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New Finding and Optimal Production of a Novel Extracellular Alkaline Lipase from *Yarrowia lipolytica* NRRL Y-2178

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Abstract Lipases are industrially useful versatile enzymes that catalyze numerous different reactions including hydrolysis of triglycerides, transesterification, and chiral synthesis of esters under natural conditions. Although lipases from various sources have been widely used in industrial applications, such as in food, chemical, pharmaceutical, and detergent industries, there are still substantial current interests in developing new microbial lipases, specifically those functioning in abnormal conditions. We screened 17 lipase-producing yeast strains, which were prescreened for substrate specificity of lipase from more than 500 yeast strains from the Agricultural Research Service Culture Collection (Peoria, IL, U.S.A.), and selected *Yarrowia lipolytica* NRRL Y-2178 as a best lipase producer. This report presents new finding and optimal production of a novel extracellular alkaline lipase from *Y. lipolytica* NRRL Y-2178. Optimal culture conditions for lipase production by *Y. lipolytica* NRRL Y-2178 were 72 h incubation time, 27.5°C, pH 9.0. Glycerol and glucose were efficiently used as the most efficient carbon sources, and a combination of yeast extract and peptone was a good nitrogen source for lipase production by *Y. lipolytica* NRRL Y-2178. These results suggested that *Y. lipolytica* NRRL Y-2178 shows good industrial potential as a new alkaline lipase producer.

Keywords: Alkaline lipase, *Yarrowia lipolytica*, fermentation, optimization

Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3.) is one of the well-known industrially useful enzymes because it can catalyze numerous different reactions including hydrolysis of triglycerides, transesterification, and chiral synthesis of esters under natural conditions. Based on the versatile reaction properties of lipase, they have been widely used in industrial applications, such as in food, chemical, pharmaceutical, and detergent industries [8, 15]. Although lipases are found widely in animals, plants, and microbes, microbial lipases have gained special industrial attention because of their versatility and availability. Many microorganisms are known as good producers of extracellular lipases [4, 16]. However, although microbial lipases have so far been utilized in many industrial applications, there are still great interests in developing new enzymes with commercially useful properties, such as high activities under extreme conditions. Specifically, alkaline lipases are useful in the detergent industry, since triglycerides in stains on fabrics are difficult to remove because they are hardly saponified compared with fatty acids. At the National Center for Agricultural Utilization Research (NCAUR, Peoria, IL, U.S.A.), they conducted a large-scale screening for lipase activity with selected cultures from the Agricultural Research Service (ARS) Culture Collection (Peoria, IL, U.S.A.) and characterized 25 highly active yeast lipases with respect to their positional specificity against triglyceride [10].

In this study, we expanded our research more specifically with respect to high production and novel properties of lipase from *Yarrowia lipolytica* NRRL Y-2178, which was selected as a best lipase producer among yeast strains tested.

Y. lipolyica NRRL Y-2178 and other yeast strains tested in this study were kindly provided by Dr. Hou in NCAUR. The stock culture was maintained in a cryogenic vial containing 0.4 ml glycerol and 0.6 ml YM medium (1% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, w/v) in a deep freezer at −70°C prior to use. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), unless otherwise mentioned. Seed culture with 2% inoculum

