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RESEARCH ARTICLE

Purification and characterization of a mesophilic organic solvent tolerant lipase produced by *Acinetobacter* sp. K5b4

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ABSTRACT

The extracellular lipase produced by *Acinetobacter* sp. K5b4 was purified to homogeneity using ultrafiltration (cutoff 30 KDa) followed by gel filtration chromatography on Sephadex G-50. The enzyme was purified to homogeneity with an apparent molecular mass of 133 KDa by SDS-PAGE. This purification resulted on 10.24 fold with 18.3% recovery. The K_m and V_{max} of purified enzyme when using *p*NPL hydrolysis were 4.0 mM and 73.53 nmol/ml/min, respectively. The pure enzyme was greatly stimulated in the presence of 20, 40 and 60% (v/v) methanol, DMSO and acetone whereas, ethanol, acetonitrile and propanol decreased the enzyme activity. Maximum enzyme activity was achieved at pH 7.0 and incubation temperature of 27 °C. The enzyme was stable within a pH range of 6.5 to 7 at 27 °C for 1 h. The enzyme activity was enhanced up to 36% by KCl, $BaCl_2$, $MgCl_2$ and $CaCl_2$ while obviously inhibited (10–20%) by $CoCl_2$, $ZnCl_2$, $MnCl_2$ and $CuCl_2$. No inhibitory effects were observed with 1.0 and 5.0 mM of 2-mercaptoethanol and EDTA. Similarly, SDS at 1.0 mM does not affect the enzyme activity while high reduction (80%) was observed at 5.0 mM SDS concentration. The enzyme was active against *p*-nitrophenyl esters of C8, C12 and C16 with highest preference to the medium carbon chain *p*-nitrophenyl caprylate (C8). The fact that the enzyme displays distinct stability in the presence of methanol, DMSO and acetone suggests that this lipase is suitable as biocatalyst in organic synthesis where such hydrophilic organic solvents are used as a reaction media.

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KEYWORDS
Extracellular lipase; *Acinetobacter* sp. K5b4; purification; characterization; pNPL hydrolysis

1. Introduction

Enzymes are biocatalysts playing an important role in metabolic and biochemical reactions. Microorganisms in addition to plants and animals produce lipases. Commonly, microbial lipases confirmed to be more preferable compared to other higher organisms lipases due to their mass production and versatility of their properties (Andualema and Gessesse 2012). Recently, more and more carefulness is being paid to lipases produced by bacteria and fungi.

Lipases are hydrolytic enzymes (triacylglycerol hydrolases E.C. 3.1.1.3) that catalyze the hydrolysis of triacylglycerols (TAGs) to glycerol and free fatty acids (FAs) (Snellman et al. 2002; Ahmed et al. 2010; Kim et al. 2012). The biotechnological applications using lipases require suitable enzymes as a biocatalyst with complementary characteristics. These lipases usually obtained from the existing commercial lipase preparations or the biodiversity of their natural sources. Previously, the shortage in lipases with specific characteristics required for manufacturing processes was one of the limiting factors of their use (Yuan et al. 2016). Microbial lipases are to some extent stable and are able of catalyzing different reactions; they are of potential concern for various industrial use.

Based on their remarkable characteristics lipases now categorized in literature as thermostable (Yuan et al. 2016), cold active (Čanak et al. 2015), alkaline (Wang et al. 2012), acidic (Panizza et al. 2015) and organic solvent tolerant lipases (Li et al. 2014). Their remarkable characteristics were found individually or as a combination of two or more of them such as thermostable alkaline lipases (Bhosale et al. 2016) cold active alkaline lipase (Wang et al. 2011) thermostable organic solvent tolerant lipases (Mo et al. 2016) and thermosatble alkaline organic solvent tolerant lipase (Ahmed et al. 2010). Obviously, biocatalysts with a

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combination of these characteristics were more preferable in industries.

