JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2014. Vol. **8**(3), p. 1901-1906

Purification and Characterization of an Intracellular Halotolerant Lipase from Moderate Halophile, *Thalassobacillus* sp. SCULCB HNA-5

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(Received: 19 August 2013; accepted: 07 November 2013)

An intracellular halotolerant lipase was isolated from the moderately halophile, *Thalassobacillus sp*. SCULCB HNA-5. The enzyme was purified by chromatography using Q-Sepharose FF and POROS-XS columns. The molecular mass of the enzyme was estimated to be 60.8kDa by SDS-PAGE. The optimal temperature for activity was 50°C at pH 8.0. It was high active over broad temperature (25–65°C), pH (6.0–8.5), and NaCl concentration (0–4.5M) ranges, indicating its thermostable, alkali-stable, and halotolerant nature. The enzyme activity was markedly enhanced by Li^+, but inhibited by Tween-80 and Cu^{2+}. Moreover the lipase kept high activity in the organic solution such as DMSO, glycerol and methyl alcohol, implying that the enzyme was able to be used in organic synthesis.

Key words: Moderate halophile, Lipase, Thermostable, Halotolerant, Intracellular.

Lipase, a class of enzyme that catalyzes the cleavage and formation of ester, is widely distributed in animals, plants as well as microorganisms[1]. Microbial lipases have been recognized as useful biocatalysts since they have wide substrate tolerance, high stereospecificity toward chemicals, and high stability in organic solvents[2]. Thus they have been widely used in food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries[3, 4, 5].

Most of the well-studied microbial lipases are inducible extracellular enzymes. They are synthesized within the cell and exported to the external surface or environment, such as, lipases from *Bacillus*[6-8], *Natronococcus*[9] etc. There are several reports on the purification and characterization of solvent tolerant lipases which have been used in the synthesis of many useful products such as synthetizing biodiesel[10] and production of eicosapentaenoic acid (EPA)[11]. However few researches study the intracellular lipases and the lipases from moderate halophile.

In this paper, we isolated a lipase-producing *Thalassobacillus* sp. SCULCB HNA-5 from saline soil in Shache, Xinjiang Province, northwest of China. The purification and some remarkable properties of the lipase from this bacterium were described.

MATERIALS AND METHODS

Microorganisms and media

Thalassobacillus sp. SCULCB HNA-5 was isolated from a saline soil in Shache

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(77°14.908'E, 38°25.049'N) (Fig.1).

This strain was grown in a saline medium (TSB-7.5) with a total salt mixture concentration of 10% (w/v) supplemented with 1.5% (w/v) tryptone, 0.5% (w/v)soya peptone and the medium was adjusted to pH 7.5. The bacteria were cultivated at 37°C in an orbital shaker at 200 rpm**.**

Lipase activity assay

Lipase activity was measured spectrophotometrically (at 410 nm, pH 7.5) by using an assay based on the hydrolysis of 4-nitrophenyl ester[12]. The assay was slightly modified by replacement of the chromogenic substrate p-nitrophenyl acetate (p-NPA). One unit of enzyme activity was defined as the amount of enzyme that produced1µmol of p-nitrophenol per minute under these conditions.

Purification of enzyme

All purification steps were carried out at room temperature, unless otherwise specified.

Step.1 Cell disruption

HNA-5 cells from 72h cultures, growing at 37°C, were harvested by centrifugation at 10000g for 10 min at 4°C. The culture supernatant was removed and the pellet was washed twice in 50 mM Tris-HCl buffer (pH 8.5). The cells were disrupted by ultrasonic treatment (Labsonic, Braun Biotech International) for 30 min and the cell debris was removed by centrifugation at 10000x g for 10 min at 4°C. The resulting supernatant was kept as the intracellular fraction and stored at -20°C until use.

Step.2 Anion-exchange chromatography

The samples resulting from cell disruption were applied onto a Q-sepherose FF column (1.6 cm×20 cm) previously equilibrated with 50 mM Tris-HCl buffer (pH 8.5). The column was washed with the same buffer, and eluted with 50 mM Tris-HCl buffer (pH 8.5) containing 1 M NaCl, then followed with a linear gradient of NaCl (0–1 M) of the same buffer. The activity was assayed and the active fractions were pooled, and then dialyzed against 20mM phosphate buffer (pH 6.0).

