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SCREENING AND CHARACTERIZATION OF A NOVEL ALKALINE LIPASE FROM *ACINETOBACTER CALCOACETICUS* 1-7 ISOLATED FROM BOHAI BAY IN CHINA FOR DETERGENT FORMULATION

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ABSTRACT

A novel alkaline lipase-producing strain 1-7 identified as *Acinetobacter calcoaceticus* was isolated from soil samples collected from Bohai Bay, China, using an olive oil alkaline plate, which contained olive oil as the sole carbon source. The lipase from strain 1-7 showed the maximum activity at pH 9.0 under 40 ^{o}C. One interesting feature of this enzyme is that it exhibits lipase activity over a broad range of temperatures and good stability. It is also stable at a broad range of pHs from 4.0 to 10.0 for 24 h. Its catalytic activity was highly enhanced in the presence of Ca^{2+}, Mg^{2+} and K^{+}, but partially inhibited by Cu^{2+}, Al^{3+}, Fe^{3+}, $Ba^{2+}$and Zn^{2+}. The fact that it displays marked stability and activity in the presence of TritonX-100, Tween-20, Tween-80, SDS, Hydrogen peroxide, Sodium perborate, Sodium hypochlorite, Sodium citrate, Sodium taurocholate, Glycerine and NaCl suggests that this lipase is suitable as an additive in detergent formulations.

Key words: *alkaline lipase; acinetobacter calcoaceticus; characterization; detergent.*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are one of the most important classes of hydrolytic enzymes that catalyze both the hydrolysis and synthesis of esters (9, 10). The main reason for the steadily growing interest in lipases is because of their enantioselective, regioselective and chemoselective nature (28). Lipases occur widely in nature, but only microbial lipases are commercially significant, since they can be produced at low cost and exhibit improved stability (7).

The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in household and industrial laundry (8). Washing and degreasing by using lipases allows for smaller amounts of surfactants and operation at low temperatures (18). The lipase component causes an increase in detergency and prevents scaling, since enzymes can reduce the environmental load of detergent products (7). In 1994, Novo Nordisk introduced the first commercial lipase, Lipolase, which originated from the fungus *T. lanuginosus* and was expressed in *A. oryzae*. In 1995,

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two bacterial lipases were introduced—Lumafast from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes*, both produced by Genencor International, AU-KBC Research Center, Life Sciences, Anna University, Chennai, India (7). Lipases used as detergents also include those from *Candida* (17) and *Chromobacterium* (15). Laundering is generally carried out in alkaline media, lipases active under such conditions are preferred, and for example, the *A. oryzae* derived lipase (5, 21, 26). Alkaline Lipase produced by *Acinetobacter radioresistens* had an optimum pH of 10.0 and was stable over a pH range of 6.0-10.0; therefore have great potential for application in the detergent industry (3).

This study describes the production and characterization of a novel microbial lipase. We conducted an extensive screening of bacterial isolates collected from soils and isolated an alkaline lipase-producing strain 1-7, identified as *Acinetobacter calcoaceticus*. We provided experimental evidence that strain 1-7 produced a novel alkaline lipase capable of catalyzing the hydrolysis of esters at a broad range of temperatures and pHs in the presence of detergent ingredients.

MATERIALS AND METHODS

Enrichment of Lipase-producing Microorganisms

One gram of soil sample (collected from Bohai Bay, China) was added into a 250 mL Erlenmeyer flask containing 50 mL enriching medium with the composition (%) of yeast extract (Oxoid, England) 1, olive oil (Moreno, Spain) 2, NaCl 0.05, $MgSO_4 \cdot 7H_2O$ 0.02, K_2HPO_4 0.1, peptone (Oxoid, England) 2, initial pH 8.0. The mixture was incubated at 37 ^oC on a rotary shaker (HYG Ⅱ, Xinrui Co., China) at 180 rpm for 72 h.

