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Identification of bacteria producing a thermophilic lipase with positional non-specificity and characterization of the lipase

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Abstract - A bacterial isolate producing lipase, named S31, was isolated from soil and identified as a strain of *Burkholderia cepacia.* S31 produced high activity of lipase which reached a maximum of 226.1 U/ml by fermenting at 30 °C for 60 h under the induction of olive oil. After purification, the lipase showed a single band of about 35 kDa in SDS-PAGE. The optimum temperature of the lipase was 70 °C and the optimum pH was 9.0. S31 lipase was stable at 40-70 °C and pH 0.5-10.0, as well as in several organic solvents, such as methanol, n-hexane, n-butanol, toluene and ethyl acetate. The presence of some metal ions (Ca^{2+}, Mn^{2+}, K^{+}, Na^{+} and Mg^{2+}) could activate the enzyme whereas Fe^{2+} and Cu^{2+} were found to be inhibitory. The lipase could cleave all of the three ester bonds of triglycerides. We conclude that S31 lipase is an alkaline lipase with a variety of highly desirable characteristics for research and industrial application.

Key words: *Burkholderia cepacia*; lipase; positional non-specificity; solvent tolerant; thermophilic.

INTRODUCTION

Lipases (EC 3.1.1.3), a series of enzymes that catalyze the hydrolysis of various fatty esters, are widely distributed in animals, plants and microorganisms (Jaeger and Eggert, 2002). Lipases also catalyze ester synthesis, transesterification and interesterification without cofactors. The lipases from bacteria, especially from *Pseudomonas* species and *Burkholderia* species, exhibit the highest versatility, reactivity and stability in catalyzing reactions (Iizumi *et al*., 1990; Sugihara *et al*., 1992; Yang *et al*., 2007). As such, microbial lipases are a class of favourable enzymes with their actual and potential applications in the detergent, food processing, bioenergy, organic synthesis and pharmaceutical industries (Gupta *et al*., 2004).

A lipase suitable for industrial applications is required to have excellent characteristics. For example, the lipase used in biodiesel production should be stable in organic solvents, and the trait of positional non-specificity could remarkably improve the efficiency of transesterification. The food industries such as bakery need lipases that are stable in high temperature. In addition, high productivity is the most desirable property of lipase producing strain for industrial uses. Screening of strains from nature that produce lipases with these desirable traits is an important approach to accelerate the study and application of lipases.

In this paper, we describe the isolation and characterization of a novel lipase from a *Burkholderia* strain. The strain, identified as *B. cepacia* S31, had high lipase productivity in the presence of olive oil in the medium. The lipase showed tolerance to high temperature, organic solvents, and metal ions and could cleave the ester bonds of glycerides non-specifically. The S31 lipase has high potential to be used in food processing, biodiesel production and pharmaceutical industries.

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MATERIALS AND METHODS

Screening of lipase producing bacteria. The soil samples were collected from lipid-contaminated grounds around the dining hall of Nanjing Agricultural University and the cole plantation of Jiangsu Academy of Agricultural Sciences.

Five grams of soil sample were suspended in 45 ml sterilized water. After shaken with glass beads, 5 ml suspension was added in a 250 ml flask containing 45 ml of enrichment medium with the composition of 0.2% yeast extract, 0.5% olive oil, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, and 0.05% NaCl. The mixture was incubated at 37 °C on a rotary shaker at 180 rpm for 2-4 days, after which 1 ml aliquot was diluted to a suitable concentration using sterilized water.

The dilution of the enrichment culture was spread on the first-round screening plates containing 1%emulsified olive oil, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.1% $(NH_4)_2SO_4$, 0.05% KCl and 1.5% agar. The colonies with clear hydrolysis zones were collected and inoculated to the second-round screening agar plates including Tween-80 plates (4% Tween-80, 0.8% peptone, 0.01% $CaCl_2$, 2% agar) and Rhodamine B plates (LB solid medium supplemented with 0.001% Rhodamine B and 1% emulsified olive oil).