However, some commercial applications like biodiesel production do not require pure lipase preparations. Suitable lipase purity is mostly required in the areas such as food processing, detergent, paper and pulp industry. Therefore, it is necessary to remove some unwanted impurities like proteins because of their antagonistic effects on the desired enzyme's activity (Moradi et al. 2015). However, purified lipase preparations are needed for the biocatalytic production of fine chemicals, pharmaceuticals and cosmetics (Costa-Silva et al. 2014). This study aimed to purify and characterize the extracellular lipase enzyme produced by *Acinetobacter* sp. K5b4 that was isolated from hydrocarbon contaminated soil samples, Al-karak province, south of Jordan.

2. Materials and methods

2.1. Bacterial isolate

The bacterial strain used in this study was isolated from hydrocarbon contaminated soil samples collected from Al-karak province, south of Jordan. The bacterial strain was identified based on the biochemical tests (RapID^{TM} ONE System, Remel, USA) and 16S rRNA gene sequencing (GENEWIZ, USA) as *Acinetobacter* species (Allimoun et al. 2015).

2.2. Culture medium and growth conditions

For extracellular lipase production from *Acinetobacter* sp. K5b4, the bacterial strain was grown in the optimized culture medium and growth conditions reported earlier (Allimoun et al. 2015). The optimized culture medium (pH 7.0) composed of (per 100 ml); 1.0% (w/v) olive oil, 0.2% (w/v) glycerol, 0.15% (w/v) yeast extract and 0.05% (w/v) NaCl. The culture medium was inoculated with 0.4% (v/v) of 0.2 OD_{600} seed culture and incubated at 30 °C and 150 rpm for 48 h.

2.3. Purification of extracellular lipase produced by Acinetobacter sp. K5b4

Before the gel filtration step, the culture broth was harvested and centrifuged at 5000 rpm for 5 min at room temperature. Fifteen milliliter of the cell free supernatant (total activity of 187 mU/ml) were concentrated 3X to a final volume of 5 ml using Amicon Ultra-15 centrifugal filter units (Sigma-Aldrich, USA) cut off 30 KDa in refrigerated centrifuge (Combi 514 R, Hanil Science Industrial CO., LTD, South Korea) for 5 min at 5000 rpm.

One milliliter of the concentrated enzyme was applied to a 50 ml Sephadex G-50 column (1.0 × 65 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.0. The column was eluted with equilibration buffer at a flow rate of 0.2 ml/min. Fractions of about 2 ml were collected and assayed for enzyme activity (Winkler and Stuckmann 1979) and protein content at 280 nm. Fractions with the highest lipase activity obtained from gel filtration chromatography were chosen. Protein samples were analyzed by SDS-PAGE as described by Laemmli (1970). SDS-PAGE was carried out in 10% (w/v) homogenous gel consisting of a resolving gel (pH 8.8, 10% w/v, acrylamid) and a stacking gel of 4% (w/v) acrylamide (pH 6.8). Reagents, buffers, broad range protein markers in the range of 6.5–200 KDa (Sigma-Aldrich) and the enzyme sample were prepared according to Bollag et al. (1996). Electrophoresis was carried out at constant voltage of120V for 1 h using Mini-Protein electrophoresis apparatus (Cleaver Scientific Ltd, UK). The gel was stained with silver staining method as described by Bollag et al. (1996).

2.4. Characterization of pure lipase of Acinetobacter sp. K5b4

2.4.1. Substrate specificity

Lipase specificity toward *p*-nitrophenyl fatty acid esters with different carbon chain length was measured spectrophotometrically (SPUV-19, Sco-TECH, Germany) using the method of Winkler and Stuckmann (1979). *p*-nitrophenylcaprylate (pNPC), *p*-nitrophenyl laurate (pNPL) and *p*-nitrophenyl palmitate (pNPP) (Sigma-Aldrich) as lipase enzyme mono-ester substrates was alternatively changed in the assay reaction solution. Hundred microliters of the purified enzyme was added to 1.90 ml of the assay reaction mixture of each substrate, and the reaction was allowed to proceed for 10 min at 30 °C. The *p*-nitrophenol formed from the hydrolysis action of lipase in each substrate mixture was measured at 410 nm immediately. Relative activity was calculated by comparing the enzyme activity in the three substrates reaction mixtures.

