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Novel extremely acidic lipases produced from *Bacillus* species using oil substrates

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Abstract The extremely acidophilic microorganisms *Bacillus pumilus* and *Bacillus subtilis* were isolated from soil collected from the commercial edible oil and fish oil extraction industry. Optimization of conditions for acidic lipase production from *B. pumilus* and *B. subtilis* using palm oil and fish oil, respectively, was carried out using response surface methodology. The extremely acidic lipases, thermo-tolerant acidic lipase (TAL) and acidic lipase (AL), were produced by *B. pumilus* and *B. subtilis*, respectively. The optimum conditions for *B. pumilus* obtaining the maximum activity (1,100 U/mL) of TAL were fermentation time, 96 h; pH, 1; temperature, 50 °C; concentration of palm oil, 50 g/L. After purification, a 7.1-fold purity of lipase with specific activity of 5,173 U/mg protein was obtained. The molecular weight of the TAL was 55 kDa. The AL from *B. subtilis* activity was 214 U/mL at a fermentation time of 72 h; pH, 1; temperature, 35 °C; concentration of fish oil, 30 g/L; maltose concentration, 10 g/L. After purification, an 11.4-fold purity of lipase with specific activity of 2,189 U/mg protein was obtained. The molecular weight of the extremely acidic lipase was 22 kDa. The functional groups of lipases were determined by Fourier transform-infrared (FT-IR) spectroscopy.

Keywords *B. pumilus* · *B. subtilis* · Palm oil · Fish oil · Thermo-tolerant acidic lipase · Acidic lipase

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Introduction

Industries and restaurants are confronted with disposal problems due to the inefficiency of the existing anaerobic and aerobic biological methods for the treatment of lipid-containing wastewater, which has hydrophobic characteristics. Thus, there has been constant research on bioremediation of lipid-rich wastewater, either aerobically or anaerobically [1]. The main pollutants in fish processing industry wastewater include particulate and dissolved organic matter, and oil and grease residues. High concentrations of these pollutants produce indirect impacts as they require large amounts of oxygen for their oxidation. In shallow waters with little movement, they may produce suboxic or even anoxic conditions [2]. The treatment of lipid-rich wastewater is still a challenge. The conventional methods are discouraged owing to their disadvantages [3]. In recent years, enzymatic hydrolysis has been considered an efficient method because of the advantages of high selectivity and specificity to yield high-purity products. Lipases catalyze the hydrolysis of triglycerides at the oil-water interface [4]. Thermophilic enzymes are active at high temperatures by restricting the active-site flexibility entropically. A rigidifying salt bridge favors the activity of thermophilic enzymes at high temperatures [5].

In the present study, oil substrates (palm oil and fish oil) were used for the production of extremely acidic lipases from *B. pumilus* and *B. subtilis*, respectively.

Materials and methods

Sample collection

The palm and fish oils were obtained from a commercial oil extraction factory in Chennai, India. The composition of

palm oil was palmitic acid (44 %), oleic acid (39 %), linoleic acid (10 %), and others (7 %). The fish oil contained 13.3 % EPA, 8.9 % DHA, and 15.6 % oleic acid.

Isolation of lipolytic microorganisms

Oil-contaminated soil was serially diluted, and the organisms were isolated, followed by incubation for 24–48 h at 35° and 50 °C for the growth of microorganisms. Lipase-producing microorganisms producing a clear zone of hydrolysis on the tributyrin agar plates were incubated at 35° and 50 °C. The strain that showed the maximum lipolytic activity was identified by 16S ribosomal DNA (16S rDNA) sequencing and phylogenetic analysis [6].

Response surface methodology

Optimization experiments

The culture for the optimization of lipase production was maintained by growing the organisms in the medium containing palm and fish oil. Lipase production was optimized by varying the time (24–120 h), pH (1–10), substrate concentration (1, 3, 4, 6, 7, 8, and 10 %), and temperature (20–60 °C). The significant factors were optimized by response surface methodology.

