 17.7
safflower	189.8 ± 10.2	hemp	725.7 ± 22.5
red pepper seed	934.5 ± 21.1	mustard	321.8 ± 15.2
corn	497.5 ± 12.2	flax seed	295.5 ± 18.6
palm	1445.7 ± 17.3	black seed	741.1 ± 14.8
hazelnut	152.7 ± 12.6	melon seed	539.2 ± 15.2

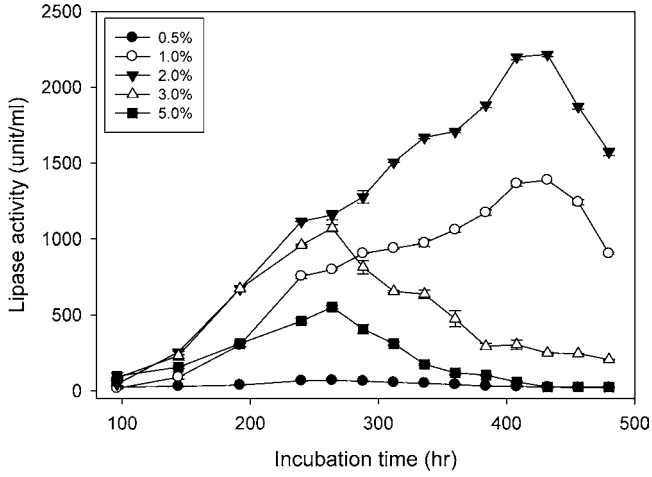

Figure 5. Effects of different amounts of palm oil on lipase production by *P. lynferdii* NRRL Y-7723. Corresponding amounts of palm oil were added to YM medium supplemented with 0.7% fructose prior to microbial inoculation, and the time course of lipase production was determined at 15 °C under standard conditions.

production with 2% palm oil was slightly lower than that obtained with 3% safflower oil (Figure 4), and the time dependency of lipase production was quite different from that of safflower oil. The incubation time for maximum lipase production at a given concentration of palm oil changed significantly according to oil concentration, whereas safflower oil showed almost the same incubation time for maximum lipase production at all concentrations tested. Although the effects of various natural oils on the production of normal lipase have been thoroughly investigated using several microorganisms,[20−22] the dose dependency and time course of production of cold-active lipase have rarely been studied. The results of the present study suggested that safflower oil was more appropriate for the production of a cold-active lipase from *P. lynferdii* NRRL Y-7723 than palm oil and that concentration as well as species of oil could be important factors influencing lipase production.

In conclusion, important nutritional and environmental conditions were determined for the optimum production of a novel cold-active lipase from the mesophilic yeast strain *P. lynferdii* NRRL Y-7723 in the present study. Cultivation under the optimum conditions resulted in the maximum lipase production increasing by >10 times when compared to that obtained under standard conditions. Future studies should

dx.doi.org/10.1021/jf304919y | J. Agric. Food Chem. 2013, 61, 882−886

include a biochemical investigation of this enzyme after purification.

AUTHOR INFORMATION

Corresponding Author

*Phone: +82-53-950-5754. Fax: +82-53-950-6750. E-mail: hakrkim@knu.ac.kr.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0023701).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Harwood, J. The versatility of lipases for industrial uses. *Trends Biochem. Sci.* **1989**, *14*, 125−126.

(2) Gerday, C.; Aittaleb, M.; Arpigny, J. L.; Baise, E.; Chessa, J. P.; Garsoux, G.; Petrescu, I.; Feller, G. Psychrophilic enzymes: a thermodynamic challenge. *Biochim. Biophys. Acta* **1997**, *1342*, 119−131.

(3) Marshall, C. J. Cold-adapted enzymes. *Trends Biotechnol.* **1997**, *15*, 359−364.

(4) Feller, G.; Narinx, E.; Arpigny, J. L.; Zekhnini, Z.; Swings, J.; Gerday, C. Temperature dependence of growth, enzyme secretion and activity of psychrophilic Antarctic bacteria. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 477−479.

(5) Feller, G.; Gerday, C. Psychrophilic enzymes: molecular basis of cold adaptation. *Cell. Mol. Life Sci.* **1997**, *53*, 830−841.