2.4.2. K_m and V_{max} determination

The Michelis–Menten constant (K_m) and the maximum velocity (V_{max}) were determined by measuring enzyme activity of the purified lipase at various concentrations of *p*-nitrophenyl laurate (pNPL) (300–900 μM) over 40 min. Lipase assay was conducted at various

substrate concentrations at pH 7.0 and temperature of 25 ± 1 °C. The initial linear rate of product formation, initial velocity (v_o), of the purified lipase calculated by adding 100 µl of the purified lipase into 1.9 ml of the assay solutions. The absorbance at 410 nm was measured every 2 min for a 40 min in a spectrophotometer (SPUV-19, Sco-TECH, Germany) to determine the amount of *p*-nitrophenol formed. The actual amount of *p*-nitrophenol formed per unit time, given as nanomoles per minute (nmol/min), was calculated by converting the absorbance value at 410 nm to an actual concentration (nmol) by comparing the absorbance at 410 nm wave length with the *p*-nitrophenol standard curve. The concentration of *p*-nitrophenol formed during the course of the reaction was then graphed as a function of time. Initial velocity (v_o) was then obtained from the slope on *p*-nitrophenol concentration versus time graph. The kinetic parameters (K_m and V_{max}) were calculated by plotting $1/V_o$ against 1/[S] in Lineweaver Burk plot.

2.4.3. Effect of hydrophilic organic solvents on lipase activity

The effects of different concentrations of different hydrophilic organic solvents on the activity of the pure lipase preparation of *Acinetobacter* sp. K5b4 were studied. Different concentrations (20, 40 and 60% (v/v) of dimethyl sulfooxide (DMSO), methanol, ethanol, acetone, acetonitrile and propanol were used in the assay reaction mixture. Hundred microliter of the pure enzyme was added to the assay reaction mixture and the reaction was allowed to proceed for 10 min at 30 °C. The *p*-nitrophenol formed from the hydrolysis action of lipase was measured at 410 nm immediately. Relative activity was calculated by comparing the enzyme activity of the control experiment with the enzyme activity in the presence of the tested organic solvents.

2.4.4. Effect of pH on the activity and stability of the purified lipase

The effect of pH on the purified lipase activity was investigated at various pH values; 6, 6.5, 7, 7.5 and 8. The pH of the assay reaction mixtures were varied using different buffers, 100 mM citrate buffer for pH 6–6.5, phosphate buffer for pH 7–7.5 and Tris-HCl buffer for 7.5–8. Hundred microliter of the purified enzyme was added to the assay reaction mixture, and reaction was allowed to proceed for 10 min at the respective pH. Relative activity was calculated based on the maximum enzyme activity at the optimum pH being 100%.

The pH stability was determined by incubating the enzyme solution separately at different pH values covering the range of pH 6.5–8.5. The pH of the pure enzyme preparation was adjusted to pH 6.5, 7.0, 7.5, 8.0 and 8.5 using 1 N HCl or NaOH. The pure enzyme preparations was then incubated at 30 °C for 1 h and sample of 100 µl was withdrew every 20 min to determine the residual lipase enzyme activity.

2.4.5. Effect of temperature on the activity and stability of the purified lipase

In order to determine the optimum temperature of *Acinetobacter* sp. K5b4 pure lipase activity, the assay reaction mixture was incubated for 10 min at (24, 27, 30, 33 and 35 °C). Hundred microliter of the purified enzyme was added to the assay reaction mixture, and reaction was allowed to proceed for 10 min at the respective temperatures. The thermostability of the purified enzyme was investigated at different temperatures covering the range from 30 to 60 °C. 100 µl of the purified lipase was incubated separately at the temperature of 30, 35, 40, 45, 50, 55 and 60 °C for 1 h. Hundred microliters of the samples were withdrawn every 20 min to determine the residual activity of the purified enzyme.