Assay of lipase

Lipase activity was measured by titrimetric assay using an olive oil emulsion as described in our work [6]. A unit of lipase activity was defined as the amount of enzyme that released 1 μmol of fatty acid per minute under assay conditions.

Purification of lipase and molecular weight determination

The acidic lipase was purified using the same steps as reported in our earlier study [6]. Protein concentration was determined by the Lowry method [7]. The molecular weights of TAL and AL were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [8].

Characteristics of purified TAL and AL

Effect of pH and temperature of purified lipases

The effect of pH on the purified lipase was determined by incubating the enzyme and substrate at different pHs ranging from 1 to 10 using buffer solutions: 0.1 M HCl/KCl buffer (for pH range 1–2), 0.1 M acetate buffer (for pH range 3–6), 0.1 M phosphate buffer (for pH 7 and 8), and 0.1 M Tris buffer (for pH 9 and 10). The activity of the pH was determined by lipase assay. The stability of pH was determined by incubating the lipase at different pH solutions and various time intervals (1–4 h), and then the lipase activity was estimated.

The effect of temperature on the purified TAL and AL was determined by incubating the enzyme and substrate at different temperatures ranging from 20 to 70 °C at pH 1. The stability was determined by incubating the lipase at different temperatures and time intervals (1–4 h), and the lipase activity was estimated.

FT–IR analysis of acidic lipases

The functional groups present in TAL and AL were identified using a FT-IR spectrophotometer (Perkin Elmer). The samples were made in the form of a pellet with 1-mm thickness and 13-mm diameter, using spectroscopic grade KBr. The spectrum was analyzed in the spectral range of 400–4,000/cm.

Results

Isolation and identification of microorganisms

Among the various microorganisms, isolates from the soil contaminated with palm oil and fish oil that showed maximum lipase activity (1,100 U/mL) and (214 U/mL), respectively, were used in further studies. The 16S rDNA sequencing data showed that the isolated organisms were *B. pumilus* and *B. subtilis.* The nucleotide sequence has been assigned accession no. KC921219 and was KC921218 from the NCBI GeneBank database for *B. pumilus* and *B. subtilis*, respectively. *B. pumilus* and *B. subtilis* were grown at extremely acidic pH 1 and at temperatures of 50° and 35 °C, respectively.

Response surface methodology (RSM)

RSM using the central composite design was employed to determine the optimal levels of the significant

Table 1 Coded and real values of the factors tested in the RSM experimental design

Factor		TAL		AL	
		Levels of factors		Levels of factors	
X1	Concentration of substrate (mL/L)	40	60	20	40
X2	pH	0	2	0	2
X3	Temperature (°C)	40	60	30	40
X4	Time	72	120	48	96
X5	Maltose concentration(g/L)			5	15

factors that affected lipase activity. The high and low levels with the coded levels for the factors are shown in Table 1. Based on the regression analysis of the data, the effects of significant factors on lipase activity were predicted by the second order polynomial function as

$$\begin{aligned}\text{Lipase activity (U/mL)} &= +1,100.00 - 98.54 * A + 67.79 * B + 35.13 * C \\ &\quad - 30.29 * +148.94 * A * \text{B} + 49.69 * A * \text{C} \\ &\quad + 20.94 * A * D + 45.69 * B * C + 3.19 * B * D \\ &\quad + 33.69 * C * D - 145.99 * A^2 - 169.24 * B^2 \\ &\quad - 106.74 * C^2 - 7.36 * D^2 \end{aligned} \quad (1)$$

$$\begin{aligned}\text{Lipase activity (U/ml)} &= +246.36 + 3.76 * A + 8.87 * B - 8.69 * C \\ &\quad - 1.26 * D + 3.42 * E + 4.53 * A * B - 10.72 * A * C \\ &\quad - 1.34 * A * D - 2.91 * A * E + 4.97 * B * C \\ &\quad - 2.53 * B * D - 6.47 * B * E + 7.97 * C * D \\ &\quad + 11.78 * C * E + 5.41 * D * E - 5.03 * A^2 \\ &\quad - 24.23 * B^2 - 11.59 * C^2 - 7.26 * D^2 - 20.43 * E^2 \end{aligned} \quad (2)$$

where A, B, C, D, and E are time, pH, temperature, substrate concentration, and maltose concentration, respectively: Eq. (1) for TAL from palm oil and Eq. (2) for AL from fish oil, respectively.

