Full Length Research Paper

Isolation and identification of a cold-adapted lipase producing strain from decayed seeds of *Ginkgo biloba* L. and characterization of the lipase

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A cold-adapted lipase producing strain of mesophilic bacterium, named SYBC LIP-Y, was isolated from the decayed seeds of *Ginkgo biloba* L. by screening with plates containing Victoria Blue B and with the flask-shaking fermentation. It was identified as a novel *Burkholderia* species. The properties of its lipase after purification by PEG1000/ potassium phosphate aqueous two-phase system were characterized. The optimal pH and temperature of the enzyme was 10.0 and 30°C, respectively. About 80% of the original activity is maintained by heating at 40°C for 60 min. The lipase could also retained 70% of the maximal activity at the temperature of 0°C and suggested that it may be belonged to the cold-adapted lipase. Thus, it was proved to have good temperature stability and might have wide applicating fields.

Key words: Cold-adapted lipase, characterization, *Burkholderia* species.

INTRODUCTION

Lipases (EC 3.1.1.3) catalyze a variety of chemical reactions which are found in a wide range including animals, plants and microorganisms (Jaeger and Eggert, 2002). Among them, microbial enzymes are often more stable and their production is more convenient and safer (Wiseman, 1995).

Microbial lipases have been a kind of favorable enzymes with their actual and potential applications in household detergents, synthesis of pharmaceuticals or agrochemicals, processing of fats and food ingredients, dairy and textile industries and production of surfactants (Gupta et al., 2004). Margesin and Schinner, (1999) had defined the cold-adapted enzyme as enzymes in which the optimum temperature is about 30°C and high activity is retained at 0°C. Cold-adapted lipases have attracted attention due to their low optimum temperature and high activity at low temperatures. The cold-adapted enzymes are expected to be applicable as additives to detergents used at low temperatures and biocatalysts for biotransformation of labile compounds at cold temperatures (Margesin and Schinner, 1994).

Thermostability of lipases has been regarded as the most important factors affecting the application of lipase. However, cold-adapted enzymes from psychophiles are generally labile; they often rapidly lose their activity *in vitro.* But there were few reports about high production of cold-adapted lipase, and the cold tolerance of current cold-adapted lipase was found to be not stable. Only a few bacteria and yeast were exploited for the production of cold-adapted lipases (Joseph, 2006). Thus, to screen microorganism strains for production of thermostable cold- adapted lipases is one of the valid approaches that accelerates the study and application of lipases.

In the investigation to screen lipolytic microorganisms, we obtained a unique mesophilic bacterial strain SYBC LIP-Y from the outer layer of decayed seeds of *Ginkgo*

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Abbreviations: **p-NP**, p-nitrophenyl palmitate; **PCR**, polymerase chain reaction; **HPLC**, high performance liquid chromatography; **ATPSs**, aqueous two-phase systems; **PEG**, polyethylene glycol.

biloba L., which could secrete cold-adapted lipase with high thermostability, but its taxonomy needs further investigation and the factors that influence its lipase production remains also unclear. The purpose of this paper was to identify the strain, and to detect the influences of temperature and pH value in the lipase activity and stability.

MATERIALS AND METHODS

Sample collection and the screen of lipase producing bacteria

The decayed seeds of *G. biloba* L. were collected from Huishan Mount in Wuxi of China. The enrichment medium containing (per liter): peptone 5 g, yeast extract 3 g, olive oil 40 ml. The preliminary screening medium contains (per liter): peptone 10 g, yeast extract 3 g, NaCl 3 g, agar 20 g, olive oil 40 ml, Victoria Blue B 0.1 g, and Triton X-100 10 ml. The duplicate screening medium contains (per liter): peptone 10 g, yeast extract 5 g, NaCl 10 g, olive oil 40 ml and Triton X-100 10 ml. pH was adjusted to 7.2 using 1 M NaOH or 1 M HCl.