2.4.6. Effect of metal salts on the purified enzyme activity

One mM of $CoCl_2$, KCl, BaCl, $CaCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$ and $CuCl_2$ metal salts were added to the assay reaction mixture in order to determine the effect of metals on the purified lipase enzyme activity. Hundred microliter of the purified enzyme was added to 1.90 ml of the assay reaction mixture, and the reaction was allowed to proceed for 10 min at 27 °C. Relative activity was calculated by comparing the activity from the control reaction with the activity from the reaction with the presence of metal salts.

2.4.7. Effect of various detergents and chemical agents on the purified lipase activity

Three chemical agents with different concentrations were investigated for their effect on lipase activity. EDTA, SDS and 2-Mercaptoethanol were added to the reaction mixture to the final concentration of 1 and 5 mM. Hundred microliter of the purified enzyme was added to 1.90 ml of the assay reaction mixture, and the reaction was allowed to proceed for 10 min at 27 °C. Relative enzyme activity was determined by

comparing the enzyme activity in the presence and absence of various agents.

2.4.8. Enzyme assay and protein content

Lipase activity was measured spectrophotometrically (SPUV-19, Sco-TECH, Germany) as described by Winkler and Stuckmann (1979). Five hundred micromolar of *p*-nitrophenyl laurate were dissolved in 10 ml of dimethyl sulfooxide (DMSO) and emulsified in 90 ml of 100 mM phosphate buffer (pH 7.0), 0.1% (w/v) polyvinyl alcohol (PVA) and 0.4% (w/v) Triton X-100. Hundred microliter of the enzyme was added to 1.9 ml of the reaction mixture. After 10 min of incubation at 27 °C, the color change was measured at 410 nm and compared with the *p*-nitrophenol standard curve. One unit (U) of lipase activity was defined as the amount of the enzyme that released 1 µmol of *p*-nitrophenol per min. Protein content in the enzyme preparation (crude and concentrated) was determined based on Lowry method (Lowry et al. 1951). While protein content in fractions obtained from Sephadex G-50 column was determined at 280 nm (SPUV-19, Sco-TECH, Germany) by comparing the readings with the BSA standard curve at 280 nm.

3. Results and discussion

3.1. Extracellular lipase purification

A principal step for concentrating and partial purification of the extracellular lipase from *Acinetobacter* sp. K5b4 was carried out using Amicon Ultra-15 centrifugal filter unit cutoff 30 KDa (Sigma-Aldrich). The results of this step showed that the crude enzyme preparation was approximately concentrated 3X as 2658.5 mU total activity of the crude lipase was detected in 5 ml concentrate as compared to the total activity of 2806.5 mU in 15 ml crude filtrate. In addition, the specific activity of the crude enzyme preparation was intensified from 256.3 to 1296.8 mU/mg protein with recovery percentage of 94.7 and 5.05 purification folds (Table 1). One milliliter of the concentrated partially pure enzyme was loaded into Sephadex G-50 (1 cm ×65 cm). The flow rate of the eluted enzyme solution was 0.2 ml per min and conducted at room temperature (25 ± 1 °C). Enzyme activities were detected in coming early fractions 8, 9, 10 and 11 (Figure 1). The specific activity was 2626.2 (mU/mg). The enzyme solutions from pooled fractions which gave the highest purification fold (F8, 9, 10 and 11) were analyzed for protein homogeneity using SDS-PAGE electrophoresis. The purified enzyme resolved into a single band on an SDS-PAGE gel (Figure 2) had an estimated molecular weight of 113 KDa. Table 1 also shows that, the specific activity of the filtrate of the enzyme solution in fractions 8–11 increased from 256.3mU/mg protein (crude enzyme) to 2626.2mU/mg protein. The results also showed that the gel filtration step was resulted in 10.2 purification fold with 18.3% recovery (Fraction 8–11).

Table 1. Purification table of the *Acinetobacter* sp. K5b4 lipase enzyme.