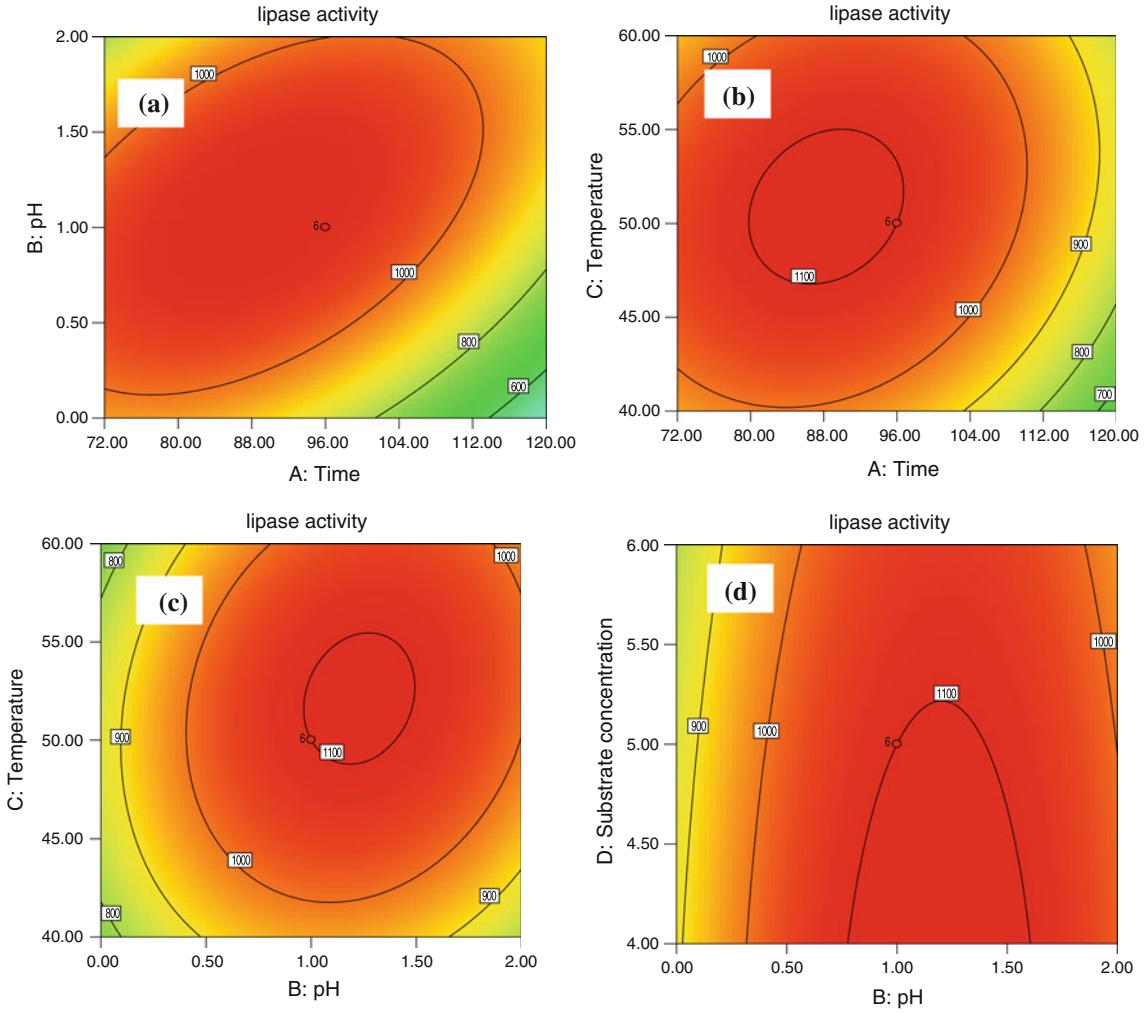

Fig. 1 Response surface curve for thermostable acidic lipase activity (U/mL) by *B. pumilus* as the function of **a** time (h) and pH, **b** time (h) and temperature (°C), **c** pH and temperature (°C), and **d** pH and substrate concentration (g/L)