Isolation, Screening and Identification of Lipase-producing Microorganisms

The grown microorganisms in the enrichment culture were isolated on the screening plates, which contain (%) K_2HPO_4 0.1, $NaNO_3$ 0.3, $MgSO_4 \cdot 7H_2O$ 0.05, $FeSO_4 \cdot 7H_2O$ 0.01, emulsion of olive oil (Moreno, Spain) (it contains 0.2% Victoria blue B (SCRC, China)) 2, agar (Solarbio, China) 2, initial pH 8.0. Plates were incubated at 37 ^oC for 24 h. First, the lipase activity was roughly estimated by Victoria Blue plate assay method (12). The growing colonies with blue zones were separately transferred to liquid medium, which contains (%) soluble starch (Shuangxuan Co., China) 1, bean flour (Shuangxuan Co., China) 2, corn flour (Shuangxuan Co., China) 1, $(NH4)_2SO_4$ 0.5, K_2HPO_4 0.1, and emulsion of olive oil (Moreno, Spain) 2, initial pH 8.0. The mixture was incubated at 37 ^oC on a rotary shaker (HYG Ⅱ, Xinrui Co., China) at 180 rpm for 36 h. After that 5 mL aliquot was performed at 60 ^oC for 30 min, a second 5 mL aliquot was performed at 80 ^oC for 15 min and another 5 mL aliquot was performed at 100 ^oC for 10 min, which were respectively added to 4-mm-diameter holes of the plates with 20 μL. Plates were incubated at 37 ^oC for 24 h. Then, in order to select the best lipase producer for enzyme purification and characterization, strains with lipolytic activity on the plates were cultured in liquid medium, and lipase activity was determined with the spectrophotometric assay using p-NPP (Sigma, USA) as a substrate (26, 27, 28).

Identification of strain 1-7 was conducted using 16S ribosomal DNA (rDNA) analysis (4). The genomic DNA was extracted according to the method described by the instruction of the Genome DNA Extraction Kit (Biofuture, China). Two primers, F (5′- AGA GTT TGA TCC TGG CTC AG -3′) and R (5′- CTA CGG CTA CCT TGT TAC GA -3′), were used for PCR (24). PCR amplification was carried out as follow: 94 ^oC for 1 min; 94 ^oC for 45 s, 55 ^oC for 45 s, 72 ^oC for 90 s, 30 cycles; 72 ^oC for 10 min. PCR was analyzed by Agarose Gel Electrophoresis and DNA was screened by gel documentation system (GeneGenius, Syngene, USA). The sequence analysis was performed by Sunnybio Corporation (Shanghai, China). A homology search to reference strains registered in DDBJ/

EMBL/ GenBank was performed using NCBI BLAST.

Lipase Production

Acinetobacter calcoaceticus 1-7 was grown in a liquid medium containing (%) soluble starch (Shuangxuan Co., China) 1, bean flour (Shuangxuan Co., China) 2, corn flour (Shuangxuan Co., China) 1, $(NH4)_2SO_4$ 0.5, K_2HPO_4 0.1, and emulsion of olive oil (Moreno, Spain) 2, initial pH 9.0. Culture conditions were 37 ^oC and 180 rpm in a rotary shaker (HYG Ⅱ, Xinrui Co., China), in the 250 mL flask containing 30 mL of medium. An aliquot of 0.6 mL of a 10 h pre-culture in the culture (containing (%) peptone (Oxoid, England) 1, yeast extract (Oxoid, England) 0.5, NaCl 1, initial pH 9.0.) was used as inoculum.

Crude enzyme was obtained by centrifugation (CR21G, Hitachi, Japan) at 10,000 rpm at 4 ^oC for 10 min. The cell-free supernatant was considered as crude enzyme (20).

Lipase Activity

Lipase activity was determined by the spectrophotometric method with p-nitrophenyl palmitate (p-NPP) (Sigma, USA) as the substrate. Solution A contained p-NPP (30 mg) dissolved in propane-2-ol (10 mL), solution B contained Triton X-100 (Biofuture, China) (1 mL) dissolved in 90 mL buffer (0.1 mol/L Gly-NaOH, pH 9.0). The assay solution was prepared by adding solution A to solution B. The assay mixture contained 900 μL of the emulsion and 100 μL of the appropriately diluted lipase (boiled for 30 minutes as blanks) solution. The reaction was performed at 40 ^oC for 15 min and terminated at 4 ^oC for 10 min. The liberated p-nitrophenol was measured at 410 nm by spectrophotometer (TU-1810, Pgeneral Co., China). One unit (U/mL) of lipase was defined as the amount of lipase that releases 1 mmol p-nitrophenol per minute at 40 ^oC, pH 9.0 (26).