	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Crude enzyme	15	2806.5	10.95	256.3	100	1
Amicon tube (3X)	5	2658.5	2.05	1296.8	94.7	5.05
G-50 Sephadex gel filtration (F8-11)	8	513.6	0.44	2626.2	18.3	10.24

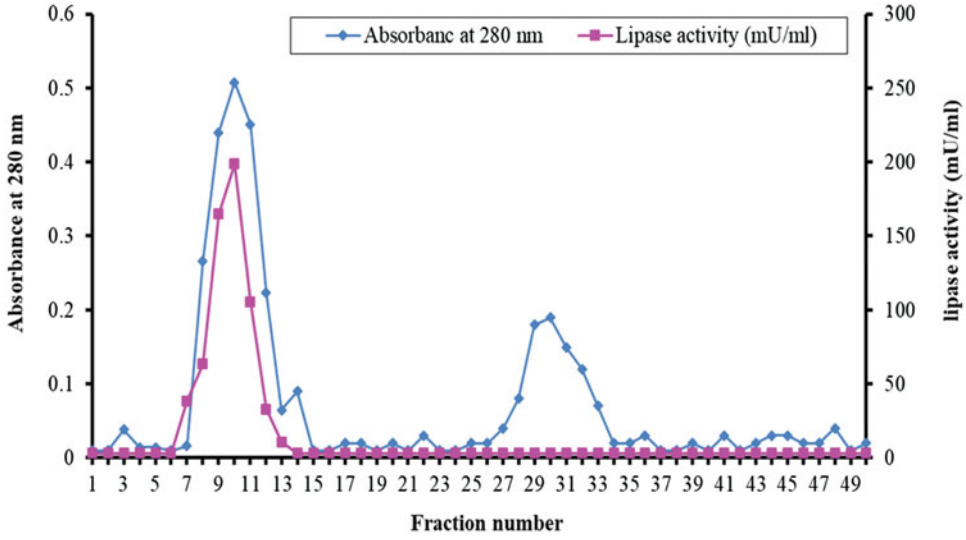

Figure 1. Chromatography of lipase from *Acinetobacter* sp. K5b4 on Sephadex G-50.

Several lipases from different *Acinetobacter* strains have been previously purified to homogeneity. In their purification process, lipases from *Acinetobacter* strains usually treated with different chromatography resins in single, two or multistep procedure. Their molecular mass were reported and generally estimated to be between 20 and 65 KDa which is much less than the molecular mass of *Acinetobacter* sp. K5b4 lipase that reported in this work. An exception is the earliest study made by Breuil and Kushner (1975) who estimated the molecular mass of the lipase enzyme of psychrophilic *Acinetobacter* sp. O16 to be more or equal to 200 KDa. A summary of the molecular weights reported in the literatures of the purified lipases from different *Acinetobacter* species is shown in Table 2.

3.2. Characterization of pure lipase of Acinetobacter sp. K5b4

3.2.1. Substrate specificity

Substrate preferences of *Acinetobacter* sp. K5b4 pure lipase for carbon chain length of acyl group in the *p*-nitrophenyl mono-acyle ester were investigated in this experiment. Substrate specificity results of the investigated lipase with various *p*-nitrophenyl esters showed that, medium carbon chain *p*-nitrophenyl fatty acid esters served as good substrates for the enzyme, and *p*-nitrophenylcaprylate (C8) was the best among all the substrates tested (Figure 3). On the other hand, the enzyme showed similar preference to the *p*-nitrophenyl mono-acyle ester *p*-nitrophenyl laurate (C12) and *p*-nitrophenyl palmitate (C16). In parallel to our results the lipase enzyme produced by *Acinetobacter* sp. XMZ-26 and *Acinetobacter* sp. RAG-1 were found to preferentially attacks medium length acyl chains C6 and C8 (Snellman et al. 2002; Zheng et al. 2011). However, due to the instability of *p*-nitrophenylcaprylate (C8) substrate, *p*-nitrophenyl laurate (C12) was used in further experiment as enzyme substrate in the assay solution.