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in 50 ml of YM media in a 100-ml flask was prepared by incubation at 25°C for 48 h with reciprocal shaking at 150 rpm. For the main culture for production of lipase, a portion (2%, v/v) of the seed culture was inoculated into 250-ml conical flasks containing 100 ml of lipase-producing media (YM media plus 1% soybean oil, v/v) and incubated for a proper time in a reciprocal shaking incubator (25°C, 150 rpm). YM medium was used as a basal medium and medium composition was modified as needed for the study of optimization of culture condition. For the study of optimal pH for lipase activity, the pH of the assay mixture was adjusted in 50 mM sodium-phosphate buffer (pH 3.0–7.0) and 50 mM Tris buffer (pH 7.0–10.0). Cell growth was determined by measurement of the absorbance of cells, after being washed and resuspended in distilled water, at 610 nm with a spectrophotometer (Jasco V-530, Tokyo, Japan). Dry cell weight (DCW) was calculated from the absorbance value using a standard curve. Lipase activity was estimated by a spectrophotometric method [20] with *p*-nitrophenylbutyrate (*p*-NPB) as the substrate. The assay mixture was composed of 0.1 ml of enzyme sample and 0.9 ml of working substrate solution. The substrate solution was prepared by a ten-times dilution of 10 mM *p*-NPB dissolved in 100% cold ethanol with 0.05 M sodium-phosphate buffer (pH 7.0). After immediate transfer of the assay mixture into a spectrophotometer cell, the increase of absorbance at 37°C was measured at 410 nm for 360 sec against an enzyme-free control (YM medium). One unit of enzyme activity was defined as the amount of enzyme that liberates 1 nmol of *p*-nitrophenol from *p*-NPB per minute. The values presented in each experiment of this study were the averages of duplicates, unless otherwise specified.

We determined the actual lipase production and cell growth of the yeast strains and selected *Y. lipolytica* NRRL Y-2178 as a best lipase producer among eight *Candida* species, eight *Pichia* species, and one *Yarrowia* species, all identified as good lipase producers by NCAUR [9]. The lipase activity of *Y. lipolytica* NRRL Y-2178 was 107 units/ml under the standard condition. Study of the time-coursed lipase production showed that the highest lipase production with the 200 units/ml was observed after 72 h incubation, which was corresponding to the late exponential phase of cell growth (data not shown). Based on this result, further environmental optimization processes were performed with 72 h incubation. The optimal incubation temperature for lipase production was determined to be 27.5°C when tested under the range of 20°C to 35°C. Lipase production at 27.5°C was almost twice higher than that at 25°C or 30°C (data not shown). Cell growth was not significantly influenced within the range up to 30°C. However, both lipase production and cell growth were almost stopped at 35°C.

The pH of a culture is one of the most important environmental parameters affecting microbial cell growth and biochemical metabolism. Most natural environments

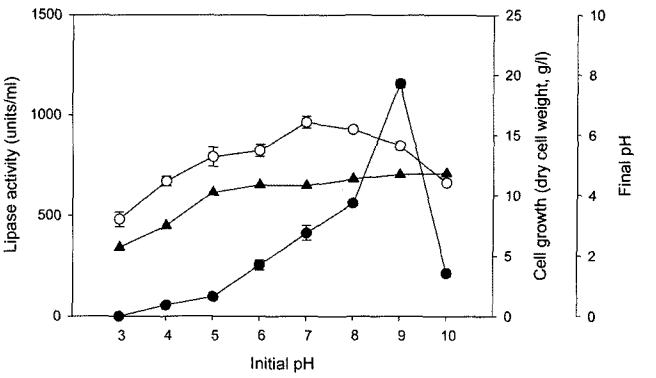

Fig. 1. Effect of initial medium pH on the production of lipase by *Y. lipolytica* NRRL Y-2178.
Control medium with 1% soybean oil was used as the basal medium and the initial pH of the medium was adjusted with 1 N HCl or 1 N NaOH. Closed and open circle represent lipase production and cell growth, respectively. Final pH (closed triangle) was determined after 72 h incubation. See the text for other experimental conditions.

have pH values between 5 and 9, and organisms with optimal pH in this range are most common. We determined the optimal initial pH of the basal medium for cell growth and lipase production over the pH range of 3–10 (Fig. 1). Total cell growth after 72 h incubation proportionally increased with pH up to pH 7.0 and decreased thereafter. However, the degrees of changes were not significant in the range of pH 5–10, indicating that *Y. lipolytica* NRRL Y-2178 showed sufficient cell growth in the broad pH range. By contrast, lipase production was very sensitive with pH values. Lipase production increased dramatically up to pH 9.0 and dropped significantly at pH 10.0. The highest value of lipase production reached 1,156 units/ml