Properties of Lipase

The optimal temperature of the lipase was evaluated by using the lipase activity assay with p-NPP (Sigma, USA) at various temperatures from 20 ^oC to 50 ^oC under pH 8.0. The stability of the lipase was determined by measuring the residual activity after 60 h of pre-incubation in sodium phosphate buffer (pH 8.0) at various temperatures.

The optimal pH of the lipase was measured by incubating the lipase substrate at various pHs from 3.0 to 10.0. The following buffers (0.1 mol/L) were sodium acetate buffer (pH 3.0-5.0), sodium phosphate buffer (pH 6.0-8.0), and Gly-NaOH buffer (pH 9.0-10.0), and were adjusted to the optimal enzyme reaction temperature.

To determine the effect of metal ions on lipase activity, the enzyme solution was pre-incubated with metal ions (1 mmol/L) such as $ZnCl_2$, $CuCl_2$, $MnCl_2$, $AlCl_3$, KCl, $FeCl_2$, $FeCl_3$, $BaCl_2$, $MgCl_2$ and $CaCl_2$ at 40 ^oC for 20 min and then the residual activity was determined.

In order to determine the potential application of lipase from *Acinetobacter calcoaceticus* 1-7 in detergent industry, its compatibility with various surfactants and oxidizing agents was investigated by respectively adding TritonX-100, Tween-20, Tween-80, SDS, Hydrogen peroxide, Sodium perborate, Sodium hypochlorite, Sodium citrate, Sodium taurocholate, Glycerine and NaCl into the activity assay mixture, then the lipase samples were incubated for 1 h at 40 ^oC and their activity was determined by the spectrophotometric assay at pH 9.0 and 40 ^oC. The residual lipase activity in each sample was determined.

RESULTS AND DISCUSSION

Screening and Identification of Lipase-producing Microorganisms

Fifty six strains with high lipase activity from 189 samples were isolated by enrichment cultures, among which 6 strains were shown to produce alkaline lipases. Strain 1-7 was selected for subsequent experiments due to its ability to produce a lipase with good stability at various temperatures

(Table 1). The taxonomic identification of the strain 1-7 was conducted. The sequence of 16S rDNA of the strain 1-7 showed 99% homology to *Acinetobacter calcoaceticus* compared with the GenBank database.

Table 1. The size of rings formed on Victoria Blue agar plate with the crude lipases of six strains

Strains	Diameter of the blue zones (mm)			
	Control	A	B	C
1-7	10.0	8.5	7.0	5.0
2-12	7.0	6.0	5.0	4.5
3-1	9.0	7.5	6.0	4.5
3-2	8.0	6.5	5.5	4.5
3-5	8.5	7.0	5.5	4.5
3-19	10.0	8.0	6.0	4.5

(A) The lipase incubation at 60 °C for 30 min, pH 8.0. (B) The lipase incubation at 80 °C for 15 min, pH 8.0. (C) The lipase incubation at 100 °C for 10 min, pH 8.0.

Effects of Temperature on Lipase Activity

The lipase exhibited optimum lipolytic activity at 40 °C and has substantial activity from 20 °C to 50 °C with the relative activity of 87 % at 20 °C, 96 % at 30 °C, 100 % at 40 °C and 85 % at 50 °C. However, at low temperature (5 °C) and high temperature (70 °C), the lipase activity was greatly reduced (Figure 1). And as shown in Figure 2, stability test at various temperatures for 60 h at pH 8.0 showed that the enzyme was relatively stable, since it retained almost 90 % of its activity at 20 °C to 50 °C.