Lipase fermentation. The colonies showing clear positive zones on the second-round screening plates were collected and inoculated into liquid medium for the production of lipase. The media used in fermentation were LB (1% tryptone, 0.5% yeast extract, 1% NaCl) and FM (1% emulsified olive oil, 1% peptone, 0.5% yeast extract, 0.2% beef extract, 0.2% glycerol, 0.3% NaCl, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4$). Single colonies were inoculated into LB and shaken at 30 °C, 180 rpm overnight and 1 ml of this precultured fluid was inoculated into a 250 ml flask containing 40 ml of LB or FM medium. The mixture was incubated by rotary shaking for 72 h and samples were taken every 6 h for lipase assay.

Measurement of lipase activity. Culture supernatant (10 µl) was added to 2.99 ml PBS buffer (pH 7.4) containing 30 µM of *p*-nitrophenyl butyrate (pNPB) as a substrate. The reaction was carried out in the colorimetric assay followed by spectrophotometry at 410 nm. The lipase activity was calculated according to the standard curve of *p*-nitrophenol measured in the same condition. One unit is the amount of lipase releasing 1 µmol *p*-nitrophenol per minute. All the assays were performed in triplicate.

Positional specificity against triacylglycerol. Two millilitres of culture supernatant were added to PBS buffer (pH 7.4) containing 100 mM of triolein and shaken at 180 rpm at 37 °C for 4 h. The reaction products were extracted with the same volume of *n*-hexane for 1 h, and then 10 µl products in *n*-hexane were analyzed by thin-layer chromatography. A silica gel plate (Silica gel G, 10 cm x 20 cm, Qingdao Haiyang, China) was developed in a mixture of petroleum ether, ethylether and acetic acid (80:20:0.5). 1,2-diolein, 1,3-diolein, oleic acid, triolein and oleic acid from Sigma were used as standards. Lipozyme was included in the experiment as a control of 1,3-specific lipase. The spots were visualized by iodine vapor.

Identification of strain S31. Strain S31 produced the highest lipase activity among all of the bacterial isolates screened from the Rhodamine B plates. Phenotypic and biochemical characterizations of strain S31 were performed as described in Bergey's Manual of Determinative Bacteriology (Bergey, 1974).

The 16 S rRNA gene of S31 was amplified by polymerase chain reaction (PCR) with the following pair of primers: 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5'-GGTTACCTTGTTACGACTT-3'). The PCR product was examined by electrophoresis and then purified and sequenced on a 3730 sequencer (Jinsite company, Nanjing, China). Homologous sequences of the 16 S sequence were obtained from the NCBI database using BLAST, and a phylogenetic tree was generated by software MEG4.

Purification of S31 lipase. Two litres of culture supernatant were precipitated with ammonium sulphate, and the precipitate was dissolved in 0.05 M Tris-HCl (pH 7.5). The fraction with high activity was dialyzed and condensed, and then loaded onto a column (1 cm diameter, 15 cm high) of DE-52 gel (Whatman, England) equilibrated in 0.05 M Tris-HCl (pH 7.5). Proteins bound to the anion exchange gel were eluted by 0-0.8 M NaCl. The elution that showed lipase activity was collected and condensed, and again chromatographed on the Sephadex® G-100 (Sigma, USA) column (1 cm diameter, 60 cm high). The solution containing lipase activity was run in SDS-PAGE, with 5% stacking gel and 12% separating gel. The protein bands were visualized using Coomassie Brilliant Blue R-250 stain.

Characterization of S31 lipase.

Effect of temperature and pH on enzyme activity and stability. The optimal temperature for activity was determined by using pNPB as a substrate at different temperatures (40–80 °C). For determination of temperature stability, residual activity of 0.5 ml of the lipase solution was measured after incubation for 1, 6 and 12 h at different temperatures (50, 55, 60, 65 and 70 °C).

The optimal pH value was investigated by measuring the lipase activity in buffers ranging from pH 6.0-11.0: Na_2HPO_4-KH_2PO_4 (pH 6.0-9.0), Na_2CO_3-$NaHCO_3$ (pH 9.0-11.0). The pH stability of lipase was measured by incubating the enzyme in the above-mentioned buffers ranging from pH 0.6-11.0 for 12 h, and the residual activity was measured.

Effects of metal ions and detergents on lipase. A variety of chemicals and detergents were respectively added to 0.5 ml lipase solution including 10 mM of $FeCl_2$, $CaCl_2$, $CuSO_4$, $MgCl_2$, NaCl, KCl, $MnCl_2$, $ZnCl_2$, EDTA, 1% (v/v) of Tween-80, SDS and Triton X-100. After incubation for 6 h, the residual activity was measured.

