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A novel alkaline and low-temperature lipase of *Burkholderia cepacia* isolated from Bohai in China for detergent formulation

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Abstract - The bacterial strain LP08 was isolated from soil collected from bay of Bohai, China. The sequence of 16S rDNA of strain LP08 showed 99% homology to *Burkholderia cepacia*. The lipase from *Burkholderia cepacia* LP08 was purified by ammonium sulphate precipitation, ion exchange chromatography and Sephadex G-75 chromatography. The characterization of the lipase exhibited maximum activity at 30 °C and pH 9.0. The lipase retained 63, 66, 74, and 95% of its maximum activity at 10, 15, 20 and 25 °C respectively. The lipase activity was promoted in the presence of commercial detergent, sodium cholate, sodium taurocholate, glycerine and NaCl, while was little inhibited in the presence of TritonX-100, Tween-20, Tween-80, SDS, saponin. The present lipase was highly stable towards oxidizing agents and was stable after 1 h at 25 °C in the presence of hydrogen peroxide, sodium hypochlorite and sodium perborate. The results suggest that the lipase from *Burkholderia cepacia* LP08 showed good potential for application in the detergent formulation.

Key words: *Burkholderia cepacia;* alkaline lipase; low-temperature lipase; purification; detergent.

INTRODUCTION

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are one of the most important classes of hydrolytic enzymes that catalyze both the hydrolysis and the synthesis of esters (Sharma *et al.,* 2002). They are ubiquitous in nature and are produced by various animals, plants, fungi and bacteria. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains. Of these, the important ones are: *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas* (Gupta *et al.,* 2004). Several kinds of lipases originating from *Burkholderia* species have been identified and their enzymatic properties and crystal structures have been elucidated (Rathi *et al.,* 2001; Mandrich *et al.,* 2005; Park *et al.,* 2007; Yang *et al.,* 2007). Because of their preference for the hydrolysis of triglycerides with a long chain length (greater than C8), excellent enantioselectivity, transesterification, esterification and tolerance to solvents and high temperature, *Burkholderia* lipases were extensively studied during the past two decades for industrial use (Maury *et al*., 2005; Orcaire *et al.,* 2006; Fernades *et al.,* 2007; Park *et al.,* 2007; Yu *et al.,* 2007; Li *et al.,* 2007).

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Low-temperature lipase might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures and unusual specificities. These properties are of interest in different fields such as detergents, textile and food industry, bioremediation and biocatalysis (Alquati *et al*., 2002). The most commercially important field of application for hydrolytic lipases is their addition to detergents, which are used mainly in industrial laundry and in household dishwashers. The use of enzyme-based detergents is preferred over the conventional synthetic ones due to their better cleaning properties, lowering of washing temperatures and reduction in pollution. Lipases improve the washing capacity of detergents as well as removal of fatty food stains and sebum from fabrics, which are difficult to remove under normal washing conditions (Hasan *et al.,* 2006; Saisubramanian *et al.,* 2006). In 1994, Novo Nordisk introduced the first commercial recombinant lipase 'Lipolase' which originated from the fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae*. In 1995, two bacterial lipases were introduced - 'Lumafast' from *Pseudomonas mendocina* and 'Lipomax' from *Pseudomonas alcaligenes* - by Genencor International (Sharma *et al.,* 2001).

At present, lipases originated from *Pseudomonas* and *Burkholderia* are most commonly used in household detergents (Park *et al.,* 2007; Ruchi *et al.,* 2007), but there is no report that *Burkholderia* produces both low-temperature and alkaline lipase. Here, we describe process for isolation of the strain LP08 producing both low-temperature and alkaline lipase and evaluation of lipase as a detergent additive.

