DOI:10.1021/jf903430t

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Production of a Novel Cold-Active Lipase from *Pichia lynferdii* Y-7723

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Lipase (triacylglycerol acylhydrolases, E.C. 3.1.1.3) is one of the most important enzymes applied to a broad range of industrial application fields. Especially, lipases with abnormal functionality such as thermostability and alkaline, acidic, and cold activities gain special attention because of their applicability in the restricted reaction conditions. In this study, 16 yeast strains prescreened for lipase induction were investigated for their actual lipase production, and we found a novel cold-active lipase produced from *Pichia lynferdii* Y-7723. The activity of lipase Y-7723 was retained by 74 and 70% at 20 and 10 °C, respectively, as compared to the maximum value at 35 °C. On the basis of an optimization study, the optimal lipase productivity was obtained at 96 h of incubation with 3% oil substrate in a medium composed of sucrose as a carbon source at pH 7.0. Among carbon sources tested, sucrose showed almost twice as high of a lipase production (184%) as the control, while the cell growth was similar (105%). Yeast extract and ammonium salts were effective as individual nitrogen sources for lipase production. This study demonstrated that the cold activity of lipase Y-7723 at 10 °C was highest among the cold-active lipases reported so far.

KEYWORDS: Lipase; cold-active; *Pichia lynferdii*; optimization; yeast

INTRODUCTION

Lipase (triacylglycerol acylhydrolases, E.C. 3.1.1.3) is one of the most important enzymes with industrial potential. Because lipases can catalyze numerous different reactions, they have been widely used in industrial applications, such as in food, chemical, pharmaceutical, and detergent industries (*1*). Several microorganisms have been well-studied as good producers of extracellular lipases (*2*). For the industrial applications, high productivity of lipase is of great importance. There are several different environmental factors such as temperature, pH, medium composition, and presence of inducers affecting productivity of lipase. During the past decade, extensive investigations have been carried out using different fermentation techniques to increase the productivity of lipase with different microbial sources (*3*−*6*).

Although lipases have so far been utilized in many industrial applications and can be produced on a large scale using different microorganisms, there is still a great interest in new enzymes with commercially useful properties. Cold-active lipases are particularly attractive for some industrial applications, such as in detergent formulations, fine chemistry catalysis, or food processing, for the consideration of product stability and energy savings (*7*,*8*). Most microbial lipases exhibit high activity between 30 and 50 °C, with a maximum around 40 °C. At lower temperatures, their catalytic activities are severely reduced and almost down to zero at 0 °C. Cold-active enzymes, however, showed relatively high specific activity in the range of 0−20 °C, with optimal activity at 30−40 °C (*7*−*10*). These properties make cold-active lipases good candidates for low-temperature processes.

Most of the cold-active lipases and esterases that have so far been characterized originate from psychrotrophic and psychrophilic microorganisms found in the Antarctic and the Antarctic seawaters (*11*−*15*). In laboratories, the Antarctic strains are commonly cultivated at around 5 °C with their generation time being from 2 to 10 h, securing the absence of cellular stress. A higher temperature (> 20 °C), although shortening the generation time, induces cellular stress, typically leading to low density of cells and poor extracellular enzyme production. These cultural conditions for the optimal growth and most efficient production of extracellular lipases from the cold-adapted microorganisms give rise to limitations for commercial production of cold-active lipases considering cost effectiveness, rendering a new search for cold-active lipase produced from mesophilic microorganisms.

In this study, as an effort to search for mesophilic microorganisms producing cold-active lipase, we screened yeast strains that were prescreened for the induction of extracellular lipase and report about a novel cold-active lipase produced from a mesophilic

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 Published on Web 12/10/2009