Effects of organic solvents on lipase. A 0.75 ml enzyme solution was mixed with 0.25 ml organic solutions of methanol, ethanol, acetonitrile, isopropanol, n-hexane, n-butanol, toluene, phenol or ethyl acetate, respectively. The mixtures were incubated at 37 °C on a shaking rotator for 6 h. The organic solutions tolerance of the enzyme was determined by comparing the residual activity with the lipase incubated without organic solutions.

Cloning of S31 lipase gene and sequence analysis. According to the *Burkholderia* sp. lipase gene data in NCBI, several pairs of primers were designed to amplify the S31 lipase gene and its chaperone gene by PCR. The PCR amplified product was gel purified, ligated with the pMD19-T vector (Takara, Ltd.), and sequenced by a 3730 sequencer (Jinsite company, Nanjing, China). The nucleotide sequence and the deduced protein sequence were analyzed using the NCBI website and the software DNAman.

RESULTS AND DISCUSSION

Isolation of strains producing lipase and lipase activity measurement

The first-round screening plates contained olive oil as the sole carbon source. Strains that can utilize olive oil are able to grow on this medium. Colonies showing clear hydrolysis zones in olive oil agar plates were inoculated onto Tween-80 plates and Rhodamine B plates. Among the many strains that are positive for olive oil utilization (Fig. 1), 40 strains with high lipase activity were collected for liquid culture in LB and FM media. Some strains produced distinctly different amounts of lipase between the LB and FM media, while others showed slightly different productivity in different media.

S31 showed the highest lipase activity among all of the isolated bacteria. After cultivation in the FM medium for 60 h, the activity in the culture supernatant reached 226.1 U/ml. When S31 was inoculated into the LB or FM medium without olive oil, the lipase activity was very low (1 U/ml), suggesting that olive oil was the inducer for lipase production. Similar results have been found in *Pseudomonas cepacia* and *Burkholderia glumae* (Sugihara *et al*., 1992; Boekema *et al*., 2007). Lipases are by and large inducible enzymes generally produced in the presence of a lipid source such as an olive oil, triacylglycerols or tweens (Rathi *et al*., 2000; Gupta *et al*., 2004).

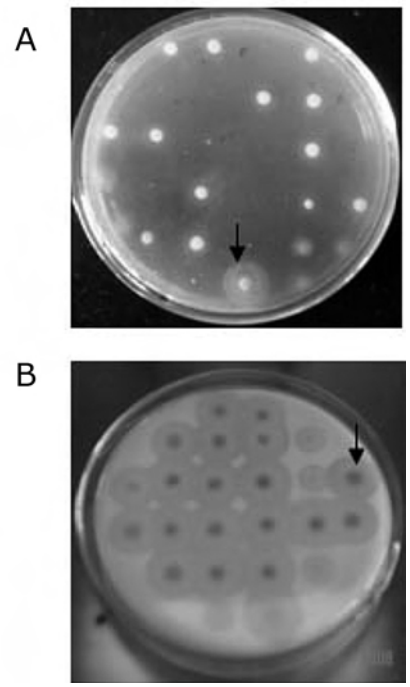

FIG. 1 - Screening of bacterial isolates producing lipase. The black arrows indicate S31 strain, which produced large positive zone after culturing at 37 °C for 2 days. A: colonies on Tween 80 plate. B: colonies on Rhodamine B plate under ultraviolet radiation.

Characterization of strain S31

The morphological, physiological, and biochemical characteristics of S31 are described in Table 1. The phenotypic and biochemical characteristics of S31 were also identical to those of *B. cepacia* according to the Bergey's Manual. Therefore we primarily identified S31 as a *B. cepacia* strain.

The 16 S rDNA sequence of S31 has 99% identity with those of *B. cepacia* in the Genbank database. The phylogenetic tree was showed in Fig. 2, which further confirmed that S31 was a strain belonging to *B. cepacia* species.