MATERIALS AND METHODS

Isolation and screening of lipase-producing microorganisms. Three hundred and fifty-four of alkaline lipase-producing microorganisms were isolated from soil collected from bay of Bohai, China with an olive oil alkaline plate, which contained olive oil as the sole carbon source. Soil samples were inoculated in 50 ml of enrichment medium, the medium contained: yeast extract 10 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 2 g l^{-1}, olive oil 20 g l^{-1}, pH 9.5. The flasks were incubated at 26 °C for 3 days under shaking 180 rpm. After inoculation, the culture liquid was used for inoculation of another set of enrichment flasks. The enriched culture was spread after serial dilution on screening medium. The screening medium contained: K_2HPO_4 1 g l^{-1}, $NaNO_3$ 3 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 0.01 g l^{-1}, emulsion of olive oil (it contained 0.2% Victoria blue B) 20 g l^{-1}, agar 20 g l^{-1}, pH 9.5. The plates were incubated at 26 °C. Growing colonies with blue zones were isolated and transferred to slants, the slants contained: peptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, pH 7.5. The lipase activity was estimated by plate assay method, as described below. By rough estimation, the lipase from strain LP08 was optimal at low-temperature and alkaline range, so strain LP08 was chosen to use for following experiments.

Bacterial strain identification. Identification of strain LP08 was conducted using 16S ribosomal DNA (rDNA) analysis (Eltaweel *et al.*, 2005). The sequence analysis was performed by TaKaRa BioTechnology Corporation (Dalian, China). A homology search to reference strains registered in DDBJ/EMBL/GenBank was performed using NCBI BLAST.

Lipase production. The seed inoculum was prepared by inoculating a loop full of culture from a slant into 35 ml the seed medium (starch soluble 10 g l^{-1}, bean flour 20 g l^{-1}, corn syrup 20 g l^{-1}, K_2HPO_4 1 g l^{-1}) and incubated for 12 h at 28 °C. Two millilitres were inoculated in 250 ml flask containing 35 ml of fermentation medium with following composition: starch soluble 10 g l^{-1}, bean flour 20 g l^{-1}, corn syrup 20 g l^{-1}, K_2HPO_4 1 g l^{-1}, emulsion of bean oil 20 g l^{-1}, the initial pH of the medium was adjusted to pH 9.0, then incubated at 28 °C for 44 h under shaking condition (180 rpm). After the incubation period, the cell-free supernatant was obtained by centrifugation at 10000 rpm at 4 °C for 20 min. The supernatant was considered as crude enzyme.

Assay of lipase activity. Lipase activity was determined by following two methods.

Plate assay method. The underlying principle employed in this method was based on change of the indicator (Victoria blue) caused by free fatty acids liberated from olive oil under proper conditions. The medium containing 10% emulsion of olive oil (it contained 0.2% Victoria blue B). The medium was adjusted to different pH using buffers: 0.067 mol l^{-1} phosphate buffer (pH 5.0-9.0) and 0.05 mol l^{-1} glycine-NaOH buffer (pH 9.0-10.6). To quantify lipase activity 4-mm-diameter holes were punched into the agar and filled with 20 µl of cell-free culture supernatant. The plate were incubated for 24 h at different temperature (15, 25, 35, 45 and 55 °C). Lipase activity was assayed by determined change of the blue zones.

Spectrophotometric method. Spectrophotometric method utilized p-nitrophenyl palmitate (pNPP) as substrate (Vorderwülbecke *et al.,* 1992). Solution 1 contained pNPP (90 mg) dissolved in propane-2-ol (30 ml); solution 2 contained Triton X-100 (2 g) and gum arabic (0.5 g) dissolved in 450 ml buffer (Tris-HC1 50 mmol l^{-1}, pH 8.0). The assay solution was prepared by adding 1 ml of solution 1 to 9 ml of solution 2 drop wise to get an emulsion which remained stable for 2 h. The assay mixture contained 900 µl of the emulsion and 100 µl of the appropriately diluted lipase solution. The liberated p-nitrophenol was measured at 410 nm. The molecular extinction coefficient of p-nitrophenol at 410 nm is 151 $mmol^{-1}$ cm^{-1}. One unit of lipase was defined as the amount of lipase that releases 1 mmol p-nitrophenol from the substrate for 1 min.