Table 1. Lipase Production from Yeast Strains

yeast strain	cell growth (DCW, mg)[a]	lipase activity (unit/mL)	productivity (unit/mL/h)
Candida apicola (NRRL Y-2481)	450 ± 17	0.22 ± 0.01	2.8×10^{-3}
Candida azyma (NRRL Y-17067)	340 ± 16	5.67 ± 0.02	77.8×10^{-3}
Candida cacaoi (NRRL Y-7302)	370 ± 21	1.60 ± 0.01	22.2×10^{-3}
Candida magnoliae (NRRL Y-2333)	400 ± 12	4.93 ± 0.02	68.1×10^{-3}
Candida magnoliae (NRRL YB-4226)	350 ± 7	1.75 ± 0.01	23.6×10^{-3}
Candida melinii (NRRL Y-1514)	350 ± 12	5.21 ± 0.04	72.2×10^{-3}
Candida quercuum (NRRL Y-12942)	380 ± 11	3.68 ± 0.01	50.0×10^{-3}
Candida silvicola (NRRL YB-2846)	380 ± 18	2.50 ± 0.01	34.7×10^{-3}
Pichia americana (NRRL Y-2156)	330 ± 9	2.86 ± 0.02	38.9×10^{-3}
Pichia bimundalis (NRRL Y-5343)	320 ± 11	4.11 ± 0.03	56.9×10^{-3}
Pichia canadensis (NRRL Y-2340)	510 ± 21	4.24 ± 0.02	58.3×10^{-3}
Pichia holstii (NRRL Y-7914)	340 ± 12	0.35 ± 0.01	4.2×10^{-3}
P. lynferdii (NRRL Y-7723)	260 ± 13	25.3 ± 1.1	347.2×10^{-3}
Pichia muscicola (NRRL Y-7005)	390 ± 18	1.90 ± 0.09	26.4×10^{-3}
Pichia muscicola (NRRL Y-7006)	420 ± 14	3.6 ± 0.2	50.0×10^{-3}
Pichia petersonii (NRRL YB-3808)	370 ± 20	1.54 ± 0.05	20.8×10^{-3}

[a] DCW represents dry cell weight per 50 mL of culture.

yeast strain. Optimal culture conditions for lipase production were also investigated for further large-scale production.

MATERIALS AND METHODS

Materials. All chemicals were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO), unless mentioned otherwise. Immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) and free lipase from *Candida rugosa* (Lipase AY) were provided by Novo Nordisk BioChem North America, Inc. (Franklinton, NC) and Amano Enzymes (Troy, VA), respectively.

Microorganism. *Pichia lynferdii* Y-7723 and other yeast strains tested in this study were obtained from the Culture Collection of National Center for Agricultural Utilization Research (Peoria, IL). 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. The seed culture was prepared by thawing the frozen stock culture in a water bath at 37 °C followed by inoculation in 50 mL of YM media in 100 mL Erlenmeyer flasks. Cultural incubation was carried out with reciprocal shaking at 150 rpm and 25 °C. For the main culture for the production of lipase, 1% (v/v) of soybean oil was added to the YM medium, and incubation was performed for 72 h. YM media were used as a basal medium, and the media composition was modified as needed for the optimization study of culture conditions. All experiments were carried out in duplicate.

Determination of Cell Growth. One milliliter of culture from the flask was collected and centrifuged (5000*g* for 10 min at room temperature). The cells were washed twice by resuspending them in the same volume of distilled water, and the harvested wet cells were dried at 80 °C in a dry oven for at least 30 min. The dry cell weight (DCW) was determined when a further weight change was not detected.

Determination of Lipase Activity. The lipase activity was determined by a spectrophotometric method using *p*-nitrophenylbutyrate (*p*-NPB) as the substrate (*16*) with slight modification. In brief, 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). An absorbance increase caused by the release of *p*-nitrophenol from *p*-NPB by enzymatic hydrolysis at 37 °C was measured spectrophotometrically for at least over 2 min at 410 nm 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 min. For the preparation of 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 collected and used for the determination of lipase activity.

RESULTS AND DISCUSSION

Screening of Lipase Production from the Selected Yeast Strains. In a previous report, the lipase induction of the yeast strains selected from the ARS Culture Collection was determined by thin-layer chromatography with respect to the relative spot intensity of the released free fatty acid (*17*). In this study, from previous results, we selected 16 strains including eight *Candida* species and eight *Pichia* species with a high potency of lipase production and determined the actual lipase production and cell growth (**Table 1**). Among those strains, *P. lynferdii* Y-7723 showed the highest lipase production (25 units/mL), although its cell growth was relatively low (260 mg/mL, DCW) under the given culture conditions. On the basis of these results, *P. lynferdii* Y-7723 was selected for further optimization study of environmental conditions for lipase production. The lipase produced from *P. lynferdii* Y-7723 was named lipase Y-7723.