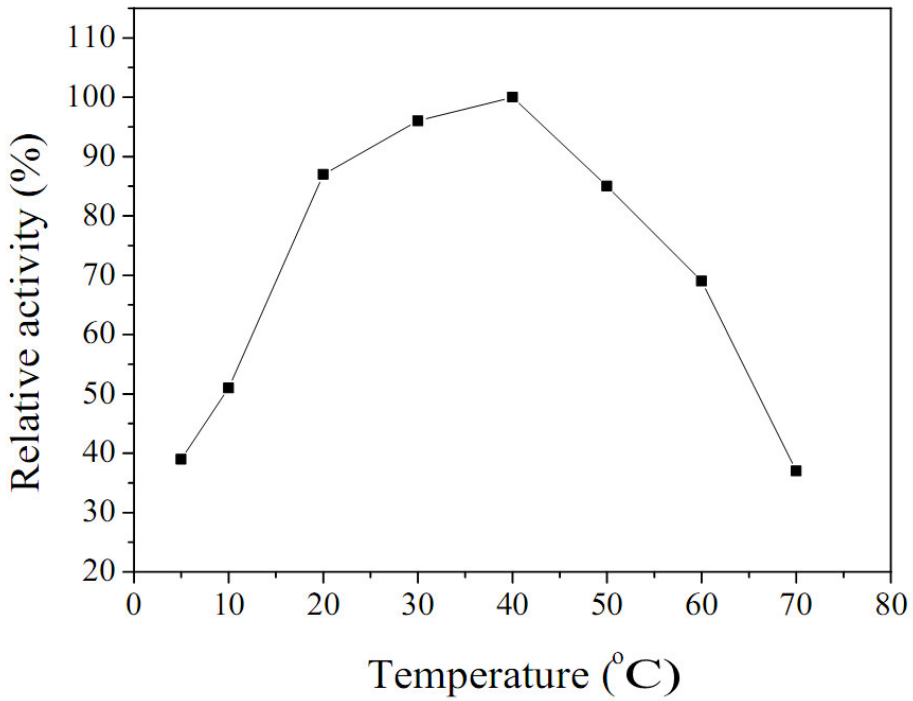

Figure 1. Effects of temperature on the activity of the lipase. The lipase reaction was incubated for 15 min at various temperatures, pH 8.0.

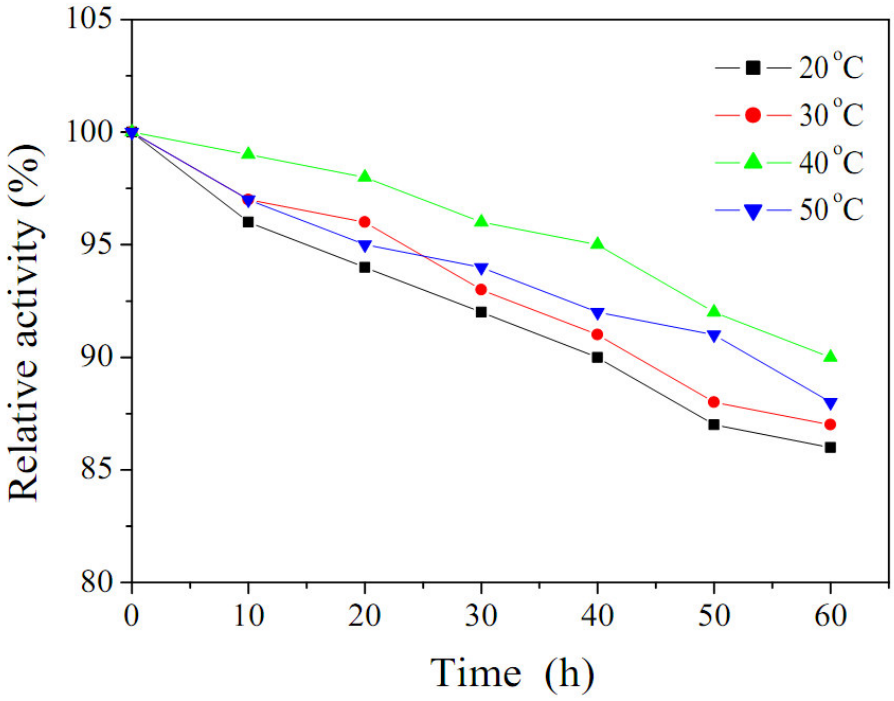

Figure 2. The stability of lipase from *Acinetobacter calcoaceticus* 1-7 was measured at 20 °C-50 °C. The relative activity was assayed after incubation at indicated temperatures for 60 h with the interval of 10 h.