Purification procedure.

Step 1: Solid ammonium sulphate was added to the supernatant with stirring to bring the saturation to 35% and standing it at 4 °C for 4 h, the precipitate was removed by centrifugation (10000 rpm at 4 °C for 20 min). Lipase activity both in precipitate and supernatant was determined. Additional ammonium sulphate was added to the supernatant to bring the saturation to 75% and the solution was left overnight. The precipitate was collected and dissolved in 0.067 mol l^{-1} phosphate buffer (pH 8.0), then the solution was dialyzed against distilled water at 4 °C for 36 h.

Step 2: The dialyzed solution was applied to a DE-52 column (2.0 cm x 16 cm). The column was previously equilibrated with three bed volumes of 0.067 mol l^{-1} phosphate buffer (pH 8.0), and bound proteins were eluted with 60 ml linear NaCl gradient (0-1.0 mol l^{-1}) in the same buffer. The flow rate was adjusted to 15 ml h^{-1}, the fraction volume of 3.5 ml was collected for lipase activity analysis. The active fractions were pooled and used for Sephadex G-75 column.

Step 3: The fraction containing lipase was chromatographed on Sephadex G-75 column (1.6 cm x 80 cm) equilibrated with 0.067 mol l^{-1} phosphate buffer (pH 8.0) and then the lipase was eluted with the same buffer. The flow rate was adjusted to 42.0 ml h^{-1} and then the fraction volume of 3.5 ml was collected for lipase activity analysis. SDS-PAGE was used to prove the purity of the lipase samples.

Lipase characterization. The effect of temperature on lipase activity was studied by carrying out the lipase reaction at different temperatures in the range of 10-60 °C at pH 8.0 using 0.067 mol l^{-1} phosphate buffer. For studying thermal stability, 1 ml of lipase was mixed with 19 ml of 0.067 mol l^{-1} phosphate buffer (pH 8.0) and incubated at 25, 30, 35 and 40°C for 84 h. Residual lipase activity was determined at different intervals from 0 to 84 h, at pH 8.0 and 25 °C.

For the determination of the effect of pH on lipase, lipase activity was measured at 25 °C in a pH range of 5.0-10.6 using different buffers, 0.067 mol l^{-1} phosphate buffer (pH 5.0-9.0) and 0.05 mol l^{-1} glycine-NaOH buffer (pH 9.0-10.6). For pH stability studies, 1 ml of lipase was mixed with 19 ml of buffers with different pH (5.0-9.0) and incubated at 25 °C for 84 h. Subsequently, the residual enzymatic activity was determined by lipase activity assay.

Evaluation of lipase as an additive for detergent formulation. Lipase from strain LP08 was biochemically characterized for its potential application in the detergent industry. The lipase sample was incubated in presence of surfactants viz. Triton X-100, Tween-20, Tween-80, SDS, sodium cholate, sodium taurocholate,

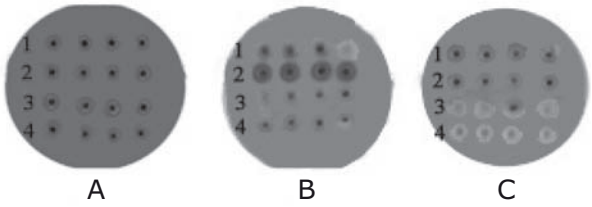

FIG. 1 - The size of rings formed on Victoria blue agar plate with the crude lipase of four different strains. In the three plates, 2 is lipase from *Burkholderia cepacia* LP08, 1, 3 and 4 are lipase from other strains. A: The lipase reaction at 25 °C, pH 8.0. B: The lipase reaction at 25 °C, pH 9.0. C: The lipase reaction at 35 °C, pH 9.0.