Burkholderi cepacia is a kind of Gram negative bacteria widely distributing in soil, water and plant surfaces. It has been used in the fields of biodegradation, bioprevention and acceleration of plant growth. Burkholder reported that this bacterium caused onion rotten and named it *Pseudomonas cepacia* in 1950 (Coenye *et al*., 2001a). In 1992, Yabuuchi *et al.* reclassified *P. cepacia* and six other *Pseudomonas* sp. as a new genus *Burkholderia* (Yabuuchi *et al*., 1992). New taxonomic approaches reveal that *B. cepacia* is not a single species but a group of bacterial species with similar genotypes and phenotypes (Coenye *et al*. 2001b; Vandamme *et al*., 1997, 2003).

Positional specificity of S31 lipase

Lipases have selectivity to the position of the ester bond in triglyceride. Some lipases can hydrolyze only the 1,3-positioned bonds while others are able to hydrolyze all three ester bonds.

According to the thin-layer chromatography analysis of the hydrolysis products of triolein (Fig. 3), S31 lipase cleaved not only the 1,3-positioned ester bonds but also the 2-positioned ester bond. Therefore, we conclude that S31 lipase is a lipase without positional specificity. Similar lipase from *P. cepacia* was reported by Sugihara *et al*. (1992) that was able to cleave all ester the ester bonds with some preference for 1,3-positioned ester bonds. Lipases of *Pseudomonas* sp. S5 and LST03 also cleaved ester bonds non-specifically (Ogino *et al*., 2000; Rahman *et al*., 2005). But many *Pseudomonas* lipases cleaved only 1, 3-positioned ester bonds, such as those from *Pseudomonas fragi* and *Pseudomonas* sp. 2106 (Mencher and Alford, 1967; Gao *et al*., 2000).

Purification of S31 lipase and determination of its molecular weight

After a combination of purification steps including ammonium sulphate fractionation, DEAE ion-exchange chromatography and two rounds of gel filtration, the lipase preparation exhibited a single band of about 35 KD in SDS-PAGE (Fig. 4), identical to what deduced from the S31 lipase gene sequence, as well as to that reported by Kordel *et al.* (1991).

During the course of purification, the lipase appear to aggregate with each other or with other proteins. An active high-molecular-weight band was visible on non-denaturing PAGE with the size about 120 KD (data not shown). Protein aggregation had created much difficulty to purification, causing low purification efficiency and yield. Addition of 1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1propanesulfonate) according to Wolfgang Stuer *et al.* (1986) led to the deposit of some active proteins and the loss of the enzyme activities. So it's important to dissolve the protein at a suitable concentration. The tendency of lipases to aggregate has been reported by others (Cleasby *et al*., 1992; Duenhaupt *et al*., 1992; Lesuisse *et al*., 1993).

TABLE 1 - Characterization of the strain S31

Morphological characteristics		Hydrolysis of	
Colony shape	Round, smooth, convex, opacity	Triolein	+
Colony color	White or light yellow	Gelatin	+
Gram stain	Negative	Tween 80	+
Shape	Rod	Starch	−
Spore	−	Casein	+
Culture temperature		Carbon source	
30-40 °C	+	Glucose	+
41 °C	−	Fucose	+
Physiological characteristics		Sucrose	+
Fluorescent pigments	−	Lactose	+
Non-fluorescent pigments	+	Glycerol	+
Pyocyanine	+	Sodium acetic	+
Cytochrome oxidase	−	L-Valine	+
Catalase	−	β-Alanine	+
Nitrate reduction	+	DL-Arginine	−