Screening of lipase producing bacteria

1.0 g sample was dissolved in 50 ml of enrichment medium and incubated for 48 h at 30°C and shaking (200 rpm). Then it was serially diluted (10^{-5} to 10^{-8}) and the diluted samples were used to daub to the preliminary screening medium and incubated at 30°C for 24-72 h. Then the dominant organisms were isolated and individually diverted to the preliminary screening medium again and cultivated for 48 h. The colonies with clear hydrolysis zones were collected and inoculated to the duplicate screening medium and fermented at 30°C for 48 h at a stirring speed of 200 rpm followed by determination of the lipase activity.

Measurement of lipase activity

Regular lipase activity assay was performed using p-nitrophenyl palmitate (p-NP) as described by Margesin and Schinner (1999) with some modifications. 100 μl culture supernatant was added to 3.9 ml 0.1 mmol/L Tris-HCl buffer (pH 9.0) containing 20 μmol of p-nitrophenyl butyrate (p-NPB) as the substrate. The activity of the lipase samples was determined spectrophotometrically at 405 nm. One unit of lipase activity was defined as the amount of lipase releasing 1 μmol p-nitrophenol per min.

Identification and characterization of lipase

The morphological and physiological characterization of the strict aerobic thermophilic isolate SYBC LIP-Y was performed according to the methods described by Holt et al. (1994). 16 S rRNA gene of SYBC LIP-Y was amplified by polymerase chain reaction (PCR) with the following pair of primers: 16SF (5'-AGAGTTTGATCCTG GCTCAG-3') and 16SR (5'- AAGGAGGTGATCCAGCCGCA -3'). The PCR product was examined by electrophoresis and then isolated and sequenced on 1471 sequencer. The 16 S rDNA sequence was blasted in NCBI and high homologous sequences were searched.

A loopful of well-grown cells was harvested and fatty acid methyl esters were prepared, separated, and identified using the Microbial Identification System. DNA G+C contents were determined by using an HPLC-based method, as described by Mesbah et al. (1989).

Purification of lipase

Aqueous two-phase systems (ATPSs) consist of two liquid phases that are immiscible beyond a critical concentration. It is produced when aqueous solutions of polymers and salts, such as PEG/ potassium phosphate, or two polymers, such as polyethylene glycol (PEG)/dextran are mixed. Lipases are usually purified with polymer/ polymer and polymer/salt ATPSs (Ooi et al., 2009).

The lipase recovery was achieved in an ATPS at the concentration of 15% (w/w) PEG1000, 15% (w/w) potassium phosphate and pH at 8.0. The phase system was prepared in 30 ml tubes by weighing a 15% (w/w) PEG1000, a 15% (w/w) salt and a 20% (w/w) crude feedstock. Distilled water was then added to the system to obtain a final mass of 20 g. After thorough mixing by gentle agitation, the system was centrifuged at 4000×g for 10 min to induce phase separation. The lipase was in the top of phase.

Effect of temperature on lipase activity and stability

The optimum temperature for lipase catalyzation was determined using p-NPB as substrate at temperature from 0 to 70°C. For determination of temperature stability, the lipase solution was incubated at a certain range temperatures from 40 to 60°C followed by measurement of the residual activity.

Effect of pH on enzyme activity and stability

The optimal pH value for lipase activity was measured in Tris-HCl buffers range from pH 6.5 - 10.5. The pH stability of lipase was determined by incubating the enzyme in the above mentioned buffers range from pH 3.0 - 10.5 for 24 h and the residual activity was measured.

RESULTS

Isolation of high yield lipase producing strains

55 strains were primarily isolated from samples by enrichment cultures at 30°C and among them, 16 isolates showed positive phenomena (Figure 1) on the preliminary screening medium. SYBC LIP-Y showed the highest lipase activity among all of the isolated bacteria. Since SYBC LIP-Y was inoculated into medium without olive oil; the lipase activity was very low, which suggested that olive oil be the inducer for SYBC LIP-Y to produce lipase. Lipases are by and large inducible enzymes generally produced in the presence of a lipid source such as an oil or triacylglycerols, tweens etc (Lotti et al., 1998; Bradoo et al., 1999; Rathi et al., 2001).