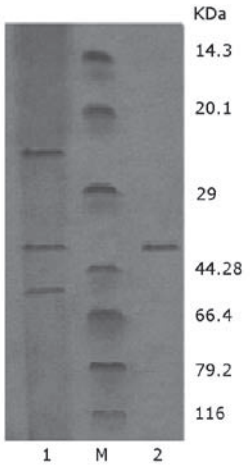

FIG. 2 - SDS-PAGE analysis of lipase from *Burkholderia cepacia* LP08 at various stages of purification. Lane 1: ion exchange, lane 2: gel filtration, lane M: molecular weight markers.

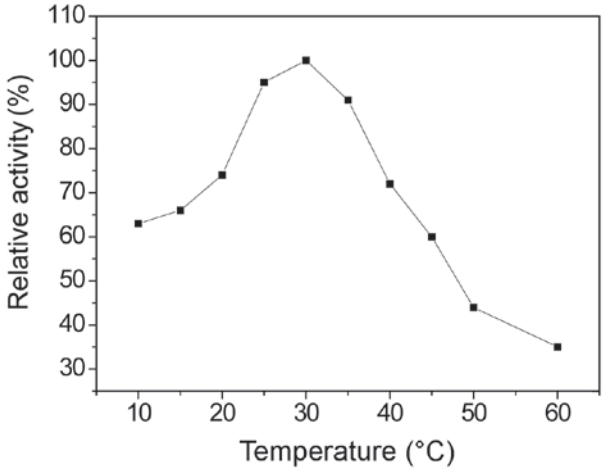

FIG. 3 - Effect of temperature on the activity of lipase from *Burkholderia cepacia* LP08.

commercial detergents, glycerine, NaCl, Borax and sodium citrate at different concentration at 25 °C for 1 h and lipase activity was determined at pH 8.0 and 30 °C.

Lipase stability in the presence of hydrogen peroxide, sodium perborate and sodium hypochlorite at 1% (w/v or v/v) at 25 °C for 1 h was checked and lipase activity was determined at pH 8.0 and 30 °C.

RESULTS

Screening of lipase-producing bacterial strains

A total of 354 bacterial isolates from soil were screened for producing lipase, among which 51 isolates were obtained based on the lipase activity, while the others were discarded based on their comparatively poor lipase activity. Of the 51 isolates, the strain LP08 had the maximum lipase activity at 25 °C and pH 9.0 (Fig. 1). The 16S rDNA sequence of strain LP08 was analysed with GenBank database, and the sequence showed 99% homology to *Burkholderia cepacia*.

Purification of lipase

The lipase from *Burkholderia cepacia* LP08 was purified employing a three-step procedure (Table 1). A 23.79-fold purification was obtained with a recovery of 13%. The specific activity of the purified enzyme was 58.53 U mg^{-1} of protein. Coomassie Brilliant Blue staining revealed the presence of a single protein with a molecular weight of 39 kDa (Fig. 2).

Characterization of lipase

As show in Fig. 3, the optimum temperature of the lipase from *Burkholderia cepacia* LP08 was 30 °C. The lipase retained 63, 66, 74, and 95% of its maximum activity at 10, 15, 20 and 25 °C respectively. The lipase retained more than 89 and 50% of its activity for 36 and 84 h respectively at 30 °C, and the half life of the lipase was 75, 72, 52 h at 25, 35 and 40 °C respectively (Fig. 4)

The lipase from *Burkholderia cepacia* LP08 showed activity in a very wide pH range (5.0-10.6). Maximal activity (100%) was observed at pH 9.0 (Fig. 5), while this was closely followed by pH 8.0 and pH 10.0 (97% and 91% of the maximum). The activity reduced drastically at pH 7.0 (84%) and was 33% of the maximum at pH 5.0. The lipase showed good stability after 84 h in an alkaline pH range, where the lipase retained 55 and 42% of the maximum activity at pH 9.0 and pH 8.0 respectively, but it was totally inactivated at acidic pH and lost 94% of activity at pH 5.0 (Fig. 6).