Analysis of variance (ANOVA) for the response surface quadratic model for TAL and AL

ANOVA (partial sum of squares—type III)

The statistical significance of the equation was checked by the *F* test and ANOVA for the second order polynomial model. The analysis of the factor (*F* test) showed that the second order polynomial model was well adjusted to the experimental data, and the coefficient of variation (CV) indicated the degree of precision of the experiment.

In general, the higher the value of the CV was, the lower the reliability of the experiment. Here, a lower value of CV, 10.12 and 7.58 for TAL and AL, respectively, indicated better precision and reliability of experiments [9]. The precision of a model can be checked by the regression coefficient (R^2). The regression coefficient was calculated to be 0.9620 for TAL and 0.9521 for AL, indicating that 96.20 and 95.21 % of the variability in the response could be explained by this model. Linear and quadratic terms were both significant at the 1 % level.

Localization of optimum conditions

The contour plots described by the regression model were drawn to illustrate the effects of the independent factors and interactive effects of each independent factor on the response factors. It also showed the optimum conditions required for the maximum production of lipase were time, 96 h; temperature, 50 °C; pH, 1.0; substrate concentration, 5 % for TAL (Fig. 1); time, 72 h; temperature, 35 °C; pH, 1.0; substrate concentration, 3 %; maltose concentration, 10 g/L for AL (Fig. 2). Each figure presented the effect of two factors, while the other factor was held at the zero level.

Purification of TAL and AL

The TAL and AL were purified by ammonium sulfate precipitation and DEAE cellulose column chromatography. The specific activity of the purified lipases were found to be 5,173 U/mg protein and 2,189 U/mg protein. The