Characterization of strain SYBC LIP-Y

The morphological, physiological, and biochemical characteristics of SYBC LIP-Y are described as in Table 1. The colonies of the candidate strain are light yellow, convex and circular with clear margins. Cells are approximately 0.4 μm wide and 1.2 μm long (Figure 2). The strain is gram-negative, strictly aerobic, rod-shaped and non-motile. It positively produces catalase and
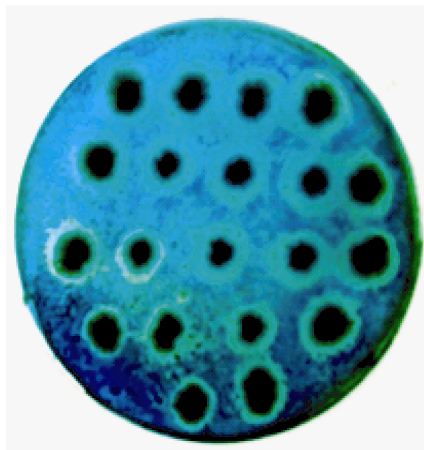
Figure 1. Colonies of *Burkholderia* sp. nov. SYBC LIP-Y cultivated in the plates containing Victoria Blue B.

Table 1. Phenotypic characteristics of strain *Burkholderia* sp. nov. SYBC LIP-Y.

Characteristic	Result	Characteristic	Result
Gram stain	-	D-glucose	+
Shape	rod	Sucrose	+
Spore	-	D-trehalose	+
Catalase	-	Starch	+
Urease	+	Citric acid	+
V - P test	-	Malonic acid	+
M - R test	-	D-alanine	+
Indole test	-	D-sorbitol	+
H_2S	-	Inositol	+
Gelatin	+	D-mannitol	+
D-fructose	+		

+, Positive; - negative.

urease, but is negative for indole and H_2S production and Voges-Proskauer test and methyl-red test. The organic compounds: D-fructose, D-glucose, D-mannitol, D-alanine, starch, sucrose, D-trehalose, citric acid, malonic acid, D-sorbitol and inositol can be used as carbon source for cell proliferation of the strain.

The phylogenetic tree (Figure 3) based on 16S rRNA gene sequences clearly indicated that strain SYBC LIP-Y was related to members of the genus *Burkholderia*. Strain SYBC LIP-Y showed the highest levels of sequence similarity (99%) with *Burkholderia cepacia* (GenBank accession number EF031062) and *Burkholderia lata* (AM905038). The main components of the cellular fatty acids of strain SYBC LIP-Y were $C_{16:0}$ (22.15 %), $C_{17:0}$ cyclo (26.88 %), $C_{19:0}$ cyclo w8c (11.04 %) and summed feature 2 ($C_{16:1}$ ISO I/ $C_{14:0}$ 3-OH, 7.45 %). With regard to the fatty acid profile, both quantitative and qualitative differences were found between strain SYBC LIP-Y and the other *Burkholderia* species investigated; the proportion of $C_{17:0}$ cyclo as a major component was higher in strain SYBC LIP-Y (Table 2). In particular, the proportion of $C_{18:1}$ cyclo w8c and summed feature 3 in SYBC LIP-Y were lower than the other *Burkholderia* species.

The DNA G+C content of strain SYBC LIP-Y was 56.4 mol %, a value similar to that of *B. cepacia* C-1 (55.1 mol %) (Adjei and Ohta, 1999), but this was different from those of majority of the genus *Burkholderia*, for example, *B. hospita* LMG 20598[T] (62 mol %), *Burkholderia terrae* KMY02[T] (62 mol %) (Yang et al., 2006) and *B. soli* sp. nov. GP25-8[T] (Yoo et al., 2007).