Lipase stability towards surfactants, detergents, oxidizing agents and proteases

Results presented in Table 2 reveal that the lipase was stable in some surfactants and retained 90.3, 91.6, 93.3, 84 and 60.4%

TABLE 1 - Purification of the lipase from *Burkholderia cepacia* LP08

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Activity yield (%)	Protein yield (%)	Purification (fold)
Crude enzyme (cell free supernatant)	765	311	2.46	100	100	1.00
Precipitation $(NH_4)_2SO_4$ (30-70%)	689	84	8.20	90	27	3.33
Ion exchange	275	11.3	24.34	36	3.6	9.89
Gel filtration	99.5	1.7	58.53	13	0.5	23.79

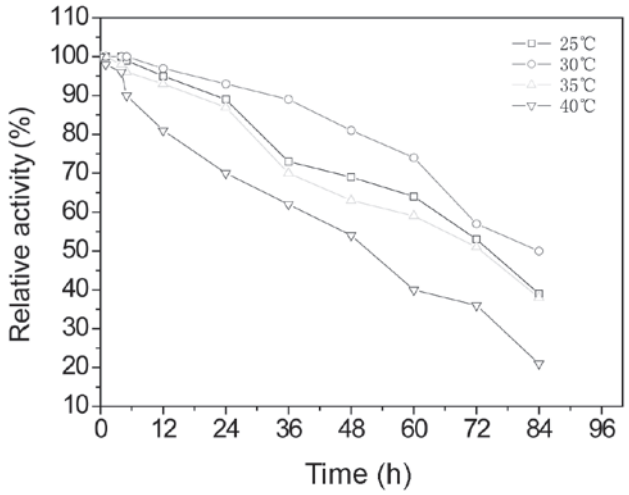

FIG. 4 - Thermostability of lipase from *Burkholderia cepacia* LP08.

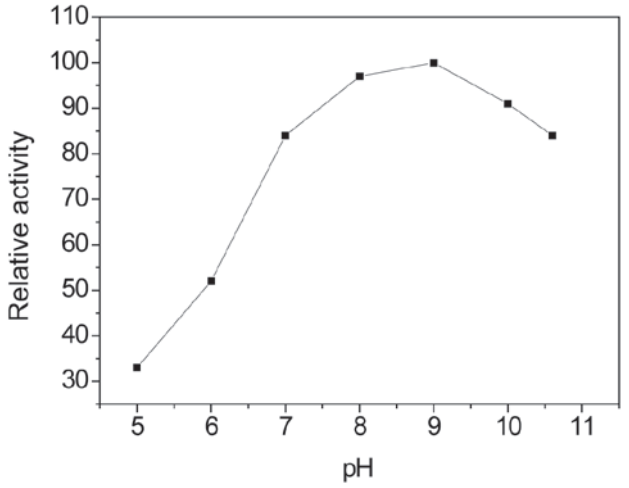

FIG. 5 - Effect of pH on the activity of lipase from *Burkholderia cepacia* LP08.

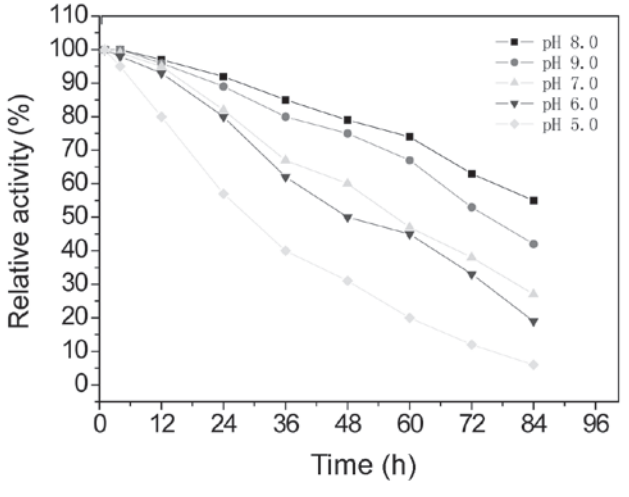

FIG. 6 - pH stability of lipase from *Burkholderia cepacia* LP08.