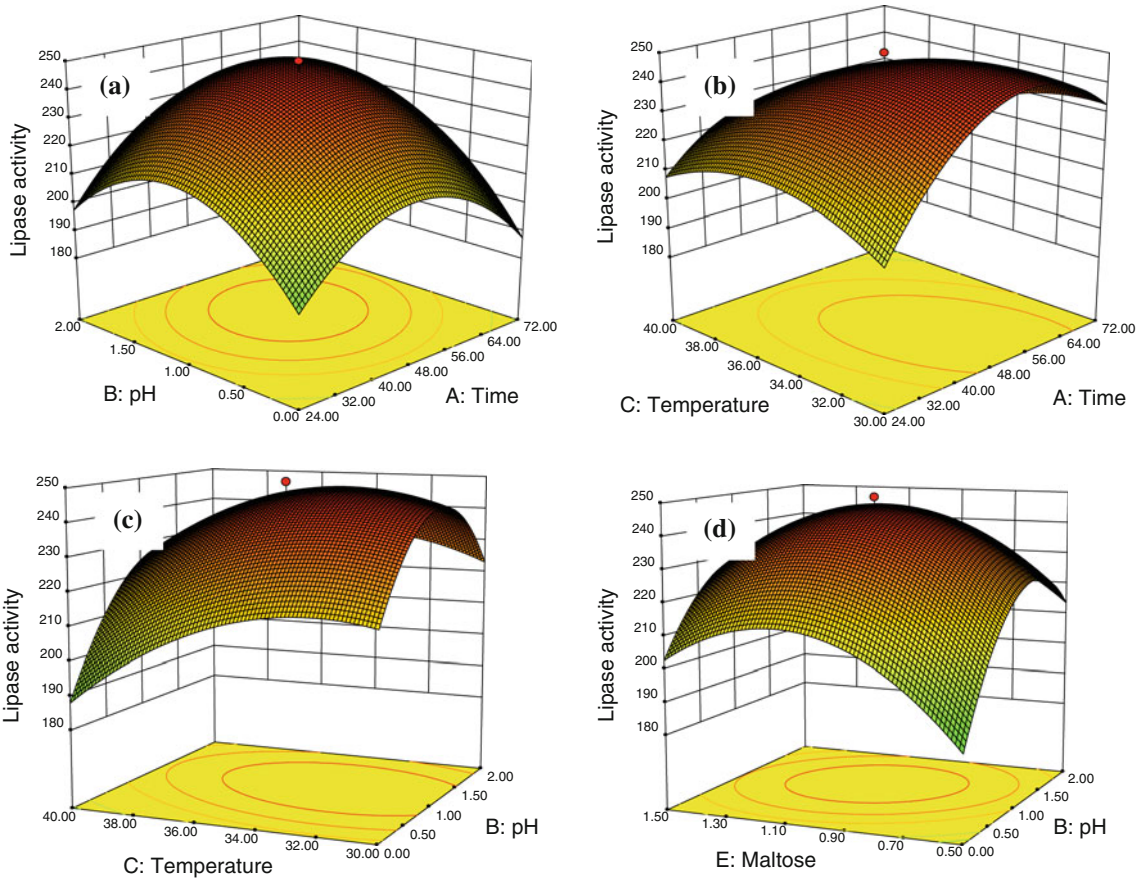

Fig. 2 Response surface curve for acidic lipase activity (U/mL) by *B. subtilis* as the function of **a** time (h) and pH, **b** time (h) and temperature (°C), **c** pH and temperature (°C), and **d** pH and maltose concentration (g/L)

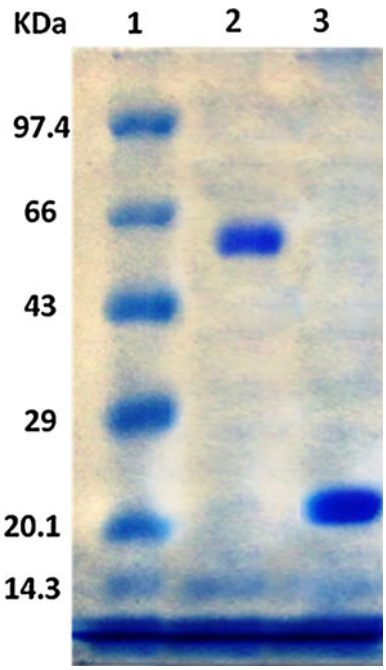

Fig. 3 SDS-PAGE showing the molecular weight of purified thermostable acidic lipases. *Lane 1* molecular weight marker, *lane 2* purified TAL (55 kDa), *lane 3* purified AL (22 kDa)

molecular mass of the protein was found to be 55 kDa for TAL and 22 kDa for AL (Fig. 3) evaluated with SDS-PAGE.

Characteristics of TAL and AL

Effect of pH and temperature on lipase activity

The TAL and AL produced by *B. pumilus* and *B. subtilis* show the maximum relative activity at very acidic pH 1. The lipase activity was reduced with an increase in pH. The study illustrated that the TAL and AL produced in the present investigation had high activity at very acidic conditions. About 100 % of relative lipase activity was observed at a very acidic pH (pH 1) (Fig. 4a). The relative lipase activity was reduced to 50 % when the pH was increased. The stability at pH 1 suggests that lipase was found to be acidic in nature (Fig. 5a).

The activity of the purified lipase was determined at different temperatures from 20 to 60 °C. The maximum activity was observed at 50 °C for TAL and 35 °C for AL; the activity was reduced at other temperatures. The TAL enzyme remains active even at higher temperature. TAL derived from *B. pumilus* was found to be active in the temperature range 50–60 °C (Fig. 4b). Stability studies show that the purified TAL was found to be highly stable (100 %) at 50 °C. The results show that TAL was highly stable at elevated temperature and at extremely acidic pH.