Step.3 Cation-exchange chromatography

The active fractions were applied onto POROS-XS column (1.6 cm×20 cm) previously equilibrated with 20mM phosphate buffer (pH 6.0). The column was washed with the same buffer, and eluted with 20mM phosphate buffer (pH 6.0) containing 1 M NaCl, then followed with a linear gradient of NaCl (0–1 M) of the same buffer. The active fractions showing lipase activity were pooled.

Determination of molecular mass

The molecular mass of the denatured protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[13]. SDS-PAGE was performed with a 15% polyacrylamide gel on a vertical mini gel apparatus (Bio-RAD) at 200V for 40min.

Characterization of lipase

Effect of pH on enzyme activity and stability

For the optimum pH determination, the activity was determined by assay at 50°C in a pH range of 3.0–10 using 50 mM different buffers: acetate buffer for pH 3.0-6.0; phosphate buffer for pH 6.0-8.0; Tris–HCl buffer for pH 8.0-9.0; borate saline buffer buffer for pH 9.0-10.0. For the pH stability, the purified lipase was incubated at room temperture in a pH range of 3.0-10 using different buffers: acetate buffer for pH 3.0-6.0; phosphate buffer for pH 6.0-8.0; Tris–HCl buffer for pH 8.0-9.0; borate saline buffer for pH 9.0-10.0, and residual activity was measured at intervals by assay.

Effect of temperature on enzyme activity and stability

For the optimum temperature determination, the activity was measured by assay at different temperatures in the range of 20-80°C at pH 8.0 in 50mM Tris-HCl buffer. For the thermostability, the purified lipase was incubated at 20-80°C for up to 30min in 50 mM Tris-HCl, pH 8.0 and residual activity was measured at intervals by assay.

Effect of NaCl concentration on enzyme activity

For the optimum NaCl concentration, the activity was measured by assay at different NaCl concentration in the range of 0-4.5 M at pH 8.0 in 50 mM Tris-HCl buffers.

Effects of various metal ions and detergents on lipase activity

The effects of various additives on lipase activity were investigated by preincubating for 3 min at 50°C, containing 1 mM of $CaCl_2$, $CuCl_2$, $MnCl_2$, $MgCl_2$, KCl, NaCl, LiCl, EDTA and 0.2% of Tween-20, Tween-80, Triton X-100.

Effects organic solvents on lipase activity

Samples of the lipase solution were mixed with different solvent solutions to yield the desired final solvent concentrations. The solvents used

were isopropanol, acetone, methanol, ethanol, acetonitrile, glycerol and dimethyl sulfoxide (DMSO). Equal volume of the purified enzyme was incubated with each of 15%(v/v) organic solvent dissolved in 50 mM Tris-HCl at 50°C for 10min.

Protein estimation

Protein concentration was determined by the Bradford method using bovine serum albumin as standard[14].

RESULTS AND DISCUSSION

We isolated several lipase-producing bacterial strains from saline soil in Shache, Xinjiang Province, northwest of China. Among them, strain HNA-5 was selected for its lipase activity, which shows high activity. On the basis of strain characteristics, strain HNA-5 was identified as a moderately halophile: *Thalassobacillus sp*. SCULCB HNA-5.

Purification of enzyme

The lipase from strain HNA-5 was purified 7.90 fold in 3 steps to a measured final specific activity of 182.93U/mg-protein with a yield of 50.93% (Table 1).The specific activhity of the enzyme was increased 7.48 fold by anion-exchange chromatography on Q-sepherose FF column (1.6 cm × 20 cm).

The next step, cation-exchange chromatography on POROS-XS column (1.6cm × 20cm), was effective for the purification of the enzyme with an increase in the specific activity of about 7.90 fold.

This enzyme showed a molecular mass of approximately 60.8KDa (Fig.2).

Effect of pH on enzyme activity and stability

The activity of lipase was investigated over a pH range of 6.0–10.0 (Fig.3). The results indicated that this enzyme presents an optimal activity at pH 8.0. The enzyme retained over 75.48% of its maximum activity at pH 6.0, but only 19.71% at pH 9.0. The activity at higher pH values (pH >10) was not tested because of spontaneous hydrolysis of p-NPA[15].

The lipase had a broad pH stability in the three different buffers covering the pH range of 5.0–8.5, but the activity decreased to 50% of its original activity after incubation at acetate buffer (pH 5.0) for 30min.

Effect of temperature on enzyme activity and stability

The effect of temperature on lipase activity was studied by assaying the lipase activity at different temperatures. The enzyme was active at a wide range of temperatures from 30 to 65°C; with an optimum value around 50°C (Fig.4) .The lipase was stable at temperature below 60°C and lost about 60% of its activity when incubated for 30min at 70°C, while the enzyme was nearly inactivated at 80°C.