3.2.2. K_m and V_{max}

Michelis–Menten constant (K_m) and the maximum velocity (V_{max}) were determined by measuring enzyme activity of the purified lipase at various concentrations

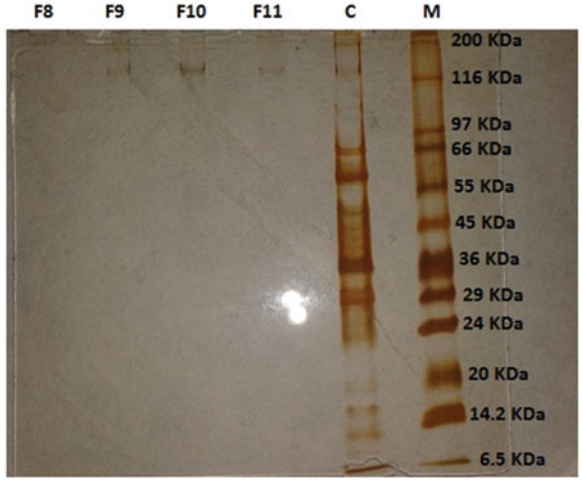

Figure 2. SDS-PAGE of the collected fractions with lipase activity from *Acinetobacter* sp. (F8) Fraction 8, (F9) Fraction 9, (F10) Fraction 10, (F11) Fraction 11, (C) crude enzyme solution and (M) Broad range protein marker (6.5–200 KDa).

Table 2. Molecular weights of the purified lipases from various *Acinetobacter* species.

Microorganism	(KDa)	References
Acinetobacter radioresistens CMC-1	45	Hong and Chang (1998)
Acinetobacter sp. KM109	62	Mitsuhashi et al. (1999)
Acinetobacter calcoacetius LP009	23	Pratuangdejkul and Dharmsthiti (2000)
Acinetobacter sp. RAG-1	33	Snellman et al. (2002)
Acinetobacter lwoffii 16C-1	45	Kim and Park (2002)
Acinetobacter sp. SY-01	37.2	Han et al. (2003)
Acinetobacter sp. ES-1	32	Lee et al. (2006)
Acinetobacter sp.	32	Saisubramanian et al. (2008)
Acinetobacter baumannii BD5	35	Park et al. (2009)
Acinetobacter baylyi	30	Uttatree et al. (2010)
Acinetobacter johnsonii LP28	53	Wang et al. (2011)
Acinetobacter venetians V28	37.186	Kim et al. (2012)
Acinetobacter sp.	60	Jagtap and Chopade (2015)
Acinetobacter sp.	46	Moradi et al. (2015)
Acinetobacter sp. O16	200	Breuil and Kushner (1975)
Acinetobacter sp. K5b4	113	In this study

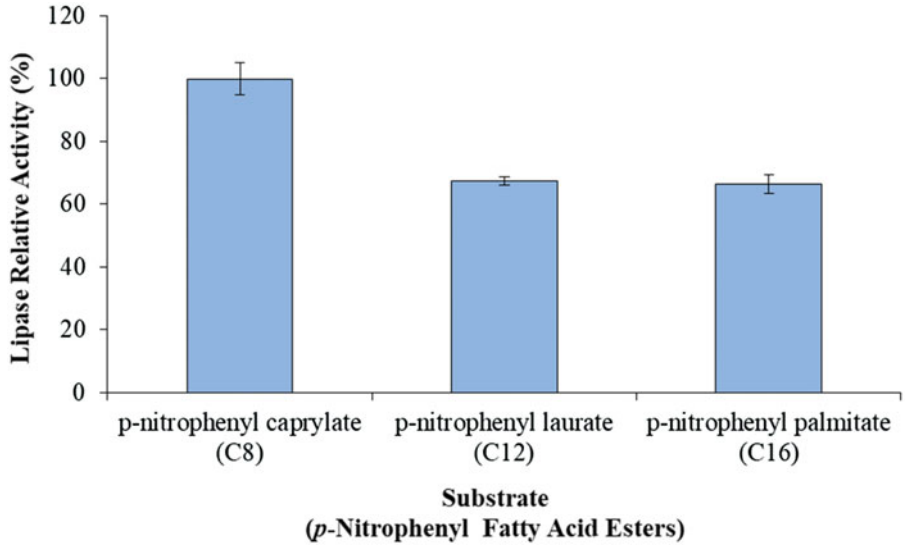

Figure 3. *p*-nitrophenyl Mono-acyl ester substrate specificity of *Acinetobacter* sp. K5b4 pure lipase enzyme.