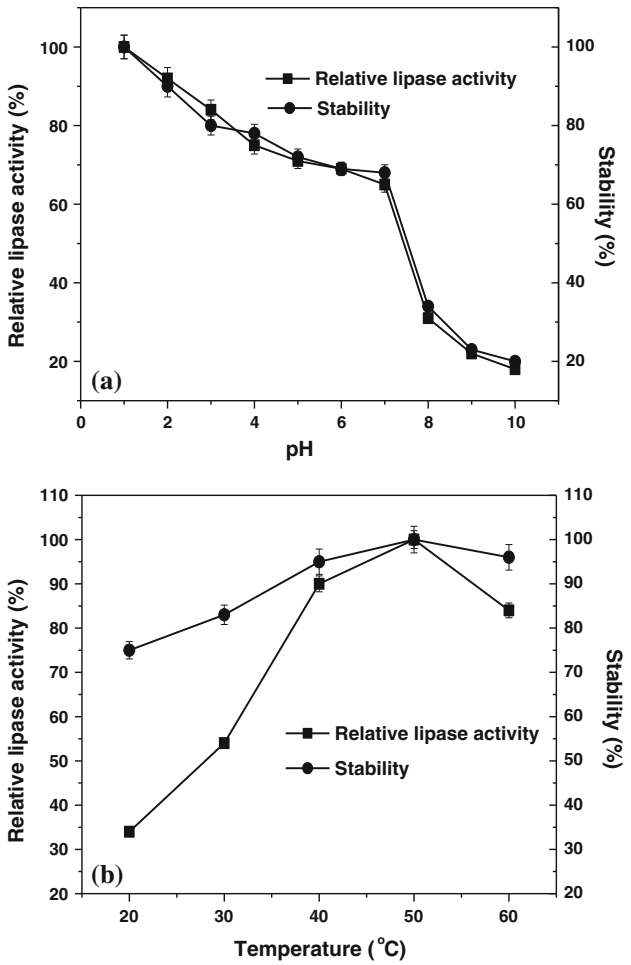

Fig. 4 Relative lipase activity and stability of purified TAL at different **a** pHs and **b** temperatures

Acidic lipase derived from *Bacillus subtilis* was found to be active in the temperature range 30–40 °C (Fig. 5b). Stability studies show that the purified acidic lipase was found to be highly stable (100 %) at 35 °C. The results conclude that acidic lipase was highly stable at 35 °C temperature and at extremely acidic pH.

FT–IR spectrum of purified acidic lipases

The FT–IR spectrum of purified acidic lipase from *B. subtilis* is shown in Fig. 6a. The spectrum shows major stretching bands owing to the peptide group occurring in the spectral region 1,200–1,700/cm. The band at 3,427.69/cm is due to the C=O stretching vibrations of amide I. The bands at 1,237.80 and 1,457.25/cm are attributed to NH bending and C–N stretching vibrations. The bands at 2,925.84 and 2,854.48/cm are attributed to stretching of methylene C–H asym./sym.

The sharp peak at 17,420.35/cm is due to the presence of alkyl carboxylate (C=O stretch), which gives the lipase its