On the basis of its morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence comparisons, it is proposed that strain SYBC LIP-Y represents a novel species in the genus *Burkholderia*, for which the name *Burkholderia* sp. nov. SYBC LIP-Y is proposed.

Enzyme purification

The enzyme was purified about 1.5-fold with a yield of 53.7% after PEG1000/potassium phosphate aqueous two-phase system (Table 3).

Effect of temperature on enzyme activity and stability

Both the activity and stability of lipase from *Burkholderia* sp. nov. SYBC LIP-Y were influenced by temperature. The optimum temperature of *Burkholderia* sp. nov. SYBC LIP-Y lipase was 30°C. Lipase had 70 and 58.8% of the activity at 0 and 70°C (Figure 4a). The lipase used in the experiment was stable at the low temperature and showed a better temperature range for enzyme activity. The lipase could retain 80.7% of its activity after being incubated at 40°C for 60 min. It retained 42% of its activity at 60°C for 60 min (Figure 4b).

Effect of pH on enzyme activity and stability

Maximum lipase activity was obtained at pH 10.0, but its activity decreased at acidic pH values. The activity of enzyme was remained stable within a pH range of 3.0 - 10.5 (Figure 5).

DISCUSSION

In this study, we have identified and isolated a novel cold-adapted lipase producing strain, SYBC LIP-Y, from decayed seeds of *G. biloba* L. of Huishan Mount in Wuxi of China. It was identified as a *Burkholderia* species. The genus *Burkholderia* contains many species which are ubiquitous (Vandamme et al., 2002; Yang et al., 2006; Kim and Ahn, 2009). Several kinds of *Burkholderia*
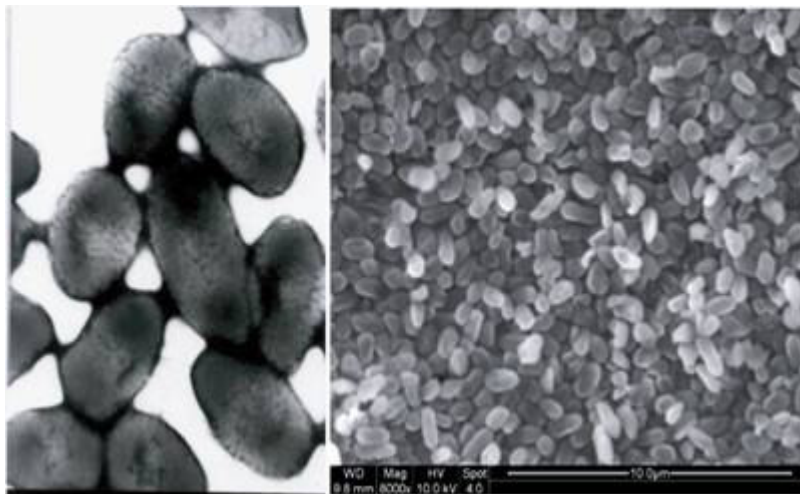
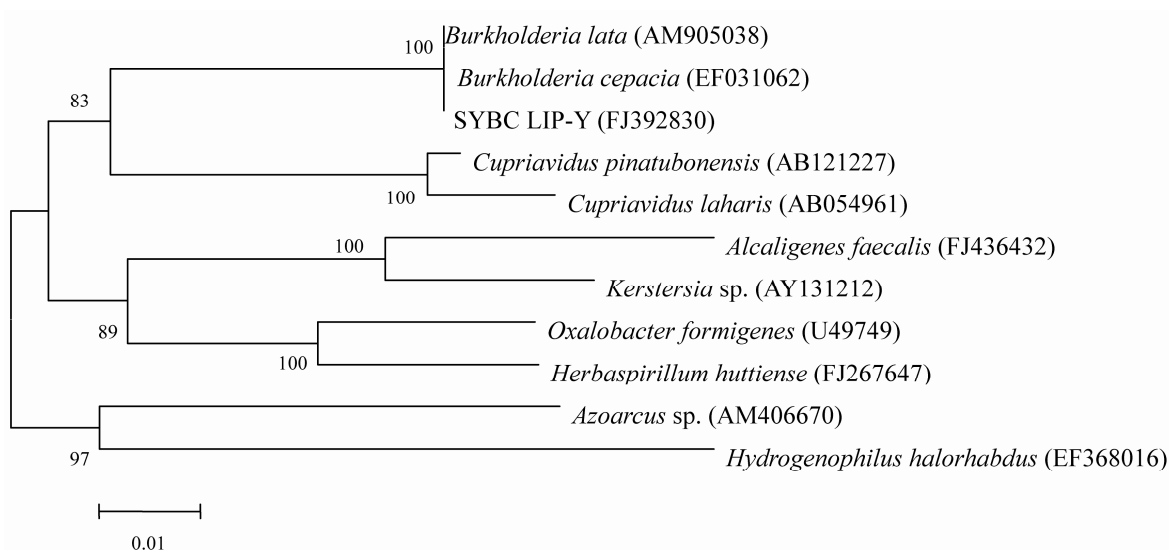**Figure 2**. The electronic microscope picture of the strain *Burkholderia* sp. nov. SYBC LIP-Y.