The effects of NaCl concentration on lipase activity

The effects of NaCl concentration on lipase activity were examined (Fig.5). The enzyme kept very stable ranging from 0 to 4.5M NaCl. These features were unique and made it a very attractive enzyme in biotechnological implication with salt concentration between 0.5–4.5M. The enzyme activity was markedly enhanced by NaCl and the enzyme showed optimal activity in 1.5M NaCl. It is known that extracellular hydrolytic enzymes produced by halophilic bacteria usually need high-salt concentration for stability and optimal activity[8], while intracellular lipase from HNA-5 show high activity in NaCl ranging from 0 to 4.5M.

Effects of various metal ions and detergents on lipase activity

Fig. 6 showed the effects of various metal ions and detergents on the lipase activity. In the cation tests, some of them, such as Mn^{2+}, Ca^{2+}, Mg^{2+} and EDTA, slightly inhibited the activity of enzyme while the enzyme activity was lightly enhanced by Na^{+}, Li^{+} and K^{+}. Comparing with high concentration (1.5 M) of Na^{+}, low concentration

Table 1. Purification of the lipase

Purification of lipase	VoL (ml)	Total protein(mg)	Total activity(U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell disruption	200	464.01	10750	23.17	1	100
Q-Sepharose FF	60	47.39	8218	173.41	7.48	76.45
POROS-XS	40	29.93	5475	182.93	7.90	50.93

(1mM) of Na^+ had no obvious effects on the lipase activity. Incubation with detergents, such as Tween-20 and Triton X-100, slightly enhanced the enzyme activity, while Tween-80 deeply decreased the enzyme activity.

Effects organic solvents on lipase activity

The effects of organic solvents on the activity were also investigated (Fig. 7). Lipase was highly stable in 15 %(v/v) ethanol, methanol, glycerol and DMSO, whereas the lipase activity was inhibited by acetone, acetonitrile and isopropanol, and completely inhibited by SDS.

Fig. 1. Location of saline-alkali soil sample used in isolating *Thalassobacillus* sp. SCULCB HNA-5

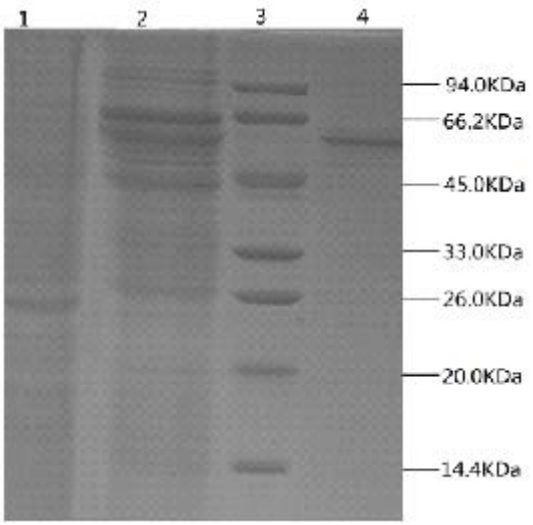

Fig. 2. SDS-PAGE of *Thalassobacillus* sp. SCULCB HNA-5 lipase. (1) Total after cell disruption. (2) Purified by anion-exchange chromatography. (3) SDS-PAGE molecular weight standards. (4) Purified by Cation-exchange chromatography

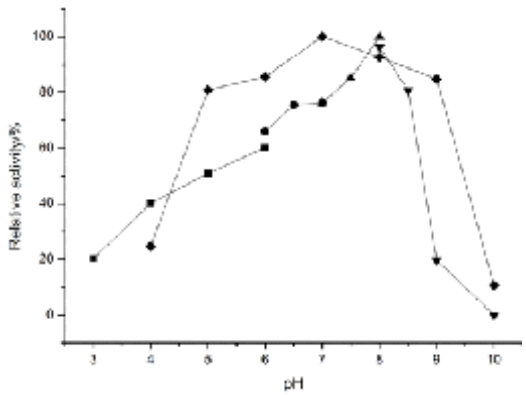

Fig. 3. Effect of pH on activity and stability (◆) of lipase. For the pH activity:acetate buffer (■, pH 3.0–6.0), sodium phosphate buffer (●, pH 6.0–7.5), Tris-HCl buffer (▲, pH7.5–8.5) and sodium carbonate buffer (▼, pH 8.5–10)