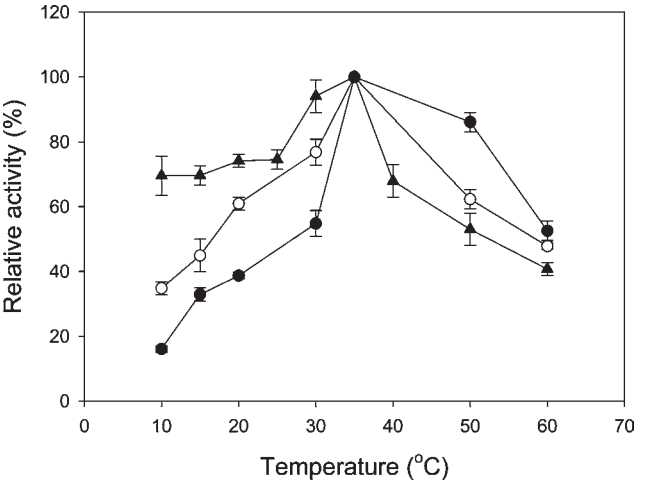

Figure 1. Effect of temperature on the activity of lipase Y-7723. Enzyme activities of crude Y-7723 lipase (▲), Novozyme RMIM (○), and Amano AYS (●) were measured at different temperatures after an incubation period of 5 min.

Production of a Cold-Active Lipase from *P. lynferdii* Y-7723. The optimal temperature for lipase activity is one of the important considerations for an industrial company for the proper application of lipase. Hence, the optimal temperature for the hydrolytic activity of lipase Y-7723 was determined at the range from 10 to 60 °C. As shown in **Figure 1**, the optimum temperature for the activity of lipase Y-7723 was determined to be between 30 and 35 °C, after which the activity dropped to 50% of the maximum at 50 °C. However, enzyme activities at low temperatures under 25 °C remained relatively high, as compared to those

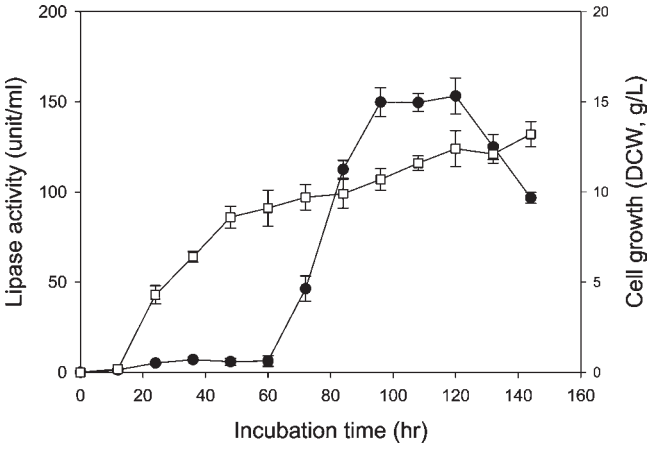

Figure 2. Time-coursed production of lipase Y-7723. The culture was harvested at a given time, and the extracellular lipase activity (●) was determined under standard conditions. Cell growth (□) was determined by calculation of the DCW obtained from the harvested wet cell. Detailed methods are described in the Materials and Methods.

at high temperatures over 40 °C. The relative activities at 20 and 10 °C were 74 and 70% of the maximum value, respectively. The relative activity of lipase Y-7723 at low temperature was much higher than other commercial lipases such as Lipozyme RM IM and Lipase AY. At 20 °C, the relative activities of Lipozyme RM IM and Lipase AY were 61 and 39% of the maximum values, respectively. However, those activities dropped to 35 and 16% at 10 °C, respectively, while lipase Y-7723 remained at 70%. Recently, several cold-active lipases were reported from various microbial strains. Cold-active lipase from *Pseudomonas fragi* showed 59% relative activity at 10 °C with an optimum temperature for activity at 29 °C (*18*). A novel psychrophilic lipase from *Pseudomonas fluorescens* represented approximately 65% relative activity at 10 °C, although its optimum temperature was 20 °C (*19*). Cold-active lipase from *Aspergillus nidulans* represented 40% relative activity at 10 °C with its optimum temperature at 40 °C (*20*). These results demonstrated that the relative cold activity of lipase Y-7723 at 10 °C was highest among the cold-active lipases reported so far.

Optimization of Environmental Conditions for Lipase Production. As lipase Y-7723 was found to be highly active at low temperature, optimal culture conditions for lipase production were investigated. Time-coursed production of lipase Y-7723 was examined in YM media containing 1% soybean oil (**Figure 2**). The cellular growth rate of *P. lynferdii* Y-7723 was relatively low as compared to the general bacterial growth. Accordingly, lipase production began to increase after 60 h of incubation, corresponding to the late exponential phase of cell growth, and reached a maximum between 90 and 120 h. After 120 h, the lipase production declined remarkably. This result was quite different from another report showing that the production of cold-active lipase from *A. nidulans* was maximized at 48 h (*20*).