Figure 3. The phylogenetic tree resulting from analysis of the 16S rDNA sequences of *Burkholderia* sp. nov. SYBC LIP-Y. (Numbers on nodes correspond to percentage bootstrap values for 1000 replicates)

species with lipase producing ability have been identified and isolated from the nature (Park et al., 2007; Yeo et al., 1998; Wei and Wu, 2008). Majority of the lipases of *Burkholderia* origin showed that they were thermophilic lipases. However, this is the first report of a *Burkholderia* strain which produce cold-adapted lipase. Cold-adapted microorganisms, which produce cold-adapted lipases, have been isolated mostly from the Antarctic and Polar Regions, which represents a permanently cold (0 ± 2°C) and constant temperature environment (Joseph et al., 2007). The deep sea bacteria are other important sources of cold-adapted lipases. These microorganisms usually cultivated at ~5°C in laboratories (Gerday et al., 2000). Mesophilic microorganism included *Geotrichum*

sp. SYBC WU-3 (Cai et al., 2009), *Serratia marcescens* (Abdou, 2003) and *Aspergillus nidulans* (Mayordomo et al., 2000), can also secrete cold-adapted lipase. In this paper, we also demonstrated that SYBC LIP-Y can secrete cold-adapted lipase at 30°C.

The enzyme from the strain *Burkholderia* sp. nov. SYBC LIP-Y retained 70% of its activity at 0°C. Compared with lipases from psychrotrophic and psychrophilic micro-organisms, the lipase had better low temperate activity (Table 4). Optimum temperatures of lipases from *Pseudomonas* Lip35 (Yu et al., 2009) and *Photobacterium lipolyticum* M37 (Ryu et al., 2006) were lower than 30°C and their relative activities at low temperature were more than 75%. However, optimum temperatures of lipases

Table 2. Cellular fatty acid content (%) of strain *Burkholderia* sp. nov. SYBC LIP-Y and phylogenetically closely related *Burkholderia* species.

Fatty acid	1	2	3	4	5
Saturated					
$C_{14:0}$	5.41	4.80	5.10	4.60	3.90
$C_{16:0}$	22.15	18.50	18.20	19.60	17.60
$C_{18:0}$	3.90				
Hydroxy					
$C_{16:0}$ 2-OH	1.82	1.80	2.60	1.00	2.20
$C_{16:1}$ 2-OH	1.01	1.50	2.10	1.60	1.80
$C_{16:0}$ 3-OH	5.17	4.80	6.30	6.60	6.10
$C_{18:1}$ 2-OH	2.63		1.10		
Cyclo					
$C_{17:0}$ cyclo	26.88	10.40	4.80	4.00	12.60
$C_{18:1}$ cyclo w8c	6.51	30.00	33.90	28.50	28.90
$C_{19:0}$ cyclo w8c	11.04	3.50	4.40	1.20	7.10
Summed features*					
2: iso - C $_{16:1}$ I/ C $_{14:0}$ 3-OH	7.45	6.90	7.30	8.70	8.20
3: C $_{16:1}$ w7c / iso - C $_{15:0}$ 2-OH	4.10	17.80	13.20	22.60	10.20