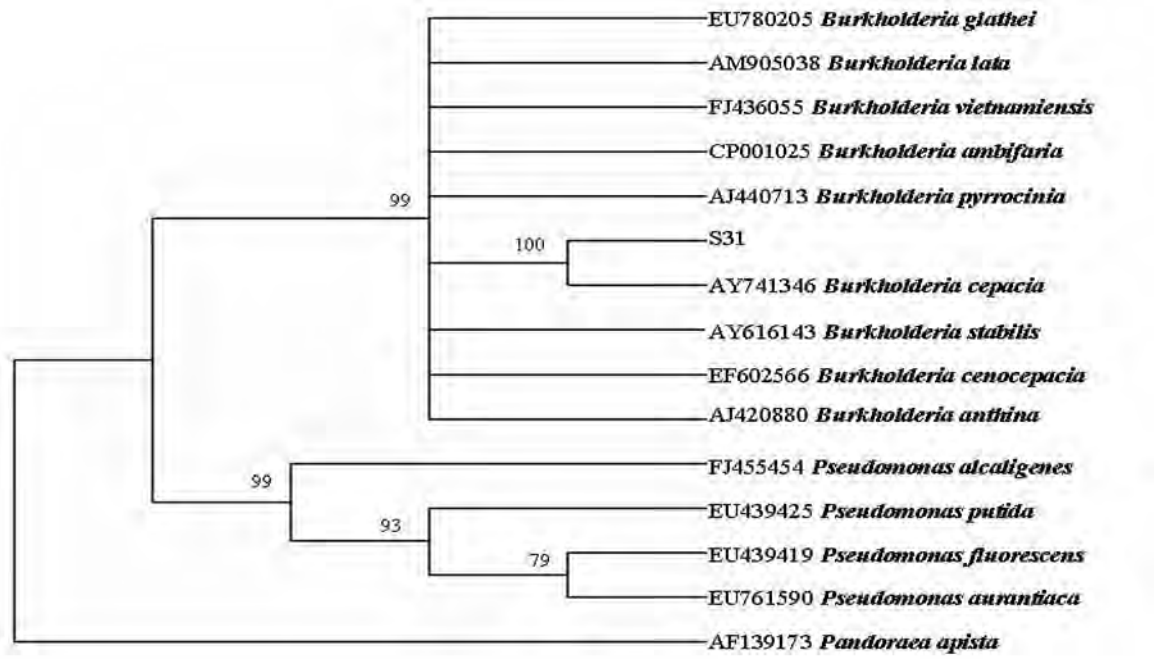

FIG. 2 - Phylogenetic tree showing the relationship of strain S31 to other *Burkholderia* species.

Effects of temperature and pH on enzyme activity and stability

Lipase activity assays were carried out at 30-80 °C and we found the optimum temperature of the S31 lipase is between 65 and 70 °C (Fig. 5A). The S31 lipase appeared to be a thermophilic enzyme, as well as a thermostable enzyme. The lipase retained 80% activity after incubation at 70 °C for 1 h, and retained 96% and 85% activity after incubation for 12 h at 50 and 55 °C, respectively (Fig. 5B).

The optimum pH value of S31 lipase was 8.5-9.0 (Fig. 5C). Its activity dropped drastically when pH is below 6.0, and it lost 50% of the maximal activity at pH 10.0. Therefore we conclude that S31 lipase was an alkaline enzyme and was different from the lipases of the *Pseudomonas* sp. KWI-56 and *P. cepacia* (lizumi *et al*., 1990; Sugihara *et al*., 1992), whose optimal pH was 5.5-7.0 and 5.5-6.5, respectively. The S31 lipase retained high activity after incubation at different pH values for 12 h, especially the residual activity retained larger than 80% after treated in buffers of pH 5.0-9.0 (Fig. 5D).

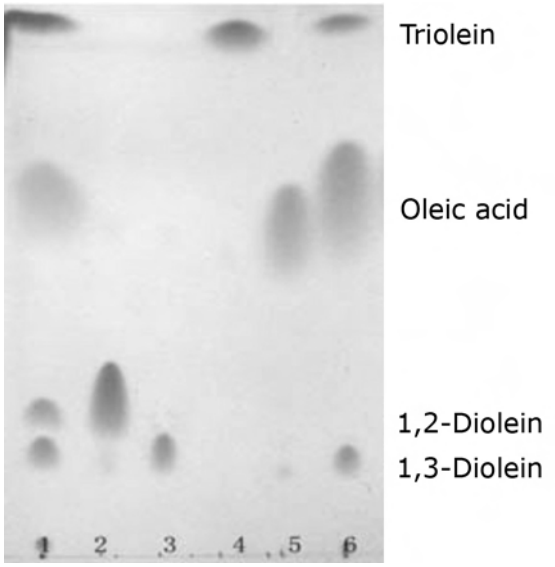

FIG. 3 - Thin-layer chromatography of the hydrolysis products of triolein catalyzed by the S31 lipase. Lane 1: the hydrolysis products of triolein catalyzed by S31 lipase, lane 2: 1,3-diolein, lane 3: 1,2-diolein, lane 4: triolein, lane 5: oleic acid, lane 6: the hydrolysis products of triolein catalyzed by lipozyme.