Effects of pH on Lipase Activity

The optimum activity of the lipase was observed at pH 9.0, consistent with the results based on the plate method. The lipase was also highly active over a wide pH range (4.0-10.0) (Figure 3). It retained 80% of its activity at pH 5.0, 86 % at pH 6.0, 92 % at pH 7.0, 96 % at pH 8.0, 100 % at pH 9.0 and 90 % at pH 10.0 after 24 h at 40 °C. It is noteworthy that the lipase retained 56 % activity even at pH 3.0. And as shown in Figure 4, stability test at various pHs for 24 h at 40 °C shown that the lipase was relatively active over a wide pH range. The remarkable wide pH range of the *Acinetobacter calcoaceticus* lipase justifies its further investigation for commercial applications.

As has been reported for other lipases, the enzyme from *Bacillus* sp. RSJ-1 showed good stability as it retained > 90 % activity at 60 °C for 1 h under alkaline conditions and also exhibited a half life of > 150 min at 60 °C and 45 min at 70 °C, respectively. The enzyme exhibited the good stability in an alkaline pH range, as it retained >95 % activity at pH 9.0, which reduced to 78 % at pH 10.0 after 1 h of incubation (23). The results showed the lipase from *Pseudomonas aeruginosa* SRT 9 that exhibited fairly stable activity ranging from 55 to 65 °C, and the enzyme was remarkably stable in the pH rang 6.0 to 7.5 retaining 68 % of the residual activity at pH 8.0 (2). The lipase from *Geobacillus thermoleovorans* was stable at temperature range 40 °C to 60 °C with no loss of activity, whereas increasing incubation temperature up to 100 °C led to 70 % loss of activity after 60 min of incubation. However, its activity of lipase was only observed at pH 7.0-8.0 (1). Actually, preferred lipases for detergent components are those which show sufficient lipase activity in washing solutions and washing conditions. Generally the pH of washing solutions is in the alkaline region. in some countries such as Japan, the temperature of washing water is 40 °C (25). Therefore, the stability of *Acinetobacter calcoaceticus* lipase in alkaline pH and at various temperatures suggests its potential utility in industrial applications.