Strains: 1, *Burkholderia* sp. nov. SYBC LIP-Y; 2, *B. hospita* LMG 20598[T] (Yang et al., 2006); 3, *B. kururiensis* LMG 19447 [T]; 4, *B. phymatum* STM815[T]; 5, *B. tuberum* STM678 [T] (Vandamme et al., 2002). Fatty acids that account for less than 1 % of the total are not shown.

Table 3. Summary of purification procedure of lipase from *Burkholderia* sp. nov. SYBC LIP-Y.

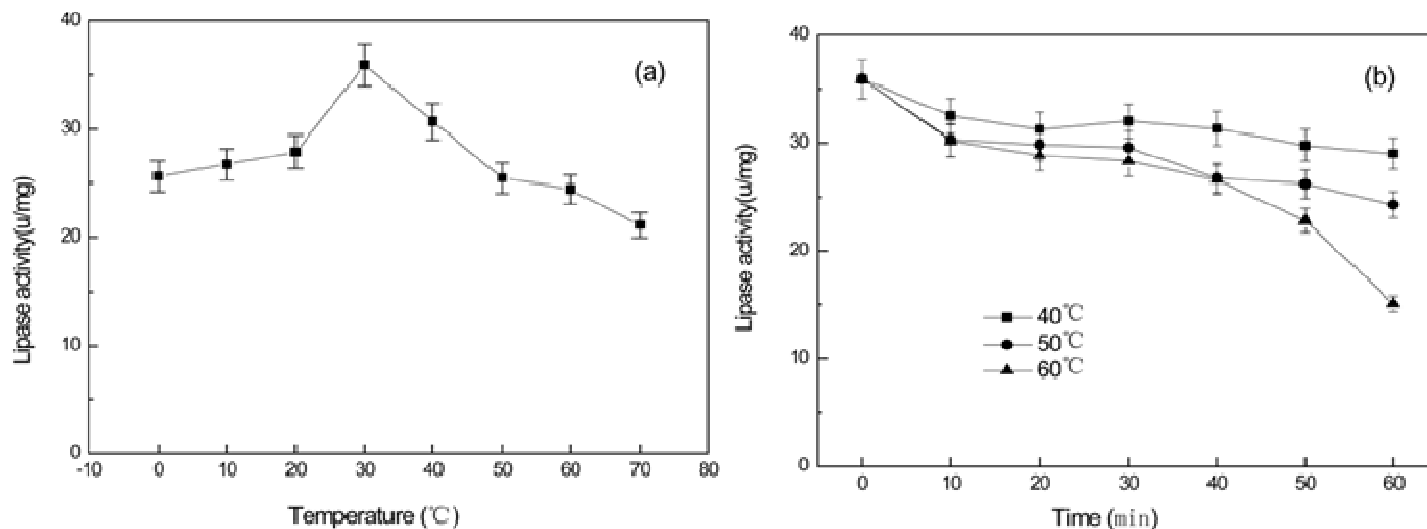| Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	382	9129.4	23.90	100	1
ATPSs	205	7377.3	35.91	53.7	1.5

Figure 4. Effect of temperature on the activity (a) and stability (b) of the lipase from *Burkholderia* sp. nov. SYBC LIP-Y.

Table 4. Partial aspects of some cold-adapted lipases.