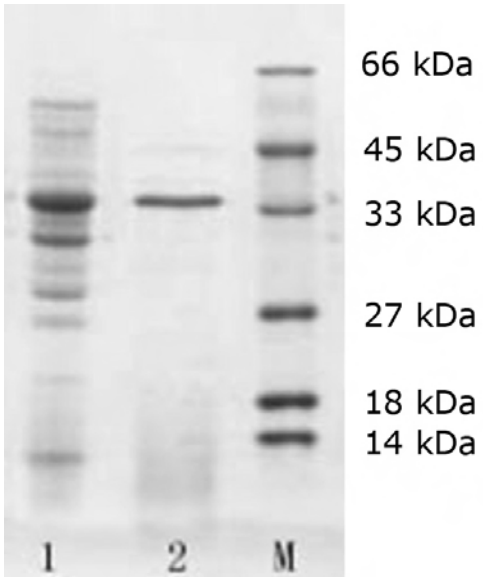

FIG. 4 - SDS-PAGE of the S31 lipase. Lane 1: crude enzyme, lane 2: purified S31 lipase, M: molecular mass markers.

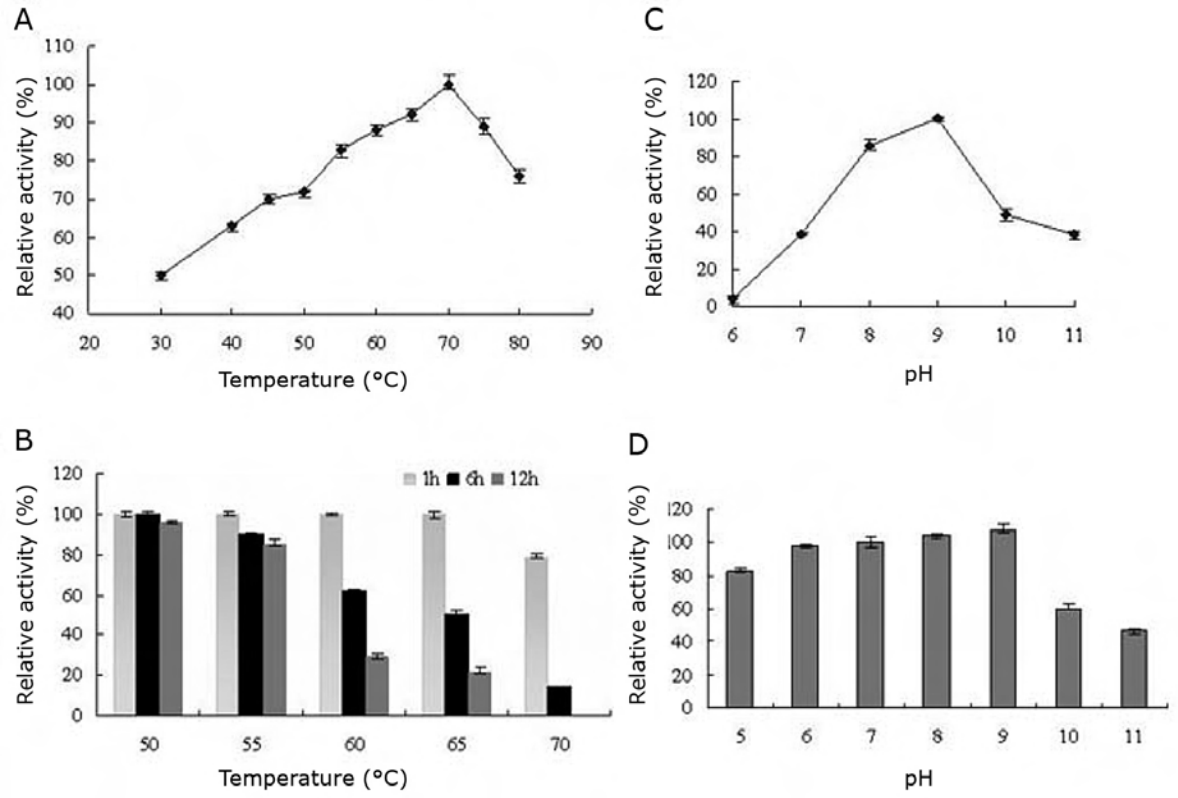

FIG. 5 - The effects of temperature and pH on S31 lipase. *p*-Nitrophenyl butyrate was used as the substrate for lipase assay. A: optimum temperature. B: thermal stability. The residual activity was measured after incubation at different temperatures for 1 h, 6 h and 12 h. C: optimum pH. D: pH stability. Lipase activity assay was carried out after incubation for 12 h in different pH solutions.