Organic carbon sources are required for microbial growth and biological functions. The influences of different carbon sources on lipase production by *P. lynferdii* Y-7723 are shown in **Figure 3**. Among eight carbon sources tested, maltose and fructose were comparable to glucose (control) in lipase production and cell growth, while lactose and xylose showed relatively low productivities. However, sucrose showed almost twice as high of a lipase production (184%) as the control, while cell growth was similar (105%). This result was quite unusual in that generally sucrose was less effective for lipase production than glucose. Lee et al. reported that disaccharides including sucrose, maltose, and

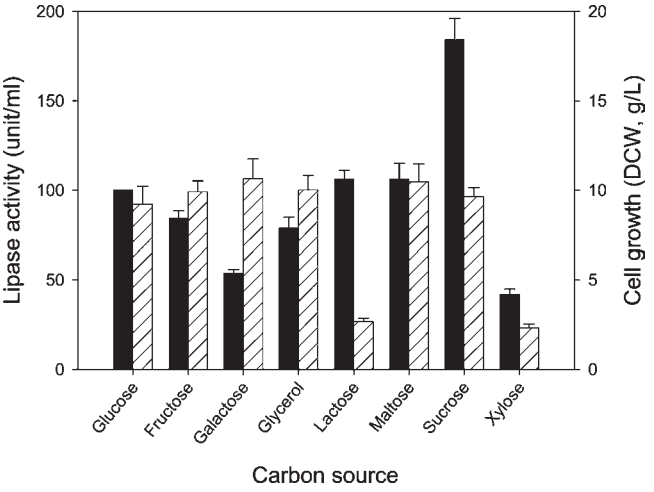

Figure 3. Effect of carbon source on the production of lipase Y-7723. Glucose of the control medium was replaced by the individual carbon source tested. The final concentration of each carbon source was fixed to be 1% of the medium. The relative lipase activities (black bar) were compared to the control (glucose), and cell growth (striped bar) was determined as described in **Figure 2**.

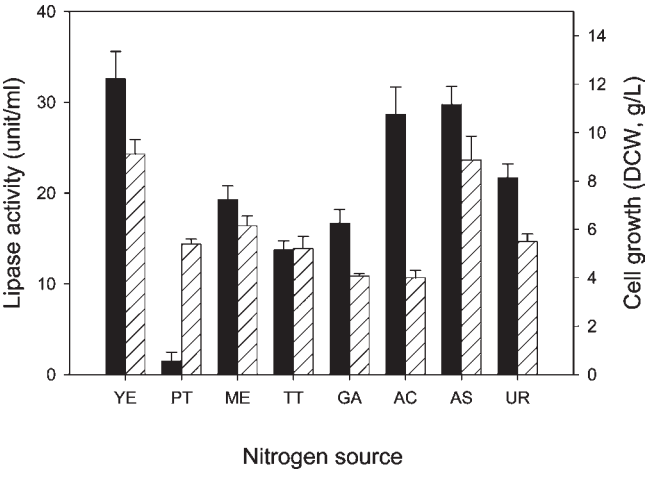

Figure 4. Effect of nitrogen source on the production of lipase Y-7723. Each nitrogen source was supplemented as a single nitrogen source to the basal medium without nitrogen source. The final concentration of nitrogen was fixed to be 0.0315% (w/v) of the medium. The lipase activity (black bar) and cell growth (striped bar) were determined as described in the Materials and Methods. YE, yeast extract; PT, peptone; ME, malt extract; TT, tryptone; GA, glutamine; AC, ammonium chloride; AS, ammonium sulfate; and UR, urea.

lactose were inhibitory for lipase production by a yeast strain *Yarrowia lipolytica* NRRL Y-2178 (*21*). Thermophilic lipase from *P. fluorescens* showed similar lower productions with disaccharides such as sucrose, lactose, and maltose than glucose (*22*). There are no specific reports about the effects of carbon sources on the production of cold-active lipases so far. Most cases dealing with cold-active lipase used glucose as a carbon source (*18*−*20*).

Eight different nitrogen sources, including organic and inorganic nitrogen sources, were tested for lipase production (**Figure 4**). Each nitrogen source was adjusted to contain 0.0315% nitrogen, which corresponded to the nitrogen concentration of yeast extract contained in YM standard medium. Yeast extract was the best nitrogen source among nitrogen sources tested for lipase production and cell growth followed by ammonium sulfate, representing 91% of lipase production and 97.3%

