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Full Length Research Paper

Biochemical properties and potential applications of an organic solvent-tolerant lipase isolated from *Bacillus cereus* BF-3

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An organic solvent-tolerant strain producing lipase was isolated. The optimal pH and temperature were 8.0 and 34°C, respectively. The lipase was stable at 50°C after 1 h incubation at pH 6 to 8. BF-3 lipase was very stable when incubated with methanol, ethanol and toluene below 30% (v/v) but was stability reduced drastically above 50%. While K^+, Na^+, Mg^{2+} and Ba^{2+} (1 mMol/L; 3 mMol/L) stimulated lipase activity, EDTA inhibited its activity indicating that the lipase from *Bacillus cereus* BF-3 was a metalloenzyme.

Key words: Organic, solvent, tolerant, lipase, *Bacillus cereus* BF-3, biochemical, properties.

INTRODUCTION

Lipases are produced by many species of animals, plants, bacteria, yeasts, and filamentous fungi. The enzymes from microorganisms are the most interesting owing to their potential industrial applications such as in food, dairy products, pharmaceuticals, detergents, textiles, biodiesel synthesis, cosmetics, and in the synthesis of fine chemicals, agrochemicals and polymeric materials (Soni et al., 2001; Jaeger et al., 2002; Ferrer et al., 2005; Hasan F et al., 2006). They have proved to be better biocatalysts for performing various reactions such as esterification, transesterification, stereospecific hydrolysis of racemic esters and organic synthesis under water-restricted environments (Schuepp et al., 1997; Soni et al., 2001; Ferrer et al., 2005).

The technological utility of enzymes can be enhanced greatly by using them in organic solvents rather than their natural aqueous reaction media. Enzyme-catalysed reactions in organic solvents, and even in supercritical fluids and the gas phases, have found numerous potential applications, some of which are already commercialized (Klibanov, 2001).The use of enzymes in organic media with low water content has been one of the most exciting facets of enzymology in recent years (Khmelnitsky et al., 1999).

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Baharum et al. (2003) reported solvent tolerant lipase produced by *Pseudomonas* sp. strain S5 that was stable in organic solvents and then studied the high-yield purification and chaperone dependent gene expression of S5 lipase (Baharun et al., 2003; Rahman et al., 2005, 2006; Baharum et al., 2010).

Hun et al. (2003) isolated organic solvent tolerant *Bacillus sphaericus* 205y producing organic solvent-stable lipase and then studied the extracellular expression of novel OST-lipase gene of *B. sphaericus* 205y (Hun et al., 2003; Sulong et al., 2006). Several authors have also reported the isolation of solvent tolerant lipase strains, purification, characterization, optimization of lipase production and/or gene expression from various sources (Zhao et al., 2008; Dandavate et al., 2009; Kawata et al., 2010). But there are no reports on *Bacillus cereus* of organic solvent stable lipase.

In this study, we reported the biochemical properties and potential applications of organic solvent tolerant *B. cereus* BF-3 lipase.

MATERIALS AND METHODS

The p-nitrophenyl palmitate (pNPP) substrate for lipase was purchased from Sigma Chemical Co., USA. The solvents and olive oil were purchased from National Pharmaceutical Group Chemical Reagent Co., Ltd, BeiJing, China. All the other chemicals used were of analytical grade. All the solvents used in the present study were

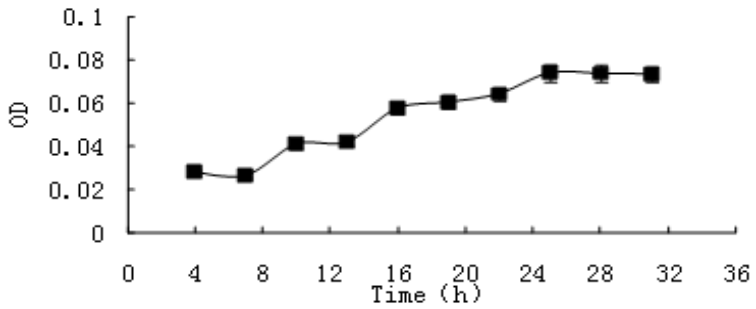

Figure 1. The growth curve of *B. cereus* BF-3.

treated with molecular sieve (4A˚).