Effects of metal ions and detergents on enzyme activity

The residual lipase activity was measured after incubation in various chemicals at room temperature for 6 h. As shown in Table 2, the lipase was inhibited by Fe^{2+}, Cu^{2+} and was activated by Ca^{2+}, Mn^{2+}, K^{+}, Na^{+} and Mg^{2+}. These two groups of ions seem to bind to the enzyme and change the conformation of the protein to either decrease or increase the stability of the enzyme, respectively (Gupta *et al*., 2004). The lipase kept 90% activity in non-ionic detergents, but lost nearly all activity in EDTA, suggesting that the S31 lipase was a metal-activated enzyme.

Effects of organic solvents on enzyme activity

The stability of S31 lipase was investigated in various polar and non-polar organic solvents. As described in Table 3, this lipase is tolerant to several organic solvents, such as methanol, n-hexane, n-butanol, toluene and ethyl acetate, some of which even enhanced the activity. The possible mechanism is that the conformation of the enzyme is prone to binding and catalyzing the substrate when the enzyme is exposed to some organic solvents. Some non-polar organic solvents could enhance the solubility and rigidity of the enzyme, and decrease the side reac-

TABLE 2 - Effects of metal ions (10 mM) and detergents (1%) on S31 lipase activity

Reagent	Relative activity (%)
Control	100
Fe^{2+}	41
Cu^{2+}	56
Ca^{2+}	142
Mn^{2+}	149
Mg^{2+}	108
Zn^{2+}	97
Na^{+}	103
K^{+}	134
Triton X-100	90
Tween-80	95
SDS	5
EDTA	5

TABLE 3 - Effects of organic solvents on S31 lipase

Organic solvents (25%)	Relative activity (%)
Control	100
Methanol	100
Glycerol	105
n-Hexane	111
n-Butanol	114
Toluene	111
Ethyl acetate	120
Isopropanol	90
Acetonitrile	71
Ethanol	43
Phenol	29