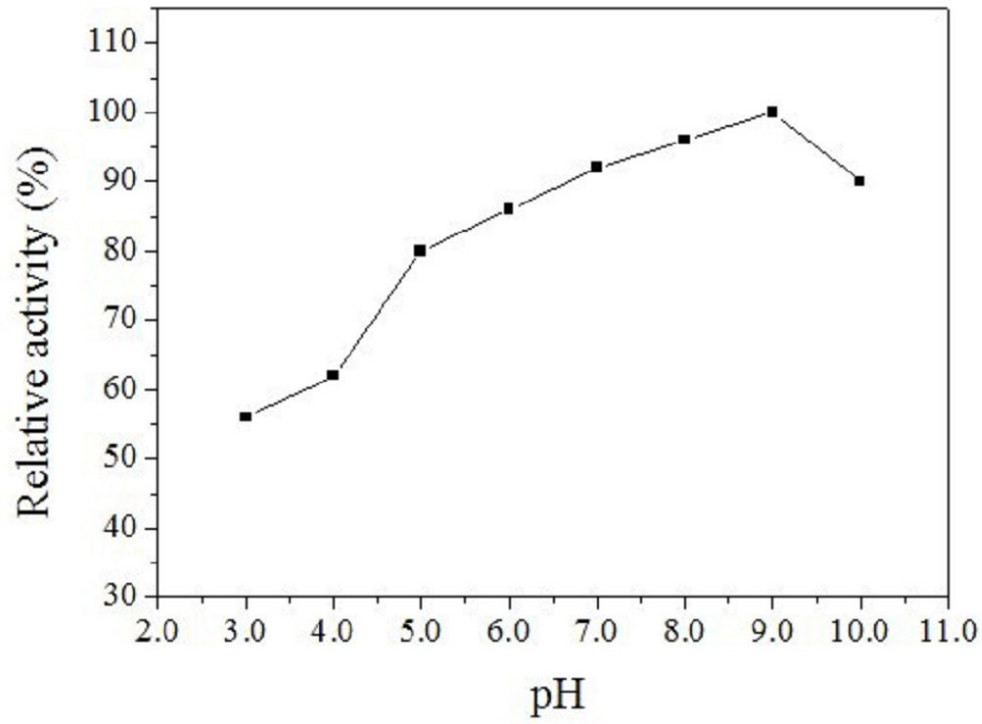

Figure 3. Effects of pH on the activity of the lipase. The reaction was determined at 40 °C under different buffers (0.1 mol/L) at various pHs from 3.0 to 10.0, sodium acetate buffer (pH 3.0-5.0), sodium phosphate buffer (pH 6.0-8.0), and Gly-NaOH buffer (pH 9.0-10.0).

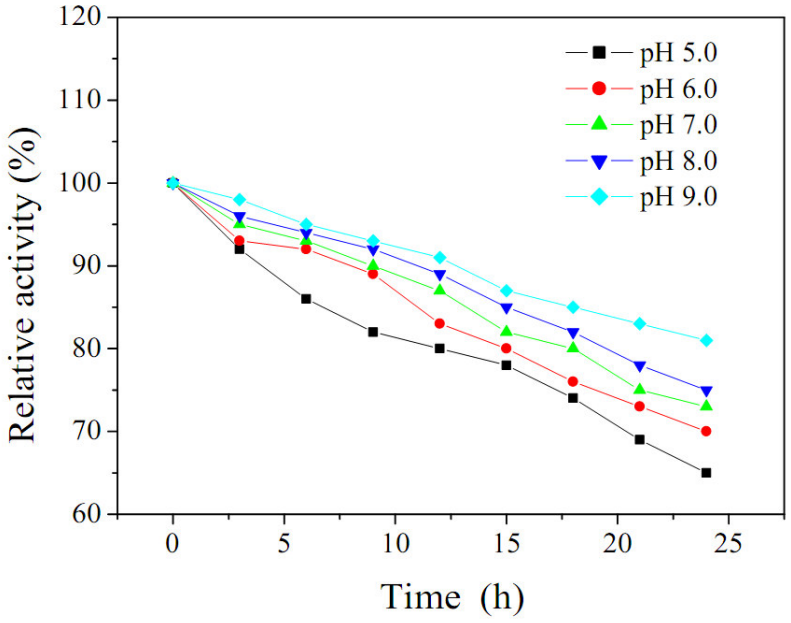

Figure 4. The stability of lipase from *Acinetobacter calcoaceticus* 1-7 was measured at pH 5.0-9.0. The lipase was incubated for 24 h at 40 °C in various buffers (0.1 mol/L), sodium acetate buffer (pH 3.0-5.0), sodium phosphate buffer (pH 6.0-8.0), and Gly-NaOH buffer (pH 9.0-10.0). The residual activity was measured with the spectrophotometric assay using p-NPP as a substrate.

Effects of Metal ions on Lipase Activity

Effects of different metal ions on the activity of the lipase were shown in Table 2. K^+, Ca^{2+}, and Mg^{2+} were found to enhance the lipase activity. While Al^{3+}, Fe^{3+}, $Ba^{2+}$and Zn^{2+} partially inhibited the enzymatic activity, Cu^{2+} completely inhibited the lipase activity. Lipase activity was not affected by the presence of Fe^{2+} and Mn^{2+}.

As has been reported for other lipases, Ca^{2+} salts increased activity immediately (59 %) and after 1 h of incubation at 30 ^oC (35 %) (6, 13, 14).The calcium-induced increase on lipase activity could be attributed to the complex action of calcium ions on the released fatty acids, and on enzyme structure stabilization due to the binding of calcium ions to the lipase, bridging the active region to a second subdomain of the protein and hence stabilizing enzyme tertiary structure (11). Another reported that Ca^{2+} showed stimulatory effect whereas Mg^{2+}, Mn^{2+}, Ba^{2+} had negligible effect on the enzyme activity. However, Fe^{2+}, Cu^{2+} and Zn^{2+} reduced enzyme activity to less than 37 % of its relative activity (2).