The solvent tolerant strain of *B. cereus* BF-3 was used. The isolation and characterization have been reported previously (Lv and Wang, 2010). *B. cereus* BF-3 was maintained at 4℃ on nutrient agar slants and subcultured at monthly interval. Inoculum was prepared by transferring loopful of this stock culture to the nutrient medium containing (g/l): glucose, 10.0; peptone, 10.0; yeast extract, 3.0; NaCl, 0.5; pH 7.0 .The cultivation was performed at 36.5℃ with shaking at 100 rpm. The ferment media contained (g/l): olive oil, 10.0; yeast extract, 5; $MgSO_4 \cdot 7H_2O$, 0.1%; NaCl, 0.25%; pH 7.5. The incubation was carried out at 100 rpm in an orbital shaker maintained at 36.5℃. Cell growth was monitored by recording A_{660}. For estimating lipase production, periodically withdrawn samples were centrifuged at 10000 g for 10 min and enzyme activity was assayed in the supernatant.

Lipase activity

Lipase activity was modified from Winkler and Stuckmann (1979) Gupta and Gupta, (2002) and Cadirci et al. (2010). 1.0 ml 1 mM p-NPP and 1.0 ml 50 mM Tris-HCl buffer (pH 8.0) was preincubated at 38℃ for 5 min. Then, 0.1 ml suitable dilution of cell-free culture supernatant (crude lipase) was added to the reaction mixture and incubated at 38℃ for 15 min. The amount of liberated p-nitrophenol (pNP) was recorded at 410 nm. One unit is defined as the amount of enzyme liberating 1 µmol of pNP under standard assay conditions.

Effect of pH and pH stability on crude enzyme activity

The effect of pH on the *B. cereus* BF-3 lipase activity was investigated by using olive oil as a substrate. Various buffer systems (pH 4.0 to 12.0) at 25 mM were added to olive oil to perform olive oil emulsion. Then, the enzyme was assayed at different pH values at 37℃ for 30 min with shaking at 200 rpm. For pH stability studies, 200 µl of purified enzyme solution was mixed with 800 µl of 25 mM buffer at specific pH. The mixture was incubated at 37℃ for 30 min, and the residual activity was determined. Buffer systems were used at a concentration of 25 mM: glycine-HCl buffer (pH 4.0 to 5.0), sodium hydrogen phosphate-NaOH buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH7.0 to 9.0), disodium hydrogen orthophosphate-NaOH buffer (pH 9.0 to 11.0), and glycine-NaOH buffer (pH 10.0 to 12.0) (Rahman et al., 2005).

Effect of organic solvents on enzyme activity

B. cereus BF-3 was grown at 37℃ in a fermentation medium. Culture medium (24 h) was centrifuged at 12000 rpm, 4℃ for 15 min and the supernatant was filtered with membrane filter (0.25 µm pore size) as crude enzyme (Hun et al., 2003). Effect of various organic solvents at the concentration of 10, 20, 30 and 50% (v/v) on the lipase activity was investigated. The reaction mixture was incubated for 30 min at 37℃ under shaking condition (200 rpm). The remaining activity was assayed under standard condition and expressed as the lipase activity with distilled water been added to the reaction mixture instead of solvent (Ogino et al., 1994).

Effect of temperature on activity and thermostability of *B. cereus* BF-3 lipase

The effect of temperature on lipase activity was studied by carrying out the enzyme reaction at different temperatures in the range of 30 to 55℃ at pH 9.0 using Tris-HCl buffer (25 mM). The reaction was carried out for 0 and 30 min. The thermostability of lipase was tested at various temperatures, ranging from 37 to 45℃, for different time intervals, ranging from 0 to 6 h. At each time interval, 500 µl samples were pippeted out and immediately frozen prior to assay.

RESULTS AND DISCUSSION

Organic solvent tolerant lipase from *B. cereus* BF-3

There are many kinds of microbes that can produce solvent tolerant lipase (Ogino et al., 1994, 2000; Amaya et al., 1995; Torres et al., 1996; Hun et al., 2003; Hernández-rodríguez B et al.; Gaur et al., 2008; Zhang et al., 2009; Ji et al., 2010). By the screening method described earlier, 80 strains of solvent tolerant lipase producers were obtained. Of these, six showed high crude lipase activity on Rhodamine B agar plates screened. These strains belonged to *B. cereus*. The crude lipase secreted by strain BF-3 among the six strains was found to be quite stable in the presence of hydrophilic organic solvents and showed higher activity in the preliminarily optimized medium. The growth curve of BF-3 is shown in Figure 1.