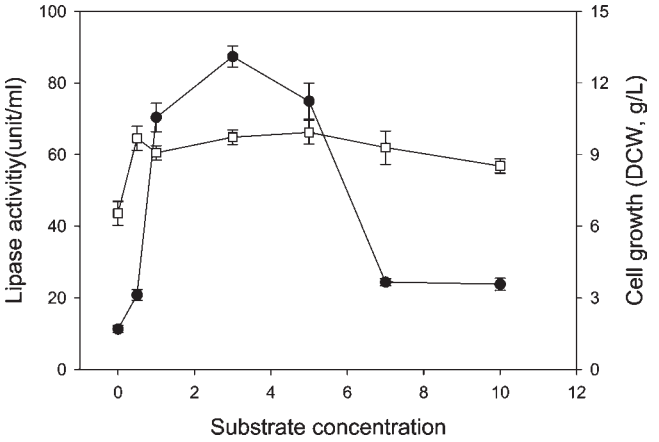

Figure 5. Effect of oil content on the production of lipase Y-7723. A corresponding amount of soybean oil was added to the basal medium prior to microbial inoculation. The lipase activity (●) and cell growth (□) were determined as described in the Materials and Methods.

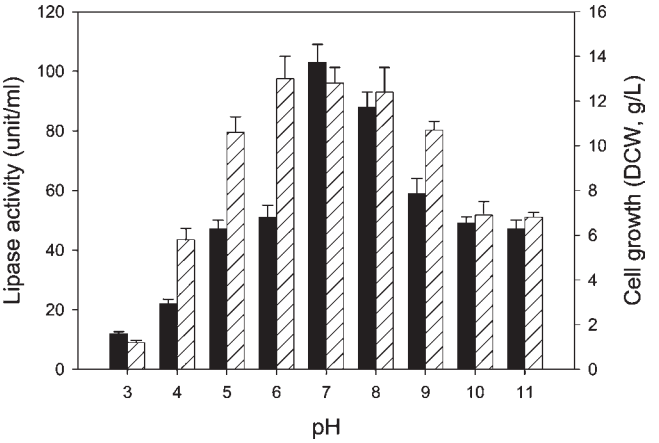

Figure 6. Effect of initial medium pH on the production of lipase Y-7723. Medium pH values were adjusted with 2 N HCl or 2 N NaOH before autoclaving. The lipase activity (black bar) and cell growth (striped bar) were determined as described in the Materials and Methods.

of cell growth as compared to yeast extract. Ammonium chloride showed 88% of lipase production, while cell growth was relatively low, representing 44% of the maximum. The optimal nitrogen source for lipase production varied according to the strains used, although yeast extract was generally accepted as a good nitrogen source. *Y. lipolytica* 681 showed 2.5 times a high value of lipase production with urea as with yeast extract (*23*), while alkaline lipase from *Y. lipolytica* NRRL Y-2178 showed very low lipase production with urea (1.6%) as compared to yeast extract (*21*). For lipase Y-7723, urea represented 67% lipase production of the maximum.

Lipase production is induced by the presence of oil substrate. Hence, the effect of oil content on lipase production needs to be verified. Lipase production was determined with the varied content of soybean oil from 0 to 10% in the YM medium (**Figure 5**). Lipase production increased hyperbolically with oil content and reached a maximum at 3% followed by a dramatic declination down to the base level over 7%. However, cell growth was not changed significantly over 0.5% oil content. Effects of various natural oils on lipase production were well-studied with several microorganisms (*22*−*24*). However, it was rarely studied about dose-dependent lipase production with natural oil. The results from this study suggested that the concentration as well as the kind of oil could be important factors for lipase production.

The pH of the culture is one of the important environmental parameters affecting microbial cell growth and biochemical metabolism. We determined the optimal initial pH of the medium for cell growth and lipase production over the pH range from 3 to 11 (**Figure 6**). Lipase production increased proportionally with pH up to pH 7.0 and decreased sharply thereafter, suggesting that pH 7.0 was optimal for lipase production from *P. lynferdii* Y-7723. Overall, alkaline condition was more efficient for lipase production than acidic condition. This result was very similar to the production of a cold-active lipase from *A. nidulans* in that lipase production from *A. nidulans* peaked at pH 6.5 (*20*). Cell growth was maximized at pH 6.0 and remained at a plateau up to pH 8.0, indicating that *P. lynferdii* Y-7723 had a broad neutral optimal pH range for cell growth. The optimal initial pH of the medium for lipase production from various microorganisms was a variety of acidic (*23*, *25*), neutral (*24*), and alkaline conditions (*26*−*28*).

In conclusion, from this study, a novel cold-active lipase was first produced from the mesophilic yeast strain *P. lynferdii* Y-7723, and some important optimal culture conditions were verified for further scaled-up production. Further work should be focused on the biochemical study of this enzyme after purification and molecular biological study to clone the corresponding gene.

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Received for review September 29, 2009. Revised manuscript received November 24, 2009. Accepted November 24, 2009. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-003-F00026).