of its control activity in the presence of Triton X-100, Tween-20, Tween-80, saponin and SDS respectively. Lipase shows higher activity than the control in the presence of sodium cholate, sodium taurocholate, glycerine, sodium citrate and NaCl, as it retained 131.5, 177.9, 281, 266 and 167% of the control activity. Interestingly, the present lipase is highly stable towards oxidizing agents and was stable after 1 h at 25 °C in the presence of hydrogen peroxide, sodium hypochlorite and sodium perborate. Alkaline protease (0.01%) had no effect on the lipase activity. The effect on the lipase activity of Borax has relation to the solution concentration: low concentrations increased the lipase activity while high concentrations inhibited the lipase activity.

DISCUSSION

Lipase used in detergents needs to be stable under alkaline pH and should be active in the presence of surfactants, bleaching agents and detergents (Sharma *et al.,* 2001, 2002)

A series of studies on characterization of lipase indicated that the optimal temperature and pH of lipase from *Burkholderia cepacia* LP08 are in low-temperature and alkaline range. At present, lots of reports show that lipases from *Burkholderia* and *Pseudomonas* has their optimum activity at high temperature and in alkaline range. Rathi *et al.* (2000, 2001) reported that optimum activity of lipase from *Burkholderia cepacia* was at 90 °C and at pH 11. Yang *et al.* (2007) found that the optimal temperature 70 °C and pH 8.0 for lipase from *Burkholderia cepacia* strain G63, and kept stable at a temperature range of 40-70 °C, after incubation at 70 °C for 10 h it remained 86.1% of its activity. Liu *et al.* (2006) reported that the optimal reaction conditions of lipase from *Burkholderia* sp. C20 were pH 9.0 and 55 °C. Park *et al*. (2007) reported that lipase from *Burkholderia* sp. HY-10 exhibited highest activities at 60 °C and pH 8.5. The lipase from *Pseudomonas aeruginosa* LP602 exhibited maximum lipase activity at pH 8.0 where it was also stably maintained. At 55 °C, the lipase had the highest activity but not stability (Dharmsthiti and Kuhasuntisuk*,* 1998). Umesh *et al*. (2003) reported that lipase from *Pseudomonas mendocina* PK-12CS was stable at room temperature for more than a month and expressed maximum activity at 37 °C and pH 8.0. Gao *et al*. (2000) reported that lipase from *Pseudomonas* had maximum activity at 45 °C and pH 9.0. Kulkarniand Gadre (2002) reported that lipase from *Pseudomonas fluorescens* NS2W had an optimal activity at 55 °C and at pH 9.0. Lin *et al*. (1996) reported that lipase from *Pseudomonas pseudoalcaligenes* F-111 was stable in the pH range of 6 to 10, and exhibited highest activities at 40 °C. There is no report about *Burkholderia* produces the low-temperature and alkaline lipase as we know so far.