Table 2. Lipase stability in presence of metal ions

Metal ions (1 mmol/L)	Relative activity (%)	Metal ions (1 mmol/L)	Relative activity (%)
Zn^{2+}	47	Fe^{2+}	93
Cu^{2+}	0	Fe^{3+}	51
Al^{3+}	39	Ba^{2+}	69
Mn^{2+}	90	Ca^{2+}	141
K^+	112	Mg^{2+}	126

Effects of Surfactants, Oxidizing Agents and Detergent Ingredients on Lipase Activity

Besides pH and temperature stability, a good detergent lipase should also be stable in the presence of various surfactants. The effects of various surfactants, oxidizing agents and detergent ingredients on lipase activity are depicted in Table 3. Addition of 1 % Triton X-100 to the lipase mixture increased the enzyme activity, but 10 % Triton X-100 inhibited its activity, while it was completely inhibited in the presence of 1 % and 10 % SDS, Tween-80 and Tween-20, the effects was apparent under increased concentrations. However, addition of 0.1 % and 1 % Sodium cholate and Sodium taurocholate were found to enhance the enzyme activity apparently. Glycerine, NaCl and Sodium citrate were also found to highly enhance the enzyme activity. All of the oxidizing agents shown in Table 3 had little effect on the lipase activity.

As has been reported for other lipases, Schmidt-Dannert et al. (22) reported a total loss of lipolytic activity in the presence of Tween-20 and Tween-80, but no effect was observed when was incubated with Triton X-100. Prazeres et al. (19) observed Tween-40, Tween-80 and surfactin inhibited up to 30 % lipase activity, but Triton X-100 and Triton X-114 showed an activating effect. Nawani et al. (16) found a total loss of activity in the presence of SDS. In contrast, activity was enhanced in the presence of TritonX-100, Tween-20 and Tween-80. Hydrogen peroxide, Sodium perborate and Sodium hypochlorite had little effect on the enzyme activity. Sodium cholate, Sodium taurocholate, Glycerine and NaCl were found to enhance the enzyme activity. Thus, the lipase from *Acinetobacter calcoaceticus* 1-7 suggested a good potential application in the detergent industry.

Table 3. Lipase stability in presence of surfactants, detergents and oxidizing agents

Treatments		Relative activity (%)
Control		100
Surfactants	1 % TritonX-100	108
	10 % TritonX-100	52
	1 % Tween-20	51.6
	10 % Tween-20	16.3
	1 % Tween-80	61.2
	10 % Tween-80	17.9
	1 % SDS	23.7
	10 % SDS	10.2
	1 % Sodium cholate	131.5
	0.1 % Sodium cholate	125.2
	1 % Sodium taurocholate	177.9
	0.1 % Sodium taurocholate	138.3
Component of detergents	4 % Glycerine	209
	0.4 % Glycerine	281
	4 % NaCl	266
	0.4 % NaCl	244
	2 % sodium citrate	116
	0.2 % sodium citrate	167
	1 % Hydrogen peroxide	91
	0.1 % Hydrogen peroxide	93
Oxidizing agents	1 % Sodium perborate	95
	0.1 % Sodium perborate	96
	1 % Sodium hypochlorite	92
	0.1 % Sodium hypochlorite	95

CONCLUSION

In aqueous solution, the lipase of *Acinetobacter calcoaceticus* 1-7 shows some interesting properties such as stability at a broad range of pHs (4.0-10.0) with the optimum activity at pH 9.0. It also showed good stability ranging from 20 ^{o}C to 50 ^{o}C with the maximum activity at 40 ^{o}C. Our experimental evidence clearly indicated that the lipase produced by the strain 1-7 is a moderate thermophilic alkaline lipase. Its activity was highly enhanced at the presence of Ca^{2+}, Mg^{2+} and K^{+}. In contrast, it was almost completely inhibited by Cu^{2+}, while Al^{3+}, Fe^{3+}, $Ba^{2+}$and Zn^{2+} partially inhibited the enzymatic activity. Moreover, it was resistant to various surfactants, oxidizing agents and enzyme inhibitors, such as Tween-20, Tween-80, Triton X-100, and SDS, while Sodium cholate, Sodium taurocholate, Glycerine, NaCl, Sodium citrate enhance its enzymatic activity. The present studies showed that this novel lipase has the potential for applications in the detergent industry.

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