Organic solvent stability of *B. cereus* BF-3 lipase

Exposure of the organic solvent tolerant BF-3 lipase to

Table 1. Stability of BF-3 lipase in the presence of various organic solvents.

Organic solvent	10%	20%	30%	50%
Methanol	126	109	82	46
Cyclohexane	112	104	97	56
Isopropyl alcohol	117	98	91	64
Ethanol	132	116	92	78
Toluene	147	132	94	80
Acetone	103	96	81	47
Isoamyl alcohol	107	101	92	67

various organic solvents for 30 min elucidated that this enzyme was stable to most of the organic solvents (Table 1).

When concentration of various organic solvents was increased from 10 to 50% (v/v), the enzyme was drastically inactivated. The BF-3 lipase was activated by methanol, ethanol and toluene (10 and 20%, v/v). Similarly, Sharma et al. (2001) reported that their AG-8 lipase was activated in the presence of ethanol and methanol. In contrast, the stability of LST-03 lipase was lower in the presence of alcohol such as *tert*-butanol, ethanol and 1,4-butanediol. This phenomenon is due to the high toxicity of the high concentrations organic solvent to the enzyme. Similarly, Sugihara et al. (1992) reported that *Pseudomonas cepacia* lipase was inactivated at all concentrations of benzene and hexane after 30 min incubation under assay conditions. In contrast, BF-3 lipase was very stable in incubation with methanol, ethanol and toluene below 30% but was reduced drastically above 50%.

The BF-03 showed the different tolerance profiles in different organic solvents environments. Ogino et al. (1999) reported that there was no clear correlation between the solubility of an organic solvent in water and stability of lipase in its presence. The stability and activation effects of the organic solvent tolerant BF-3 lipase in aqueous-organic mixtures suggested the ability of this enzyme to be used in organic synthesis and related applications

Water miscible solvents generally cause more striking enzyme denaturation. It is well known that water acts as a lubricant that affords a high conformational flexibility to enzyme molecules. Therefore, the characteristic of BF-3 lipase of organic tolerant solvent is the potential use in organic synthesis and related applications.

Effect of pH on activity and stability of the *B. cereus* BF-3 lipase

The optimal pH for lipase activity was 8.0 (Figure 2). The activity was reduced drastically at pH below 6.0 and above 9.0. Similarly, Zhao et al. (2008) reported that the lipase from *Serratia marcescens* ECU1010 had maximal activity at pH 8.0. Gilbert et al. (2010) reported that EF2 lipase had maximal activity at pH 8.5 to 9.0. *Pseudomonas aeruginosa* LX1 (Ji et al., 2010), LST-03 and F-111, have their maximal activities at pH values ranging from 6.0 to 10.0. In contrast, lipases from *Pseudomonas* sp. and *P. cepacia* showed optimal pH values at 5.5 to 6.5 and 5.5 to 7.0, respectively.

In a pH stability test, the BF-3 lipase showed good stability after 30 min at pH 6.0 to 8.0; (Figure 2).The enzyme retained 58 and 60% activity in pH 6.0 and 8.0, respectively. In comparison, Jinwal et al. (2003) reported that *Pseudomonas* lipase PK-12CS was very stable at a broad pH range (5.6 to 9.0) for 14 h at 37°C. LST-03 lipase was very stable at a pH range of 5.0 to 8.0 for 10 min at 30°C. AG-8 lipase still retained 90 to 100% activity at pH 7.0 to 10.0 for 24 h at 25°C.