(6) Feller, G.; Thiry, M.; Arpigny, J. L.; Gerday, C. Nucleotide sequence of the lipase gene Lip2 from the antarctic psychrotrophic *Moraxella* TA144 and site-specific mutagenesis of the conserved serine and histidine resides. *DNA Cell Biol.* **1991**, *10*, 381−388.

(7) Choo, D. W.; Kurihara, T.; Suzuki, T.; Soda, K.; Esaki, N. A cold-adapted lipase of an Alaskan psychrotroph, *Pseudomonas* sp. strain B11-1: gene cloning and enzyme purification and characterization. *Appl. Environ. Microbiol.* **1998**, *64*, 486−491.

(8) Russell, N. J. Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. *Adv. Biochem. Eng. Biotechnol.* **1998**, *61*, 1−21.

(9) Kulakova, l.; Galkin, A.; Nakayama, T.; Nishino, T.; Esaki, N. Cold-active esterase from *Psychrobacter* sp. Ant 300: gene cloning, characterization, and the effects of Gly/Pro substitution near the active site on its catalytic activity and tability. *Biochim. Biophys. Acta* **2004**, *1696*, 59−65.

(10) Suzuki, T.; Nakayama, T.; Kurihara, T.; Nishino, T.; Esaki, N. Cold active lipolytic activity of psychrotrophic *Acinetobacter* sp. strain no. 6. *J. Biosci. Bioeng.* **2001**, *92*, 144−148.

(11) Fischer, B. E.; Kleber, H. P. Isolation and characterization of the extracellular lipase of *Acinetobacter calcoaceticus* 69V. *J. Basic Microbiol.* **1987**, *27*, 427−432.

(12) Valero, F.; del Rio, J. L.; Poch, M.; Sola, C. Fermentation behavior of lipase production by *Candida rugosa* growing on different mixtures of glucose and olive oil. *J. Ferment. Bioeng.* **1991**, *72*, 399−401.

(13) Lin, S. F.; Chiou, C. M.; Tsai, Y. C. Effect of Triton X-100 on alkaline lipase production by *Pseudomonas pseudoalcaligenes* F-111. *Biotechnol. Lett.* **1995**, *17*, 959−962.

(14) Benjamin, B.; Pandey, A. Optimization of liquid media for lipase production by *Candida rugosa*. *Bioresour. Technol.* **1996**, *55*, 167−170.

(15) Kim, H. R.; Kim, I. H.; Hou, C. T.; Kwon, K. I.; Shin, B. S. Production of a novel cold-active lipase from *Pichia lynferdii* Y-7723. *J. Agric. Food Chem.* **2010**, *58*, 1322−1326.

(16) Vorderwulbecke, T.; Kieslich, K.; Erdmann, H. Comparison of lipases by different assays. *Enzyme Microb. Technol.* **1992**, *14*, 631−639.

(17) Alquati, C.; Gioia, L. D.; Santarossa, G.; Alberghina, L.; Fantucci, P.; Lotti, M. The cold-active lipase of *Pseudomonas fragi*. *Eur. J. Biochem.* **2002**, *269*, 3321−3328.

(18) Luo, Y.; Zheng, Y.; Jiang, Z.; Ma, Y.; Wei, D. A novel psychrophilic lipase from *Pseudomonas fluorescens* with unique property in chiral resolution and biodiesel production via trans-esterification. *Appl. Microbiaol. Biotechnol.* **2006**, *73*, 349−355.

(19) Mayordomo, I.; Randez-Gil, F.; Prieto, J. A. Isolation, purification and characterization of a cold-active lipase from *Aspergillus nidulans*. *J. Agric. Food Chem.* **2000**, *48*, 105−109.

(20) Kulkami, N.; Gadre, R. V. Production and properties of an alkaline, thermophilic lipase from *Pseudomonas fluorescens* NS2W. *J. Ind. Microbiol. Biotechnol.* **2002**, *28*, 344−348.

(21) Corzo, G.; Revah, S. Production and characteristics of the lipase from *Yarrowia lipolytica* 681. *Bioresour. Technol.* **1999**, *70*, 173−180.

(22) Tan, T.; Zhang, M.; Xu, J.; Zhang, J. Optimization of culture conditions and properties of lipase from *Penicillium camembertii* Thom PG-3. *Process Biochem.* **2004**, *39*, 1495−1502.