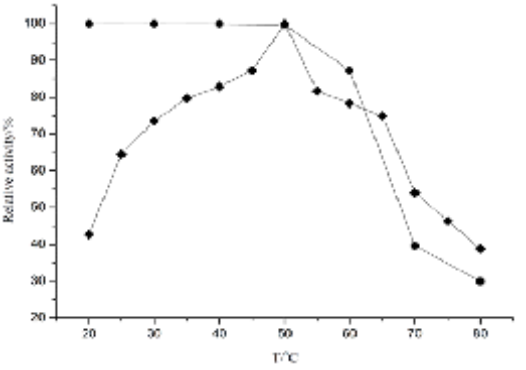

Fig. 4. Effect of temperature on activity (◆) and stability (●) of lipase

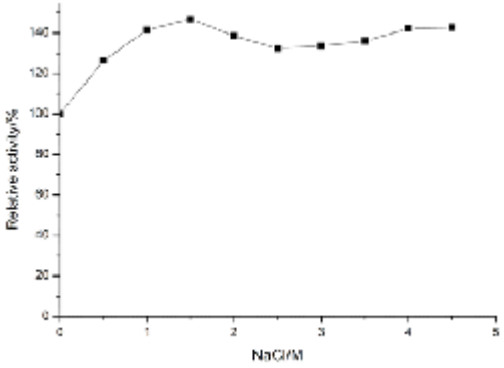

Fig. 5. Effect of NaCl concentration on enzyme activity

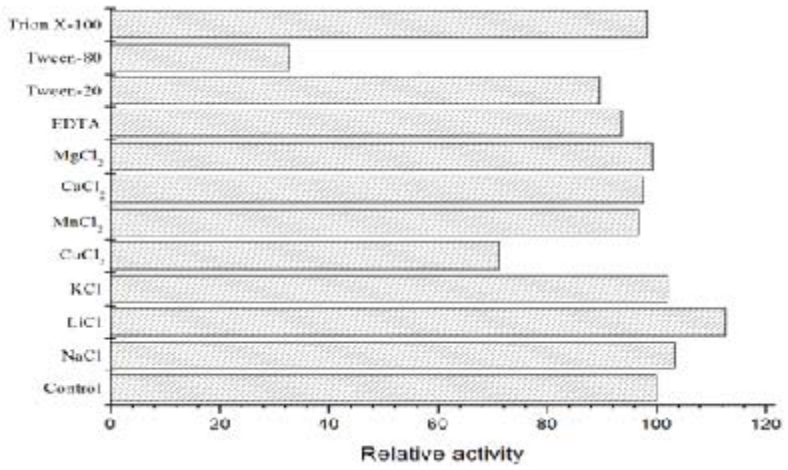

Fig. 6. The effects of various metal ions and detergents on the lipase activity

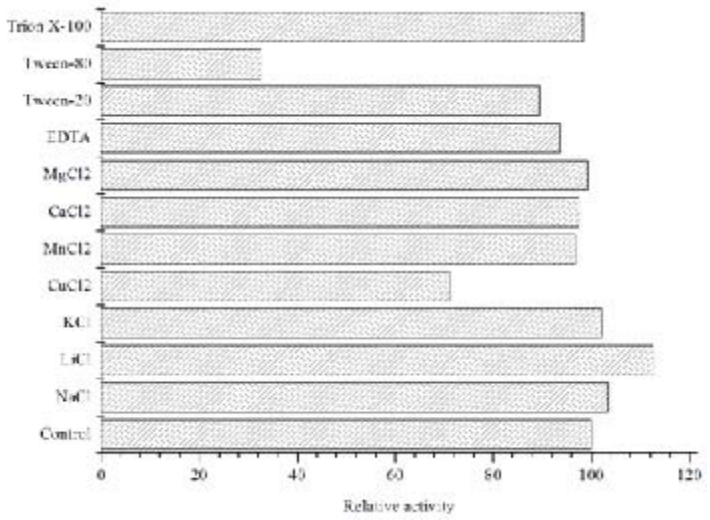

Fig. 7. The effects of organic solvents on the activity

Concluding remarks

We had isolated and characterized a lipase, isolated from the Moderate Halophile, *Thalassobacillus* sp. SCULCB HNA-5.This enzyme was characterized showing excellent biochemical properties (maximal activity at pH 8.0, 50°C, and high concentration of sodium chloride), highlighting its great stability in presence of organic solvents.

ACKNOWLEDGEMENTS

This work was supported by "the Fundamental Research Funds for the Central University".

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