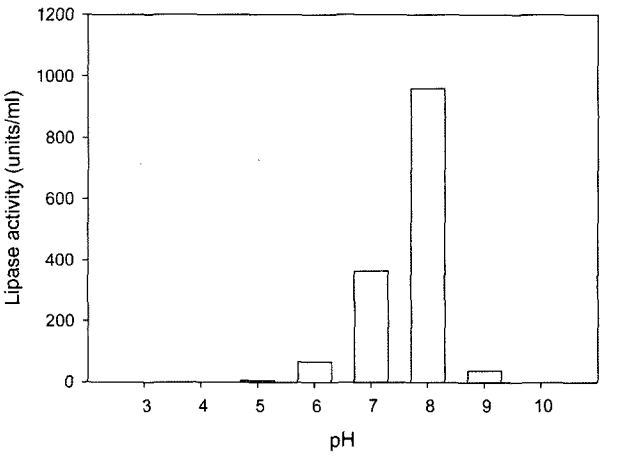

Fig. 2. Effect of pH on the activity of lipase from *Y. lipolytica* NRRL Y-2178.
Sodium-phosphate buffer (50 mM) and Tris buffer (50 mM) were used for acidic and basic pH conditions, respectively. See the text for other experimental conditions.

at pH 9.0, which was 4.6-fold higher than the control (pH 6.0). The final pH values of each culture decreased to the range of pH 4–4.8, except for the cultures with initial pH of 3.0 and 4.0. Since the lipase production under different initial medium pH was highest at pH 9.0, we determined the optimal pH for lipase activity in the same pH range (Fig. 2). The optimal pH for lipase activity was pH 8.0 with a 2.6-fold higher activity than the control (pH 7.0), indicating that the extracellular lipase of *Y. lipolytica* NRRL Y-2178 was alkaline lipase. Lipase activity at pH 9.0 dropped significantly.

The optimal initial pH values for lipase production from various microorganisms were of variety with acidic [3, 17], neutral [18, 19], and alkaline conditions [7, 11]. There were some reports about alkaline lipase from *Pseudomonas* species [11–13], *Bacillus* species [2], and from some fungal species [5, 7, 8]. However, the production of alkaline lipase from *Yarrowia* species is first reported in this study. Interestingly, similar *Y. lipolytica* strains with different origins represented different initial pH values for optimal lipase production. *Y. lipolytica* 681, from the culture collection of the Tropical Medicine Institute of Sao Paulo, Brazil, showed optimal initial pH for lipase production in the range of pH 3.0–4.5, whereas the optimal pH for activity was pH 6.0 [3]. The optimal pH for production and activity of lipase from a mutant strain of *Y. lipolytica* isolated from soil was pH 7.0 and pH 8.0, respectively [18, 21]. Extracellular lipase from the lipase overproducing mutant of *Y. lipolytica* CBS6303 showed optimal activity at pH 6.0 [6]. Another genetically modified *Y. lipolytica* strain overproducing LIP2 lipase showed maximum specific activity at pH 6.0 [1]. All these results were quite different from our results, confirming that lipase from *Y. lipolytica* NRRL Y-2178 was a novel alkaline lipase.