Effect of temperature on activity and thermostability of *B. cereus* BF-3 lipase

The lipase from *B. cereus* BF-3 was considered to be thermostable, as indicated by the optimal temperature of the activity of lipase, that is, 45°C (Figure 4). The optimal temperature was 34°C (Figure 3). The enzyme retained 86 and 52% of its maximum activity at 37 and 50°C, respectively. Ogino et al. (2000) reported that organic solvent-stable LST-03 lipase had maximal activity at 37°C. On the other hand, Sulong et al. (2006) reported that *B. sphaericus* 205y lipase had an optimal temperature of 55°C. The thermal stability profile of the lipase is shown in Figure 4. The enzyme was stable below 50°C for 1 h. It retained approximately 80% of its initial activity at 50°C for 1 h. When the time and temperature was increased, the activity decreased simultaneously. The enzyme lost all of the original activity at 70 to 80°C after 40 min incubation. The enzyme showed a higher stability than organic solvent-stable LST-03 lipase (below 40°C for 10 min).

Effect of metal ions on *B. cereus* BF-3 lipase

According to Wills et al. (1960), nearly one-third of all known enzymes require the presence of metal ions for catalytic activity. This group of enzymes includes the

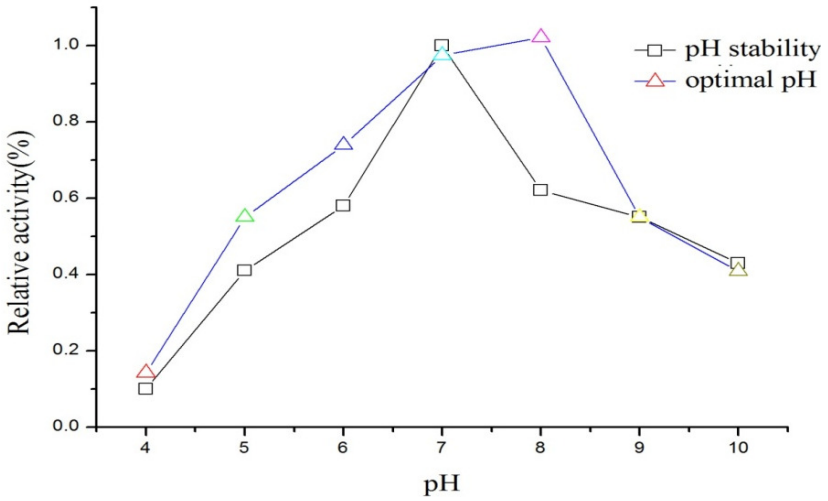

Figure 2. Effect of pH on lipase activity and stability.

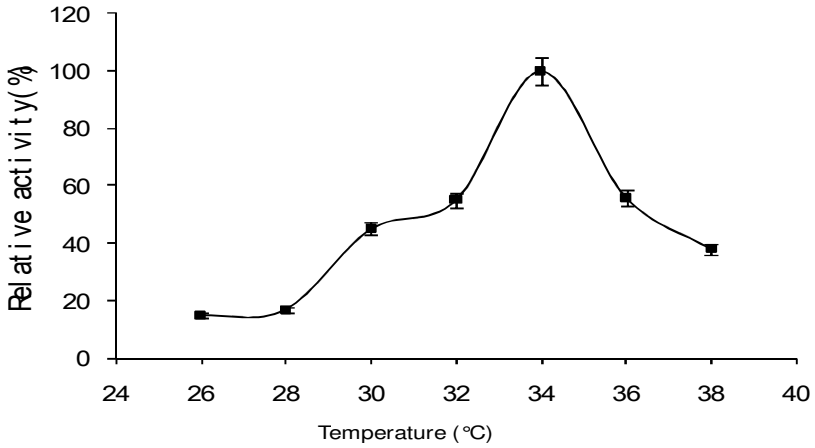

Figure 3. Effect of temperature on lipase activity of *the B. cereus* BF-3 lipase.