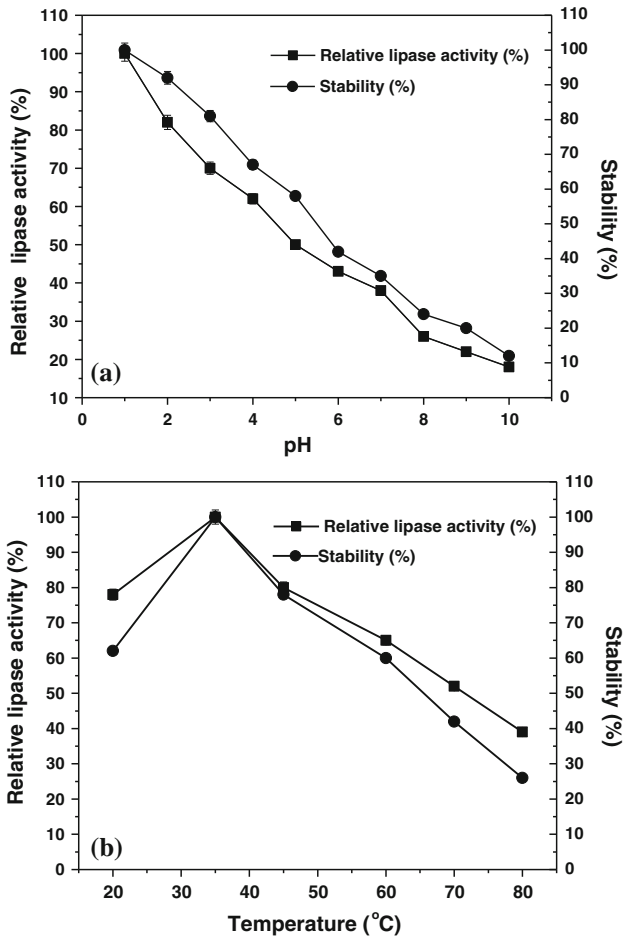

Fig. 5 Relative lipase activity and stability of purified acidic lipase at different **a** pHs and **b** temperatures

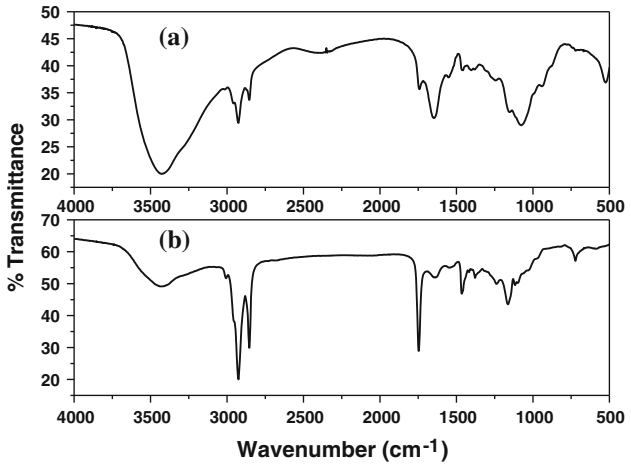

Fig. 6 FT-IR spectrum of **a** AL from fish oil and **b** TAL from palm oil

acidic nature. The bands at 1,645.22 and 1076.60/cm are attributed to NH bending and CN stretching of the primary amine, respectively.

The FT-IR spectrum of the purified TAL is shown in Fig. 6b. The spectrum shows major stretching bands owing to the peptide group occurring in the spectral region 1,200–1,700/cm. The band at 1,643.71/cm is due to the C=O stretching vibrations of amide I. The bands at 1,237.80 and 1,465.74/cm are attributed to NH bending and C–N stretching vibrations [10]. The sharp peak at 17,460.87/cm is due to the presence of alkyl carboxylate (C=O stretch), which gives the lipase its acidic nature.

Discussion

In the present study, we produced thermo-tolerant acidophilic lipase and acidic lipase from thermo-tolerant *B. Pumilus* and *B. subtilis* by utilizing palm oil and fish oil as substrates. The strain was identified using 16S rDNA sequencing and phylogenetic analysis. The strain *B. pumilus* produced 1,100 U of lipase activity per mL of medium at optimum conditions of time, 96 h; pH, 1; temperature, 50 °C; substrate concentration (palm oil), 5 % (Fig. 2). The strain *B. subtilis* produced 214 U of lipase activity per mL of medium at the optimum conditions of time, 72 h; pH, 1; temperature, 35° C; substrate concentration (fish oil), 3 %; maltose concentration, 1 % (Fig. 3). Approximately 7.1-fold purification with 10.1 % recovery of TAL and 11.4-fold purification with 38 % recovery of acidic lipase were achieved, which were higher than reported by earlier researchers [11, 12]. The purified lipase was active at extremely acidic pH 1 and at a temperature of 50 °C for TAL and 35 °C for AL (Fig. 4). The extremely acidic lipases (pH 1) produced using *Bacillus* species were novel when compared to other reported acidic lipases active at pH 1.5 [13] and pH 3.5 [14]. The functional groups of purified lipases were identified by FT–IR spectroscopy (Fig. 6).

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