Since the lipase from *Y. lipolytica* NRRL Y-2178 was found to be a novel alkaline lipase, we determined the effect of important nutritional factors on the production of the lipase and on cell growth. Table 1 showed the influences of different carbon sources on lipase production by *Y. lipolytica* Y-2178. Among eight carbon sources tested, glucose and glycerol were found to be suitable for lipase production. All monosaccharides tested, except xylose, were more effective to produce lipase than disaccharides, although cell growths were comparative. These results suggested that disaccharides were not favorable for lipase production by *Y. lipolytica* NRRL Y-2178. There are some reports about contradictory effects of glycerol on lipase production and activity. Lin *et al.* [14] reported that glycerol was the most strong stimulating factor among ordinary carbon sources that they used for lipase production from a fungal species, *Antrodia cinnamomea*. Their result was in good agreement with the results of this paper. However, lipase activity was not observed in the culture medium containing glycerol for *Yarrowia lipolytica* 681, although glycerol in the enzyme reaction medium, even with high concentration, did not significantly inhibit the activity of lipase [3]. They explained the reason for the inhibitory effect of glycerol on lipase production as that glycerol was an end product of the hydrolysis of triacylglycerols and it might repress lipase production. However, unexpectedly, glycerol was used as an efficient stimulating carbon source for lipase production by *Y. lipolytica* NRRL Y-2178, as described above. This discrepancy should be further studied.

Nine different nitrogen sources, including organic and inorganic nitrogen sources, were evaluated for lipase production (Table 2). Each nitrogen source was adjusted to contain the same nitrogen content as the control. Peptone and yeast extract, when used as a single nitrogen source, was relatively effective for lipase production. However, cell growth with peptone was very low, representing 20%

Table 1. Effect of carbon sources on the production of lipase by *Y. lipolytica* NRRL Y-2178.

Carbon sources[a]	Lipase activity (units/ml)	Cell growth (g/l DCW[b])
Glucose	375±30	13.7±0.7
Sucrose	64.3±18	9.9±0.1
Fructose	224±40	14.3±1.6
Maltose	54.8±12	10.3±0.8
Galactose	173±15	14.6±1.3
Glycerol	411±32	13.6±1.7
Lactose	77.5±20	12.7±1.2
Xylose	35.4±6	5.4±0.7

[a]Concentration of each carbon source was 1% of the medium (w/v).
[b]DCW represents dried cell weight.

Table 2. Effect of nitrogen sources on the production of lipase by *Y. lipolytica* NRRL Y-2178.

Nitrogen sources[a]	Lipase production (units/ml)	Cell growth (g/l DCW[b])
Yeast extract	25.0±2.0	14.7±0.1
Peptone	22.0±1.0	2.7±0.1
Yeast extract+Peptone	235±4.0	13.5±0.5
Malt extract	0	1.41±0.2
Tryptone	0	2.38±0.2
Glutamine	0.58±0.1	0.38±0.1
NH_4Cl	0.6±0.2	0.5±0.1
$(NH_4)_2SO_4$	0.5±0.12	0.55±0.05
Urea	0.4±0.1	0.45±0.04
NH_4NO_3	0.6±0.1	0.52±0.08
NC[c]	0	0.27±0.01
Control[d]	250±17.0	13.5±1.0

[a]Nitrogen source of YM medium was replaced with the individual nitrogen sources.
[b]DCW represents dried cell weight.
[c]NC represents negative control (no nitrogen source was used).
[d]Control represents YM medium.

of the control, whereas cell growth with yeast extract was comparative to the control. Interestingly, when peptone and yeast extract were combined in a 1:1 ratio, lipase production dramatically increased, reaching up to the control. These results suggested that yeast extract was important for cell growth, whereas peptone played a certain important role in enhancing lipase production, and that there could be a possible synergistic relationship involved between the two nitrogen sources in lipase production by *Y. lipolytica* NRRL Y-2178. Lipase production and cell growth with other organic and inorganic nitrogen sources were very low.

From this study, we confirmed that extracellular lipase from *Y. lipolytica* NRRL Y-2178 was a novel alkaline lipase and that some of the optimal culture conditions for lipase production by *Y. lipolytica* NRRL Y-2178 were quite different from other *Yarrowia* species. Glycerol and glucose were efficiently used as the most efficient carbon sources, and a combination of yeast extract and peptone was a good nitrogen source for lipase production by *Y. lipolytica* NRRL Y-2178. These results suggested that *Y. lipolytica* NRRL Y-2178 show good industrial potential as a new alkaline lipase producer.

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