Besides optimal temperature and pH are in low-temperature and alkaline range, a good detergent lipase should also be stable in the presence of surfactants. The lipase from *Burkholderia cepacia* LP08 was stable in the presence of some surfactants. The above finding was similar to that by Umesh *et al*. (2003) who found that lipase from *Pseudomonas mendocina* PK-12CS showed appreciably good stability to Triton X-100, Tween-20 and Tween-80. Ruchi *et al*. (2007) reported that lipase from *Pseudomonas aeruginosa* retained 100% activity in presence of Triton X-100, Tween-20 and Tween-80. Rathi *et al*. (2001) reported that the lipase from *Burkholderia cepacia* retained 93, 57, 40 and 53% of the activity in the presence of Triton X-100, Tween-20, Tween-80 and saponin respectively. On the other hand, Karadzic *et al*. (2006) reported lipase from *Pseudomonas aeruginosa* was strongly stimulated by Triton X-100 and Tween-

TABLE 2 - Lipase stability in presence of the surfactants, component of detergents, oxidizing agents and protease

Surfactants / Protease (1% w/v or v/v)		Relative activity (%)	Detergents/Oxidizing agents (1% w/v or v/v)		Relative activity (%)
Control		100	Control		100
Surfactants	1% TritonX-100	69	Component of detergents	4% Glycerine	209
	0.1% TritonX-100	90.3		0.4% Glycerine	281
	1% Tween-20	52.9		4% NaCl	266
	0.1% Tween-20	91.6		0.4% NaCl	244
	1% Tween-80	58.4		3% Borax	77.4
	0.1% Tween-80	93.3		0.3% Borax	287
	1% SDS	18.5		2% Sodium citrate	116
	0.1% SDS	60.4		0.2% Sodium citrate	167
	1% Saponin	53	Oxidizing agents	1% Hydrogen peroxide	91
	0.1% Saponin	84		0.1% Hydrogen peroxide	93
	1% Sodium cholate	131.5		1% Sodium perborate	95
	0.1% Sodium cholate	125.2		0.1% Sodium perborate	96
	1% Sodium taurocholate	177.9		1% Sodium hypochlorite	92
	0.1% Sodium taurocholate	138.3		0.1% Sodium hypochlorite	95
Alkaline protease	0.1%	88.4	Commercial detergent (The brand of bilang)	1%	134
	0.01%	100		0.1%	104

80. Park *et al*. (2007) reported that lipase from *Burkholderia* sp.HY-10 retained 125 and 116% of activity in the presence of Triton X-100, Tween-80. In contrast, Gao *et al*. (2000) reported that lipase from *Pseudomonas* was inactivated in the presence of Tween-20 and Tween-80.

Remarkably, the present lipase exhibited better resistance towards SDS, as compared to lipase from *Burkholderia* sp.HY-10 (Park *et al.*, 2007), *Burkholderia* sp. GXU56 (Wei *et al*., 2008) and *Aspergillus carneus* (Saxena *et al.*, 2003). SDS was strong inhibitors causing almost total inhibition of enzyme activity.
Lipase from *Pseudomonas aeruginosa* (Karadzic *et al.*, 2006) and *Pseudomonas mendocina* PK-12CS (Umesh *et al.,* 2003) exhibited only 7 and 5.1% residual activity in the presence of SDS. Thus, the current thrust for novel enzymes that tolerate SDS makes the present lipase of high commercial value.

Lipase shows higher activity than the control in the presence of sodium cholate and sodium taurocholate. Similar results were reported for lipase from *Aspergillus carneus* (Saxena *et al.,* 2003), which was stimulated by taurocholic acid. However, in another study, bile salt inhibits the most of microbial lipase, such as *Pseudomonas* (Gao *et al.,* 2000) and *Burkholderia* sp.HY-10 (Park *et al.,* 2007).

The present lipase is highly stable towards oxidizing agents. Oxidizing agents stability is an important property of an enzyme that has been achieved by site-directed mutagenesis and protein engineering for proteases and detergent compatible lipase (Lipolase®) marketed by Novo Nordisk, Denmark (Rathi *et al*., 2001).

Considering the overall properties of different lipase of microbial origin and the lipase studied from our isolate, the lipase from *Burkholderia cepacia* LP08 is better in regard to optimal temperature and pH (low-temperature and alkaline pH range), stability in the presence of surfactants, detergent compatibility and above all bleach stability, for a potential application in the detergent industry.

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