Bacterium	Source	Optimum temp. (℃)	Relative enzyme activity (%)	References
SYBC LIP-Y	Decayed seed of *Ginkgo biloba* L.	30	70 (0℃)	In this paper
Pseudomonas sp. KB700A	Subterranean environment	35	13 (0℃)	Rashid et al., 2001
Pseudomonas sp. B11-1	Alaskan soil	45	17 (0℃)	Choo et al., 1998
Pseudomonas Lip35	Refrigerator	20	90 (15℃)	YU et al., 2009
Serratia marcescens	Raw milk	37	90 (5℃)	Abdou, 2003
Photobacterium lipolyticum M37	Marine habitat	25	75 (5℃)	Ryu et al., 2006
Pseudoalteromonas sp. wp27	Deep sea sediments	30	60 (4℃)	Zeng et al., 2004
Psychrobacter sp. Ant300	Antarctic habitat	35	30 (5℃)	Kulakovaa et al., 2004
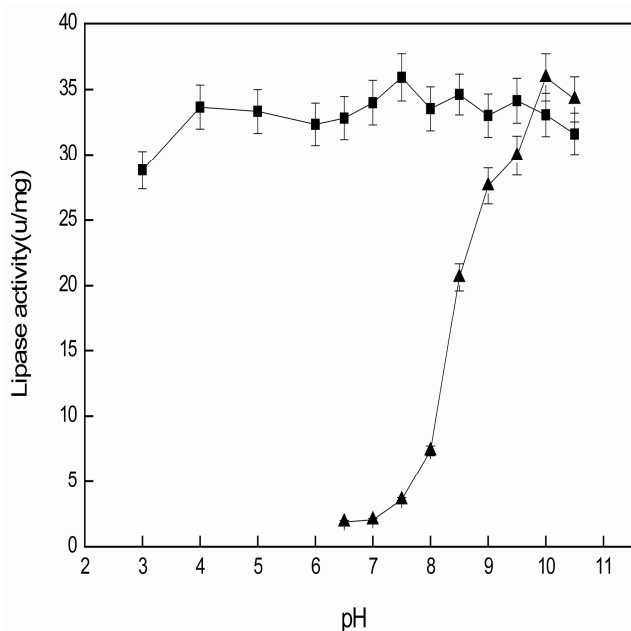
Figure 5. Effect of pH on the activity (▲) and stability (■) of lipase from *Burkholderia* sp. nov. SYBC LIP-Y.

from mesophilic microorganism *S. marcescens* (Abdou, 2003) and *Burkholderia* sp. nov. SYBC LIP-Y was 37 and 30℃, respectively. Their relative activities at low temperature were higher.

The lipase could retain 80.7% of its activity after being incubated at 40℃ for 60 min. It retains 42% of its activity at 60℃ for 60 min. Lipase from *Pseudomonas* sp. KB700A (Rashid et al., 2001) will lost 70% of its activity at 60℃ for 5 min; residual activity of lipase from *Pseudomonas* sp. B11-1 (Choo et al., 1998) is 0% after incubation at 60℃ for 60 min; lipase from *Pseudomonas* Lip35 (Yu et al., 2009) retains 10% of its activity after incubation at 60℃ for 5 min; both the lipases from mesophilic microorganism *S. marcescens* (Abdou, 2003) and *Burkholderia* sp. nov. SYBC LIP-Y retain 90% lipase activity when cultivated at 60℃ for 5 min.

The optimum pH was 10.0 and the enzyme showed stability at pH range 3.0 - 10.5. Majority of the lipases of *Burkholderia* origin showed alkaline pH optima and showed stability within wide pH range. Based on the results, we concluded that the lipase from mesophilic bacterium *Burkholderia* sp. nov. SYBC LIP-Y was cold-adapted enzyme with better thermostability. It has the potential for industrial applications. It supposed that cold-adapted enzymes produced by mesophilic micro-organisms may have higher thermostability than those by psychrophilic microorganisms.

ACKNOWLEDGEMENTS

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