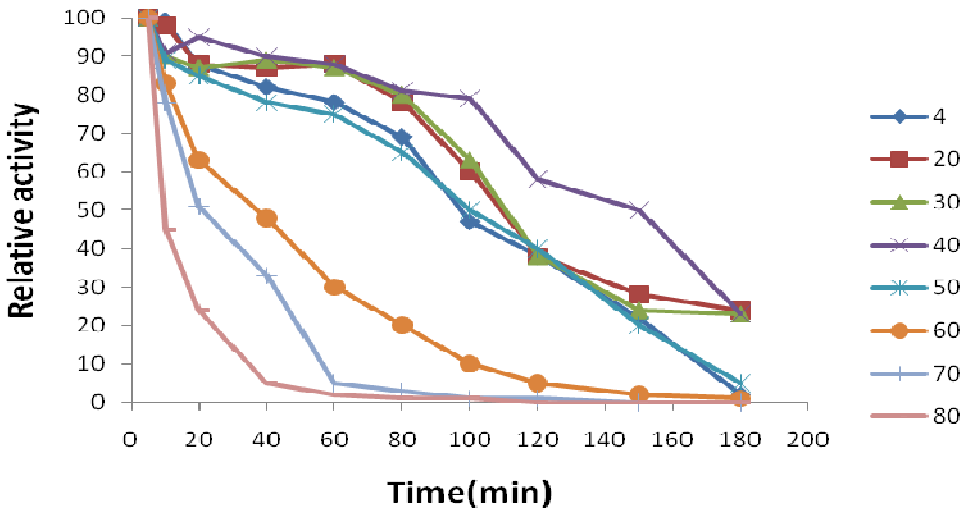

Figure 4. Thermal stability of *the B. cereus* BF-3 lipase.

Table 2. Effect of various metal ions on lipase activity.

Metal ion	1 mMol/L (%)	3 mMol/L (%)
K^+	129	134
Na^+	106	176
Mg^{2+}	122	167
Al^{3+}	50	42
Cu^{2+}	73	69
Zn^{2+}	86	79
Fe^{2+}	101	103
Ca^{2+}	90	89
Ba^{2+}	113	118

Table 3. Effect of SDS and EDTA on BF-3 lipase.

Parameter	1 mmol/L (%)	2 mmol/L (%)	5 mmol/L (%)	10 mmol/L (%)
SDS	65	31	0	0
EDTA	74	41	13	7

metal enzymes, which contain tightly bound metal ion cofactors, most common transition metal ions such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+} and Zn^{2+}. Metal-activated enzymes, in contrast, loosely bind metal ion from solution, usually the alkali and alkaline earth metal ions: Na^+, K^+ Mg^{2+}, and Ca^{2+}. The effect of metal ions was tested at 1 and 3 mM in 25 mM Tris-HCl buffer at pH 9.0 (Table 2).

The BF-3 lipase were found to stimulate the lipase activity by K^+, Na^+, Mg^{2+} and Ba^{2+}(1 mMol/L;3 mMol/L). These characteristics of BF-3 lipase illustrated that it is a mental-activated enzyme. In this group of enzymes, the ions often play a structural role rather than a catalytic one. The ions bind to the enzyme and change the conformation of the protein to counter greater stability to the enzyme. But transition metal ions change the conformation of the protein to less stable due to ion toxicity. Similar results were reported by Sharma et al. (2001) with their lipase from *Pseudomonas* sp. AG-8. They showed that Ca^{2+} ions activated the enzyme, whereas Fe^{3+} and Zn^{2+} strongly inhibited its activity. A possible explanation of this phenomenon is that Ca^{2+} has a special enzyme-activating effect that it exerts by concentrating at the fat-water interface. Therefore, calcium ions may carry out three distinct roles in lipase action: removal of fatty acids as insoluble Ca^{2+} salts in certain cases, direct enzyme activation resulting from concentration at the fat-water interface and stabilizing effect on the enzyme. Fe^{3+} and Zn^{2+}, and especially Cu^{2+}, were highly toxic to lipase even in a diluted solution.

Effect of SDS and EDTA on *B. cereus* BF-3 lipase

The activity of the lipase from *B. cereus BF-3* decreased with both inhibitor and chelator. When exposed with SDS and EDTA of 1 mmol/L, the enzyme actively retained 65 and 74%, respectively, whereas it reduced to 31 and 41%, sharply when the concentration was increased to 2 mmol/L (Table 3). These results indicated that the lipase from *B. cereus* BF-3 is a metalloenzyme. It is the same as the results of mental ions.

Conclusions

We isolate a new organic solvent tolerant strain *B. cereus* BF-3 that is capable of growing in the presence of organic solvent environments. To date, no report is available on the isolation and production of lipase by *B. cereus.* We have successfully isolate a new strain of *B. cereus* BF-3 that is stable in solvent. Therefore, it is very useful for bacterial reaction processes.

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