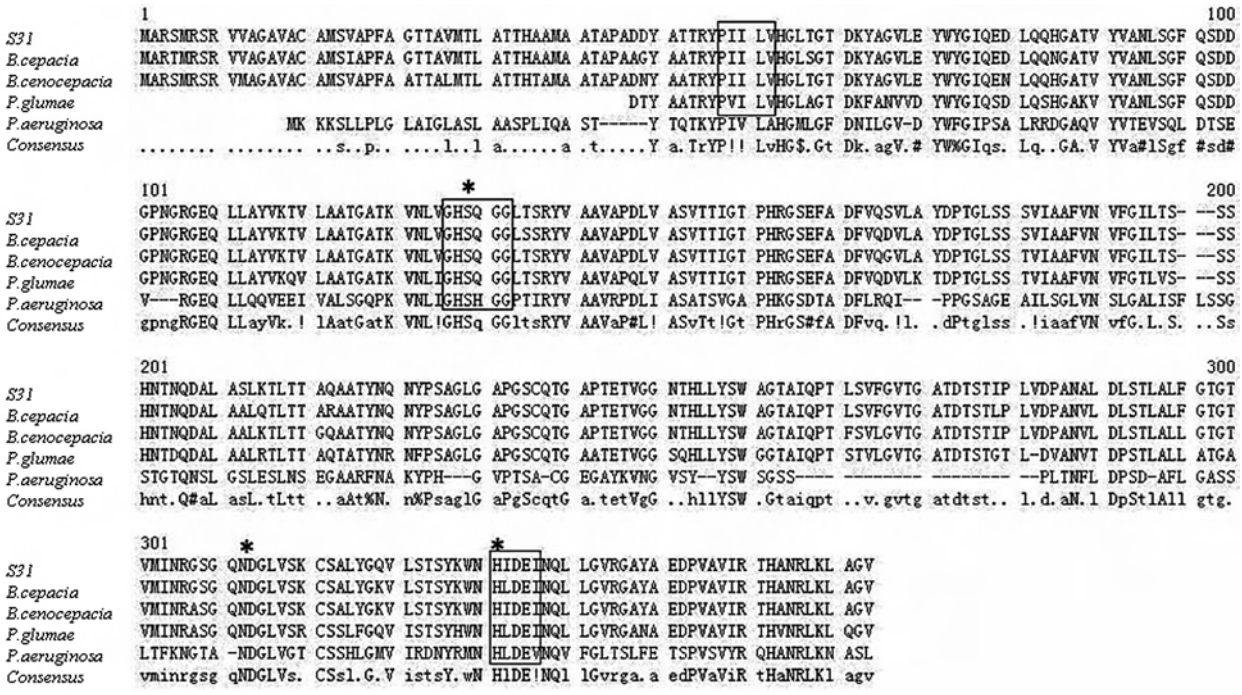

FIG. 6 - Sequence alignment between the deduced S31 lipase protein and some other *Pseudomonas* lipases. Active site residues are indicated by asterisks (*). Sequence motifs 'PIILV', 'GXSXG' and 'HLDXZ' are boxed.

tion in aqueous phase. Stability of this lipase in organic solvents suggests that it could be used as a biocatalyst in non-aqueous media, and the tolerance to methanol and glycerol is a highly desirable feature for biodiesel production (Du *et al*., 2004; Yan *et al*., 2007).

Gene cloning of S31 lipase

We designed the primers according to the most conserved regions among *Burkholderia* lipase genes. Several pairs of primers were designed and one pair of primers was able to amplify the gene successfully. The primers were F2 (GAAGATCTGGCAGCACAATAATCAGGAGAACAT) and R4 (AATACCATCGAGCAACACCTGCCTC). The GC content of the lipase gene in *B. cepacia* is very high (about 70%), resulting in the failure of amplification by standard PCR conditions. The PCR was accomplished when 5% glycerol or 8% DMSO were added. These additives may have helped the template to denature completely.

A band of about 2.1 kb was obtained from PCR. The cloned DNA fragment was sequenced and the sequence was deposited to Genbank with the accession number FJ638612. There are two open reading frames separated by three nucleotides, containing *lip A* (1095 nucleotides) encoding 364 amino acids and *lip B* (1035 nucleotides) encoding 344 amino acids. The protein encoded by *lip B* was a molecular chaperone of Lip A, helping Lip A fold and secrete correctly (Steen *et al*., 1991). The nucleotide sequence of *lip A* was aligned with *lip A* sequences from *P. cepacia* KWI-56, *B. cepacia* G63, and other *Burkholderia* strains (lizumi *et al*., 1990; Yang *et al*., 2007). There is about 90% sequence identity among these genes.

The deduced protein sequence of the S31 lipase gene was aligned with those from *P. cepacia* (M58494), *B. cenocepacia* (EAM08623), *P. aeruginosa* (AY682924), and *P. glumae* (Cleasby *et al*., 1992). Sequence identity between LipA from S31 and those from *P. cepacia*, *P. glumae* and *P. aeruginosa* is 96, 80 and 35%, respectively. According to Svendsen *et al*. (1995), the catalytic triad consists of Ser 131, Asp 308 and His 330 in the pre-protein of Lip A. Three conserved motifs were indicated in Fig. 6.

CONCLUSION

In the present study, we isolated a *B. cepacia* strain named S31 with high productivity of lipase from the soil of a cole plantation. After a series of purification steps, we obtained a single chromatographic peak with high lipase activity. The molecular weight of the purified protein was estimated to be about 35 KD by SDS-PAGE. Characterization of the lipase showed that it was a thermophilic alkaline lipase of positional non-specificity, with the optimum temperature at 65–70 °C and optimum pH at 8.5-9.0. The enzyme was stable under the incubation of high temperatures and a broad range of pH values. It had high tolerance to organic solvents such as methanol, glycerol, n-hexane, n-butanol, toluene, ethyl acetate and isopropanol, suggesting its potential to function in organic systems.

Using the S31 genomic DNA as the template, we cloned a lipase encoding gene *lip A* comprising of 1095 nucleotides that encoded 364 amino acids and its chaperone gene *lip B* comprising of 1035 nucleotides.

With a variety of desirable characteristics, S31 lipase has the potential to be applied in food processing and biodiesel production. This research has laid a foundation for the improvement, over-expression and industrial application of this lipase.

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