of *p*-nitrophenyl laurate (pNPL) (300–900 μM) over 40 min (Figure 4). The kinetic parameters (K_m and V_{max}) were calculated from a Lineweaver-Burk plot fit by linear regression. Our results showed that the K_m and V_{max} values for the pure lipase enzyme produced by *Acinetobacter sp*. K5b4, using pNPL as substrate, were 4.0 mM and 73.53 nmol/ml/min, respectively.

Practically, straightforward comparison of the kinetic parameters of other lipases from different species of the same genus reported in the literature is trivial. This is partially due to the differences in the assay conditions, the substrate used, concentration range and the nature of the enzyme. However, few reports were found in the literature reporting some of the kinetic parameters of certain pure enzymes produced by *Acinetobacter* strains. Kim and Park (2002) detected the K_m and V_{max} of the purified 45 KDa esterase enzyme produced by *Acinetobacter lwoffii* 16C-1, using *p*-nitrophenyl butyrate as substrate, as 11 μM and 131.6 μM min^{-1} mg of protein, respectively. In another study, the K_m value of the cold-adapted lipase enzyme from *Acinetobacter* sp. XMZ-26, using *p*-nitrophenyloctanoate as substrate, found to be 0.075 ± 0.008 mM (Zheng et al. 2011, 2012).

3.2.3. Effect of hydrophilic organic solvents on lipase activity

Relative activities of *Acinetobacter* sp. K5b4 pure lipase was studied in the presence of different concentrations of various water miscible organic solvents (Figure 5). In the presence of 20, 40 and 60% (v/v) of water miscible organic solvent methanol, the enzyme activity was enhanced by a 79, 169 and 49%, respectively, as compared with the control. Similar to methanol, the addition of DMSO to the enzyme assay in the concentrations of 20, 40 and 60% (v/v) caused a rise in the hydrolysis activity by 46.4, 175.6 and 267.6%, respectively relative to control. The addition of acetone to the assay solution was also showed an enhancement in the enzyme activity over the studied concentrations. At 20, 40 and 60% (v/v) concentrations, the enzyme exhibited stimulation in the enzyme activity of 223, 203 and 24% (v/v) in comparing with the control. Alternatively, the addition of ethanol, acetonitrile and propanol to the assay solution inhibit the enzyme activities (except at the 20% (v/v) of acetonitrile that cause 143% boosting over the control) in the range between 20 and 95% as compared with the control.

The extracellular lipases extracted from various *Acinetobacter* strains retain their initial activity between 70 and 75% after exposure to different organic solvents. For example, *Acinetobacter* sp. RAG-1 (Snellman et al. 2002), the thermostable alkaline lipase of *Acinetobacter* sp. EH28 (Ahmed et al. 2010), the cold adapted lipase of *Acinetobacter* sp. XMZ-26 (Zheng et al. 2011), *Acinetobacter* sp. XMZ-26 (Zheng et al. 2012) and *Acinetobacter venetians* V28 (Kim et al. 2012). Besides, several lipases produced by different *Acinetobacter* strains were reported as organic solvent tolerant enzymes. For instance, the alkaline lipase of *Acinetobacter radioresistens* (CMC-1) exhibited activation in presence of 40% (v/v) dimethylsulfoxide and 20% (v/v) dimethylformamide (Hong and Chang 1998). The lipase of the benzene tolerant *Acinetobacterbaylyi* found to be highly resistance to many organic solvents especially benzene and isoamyl alcohol

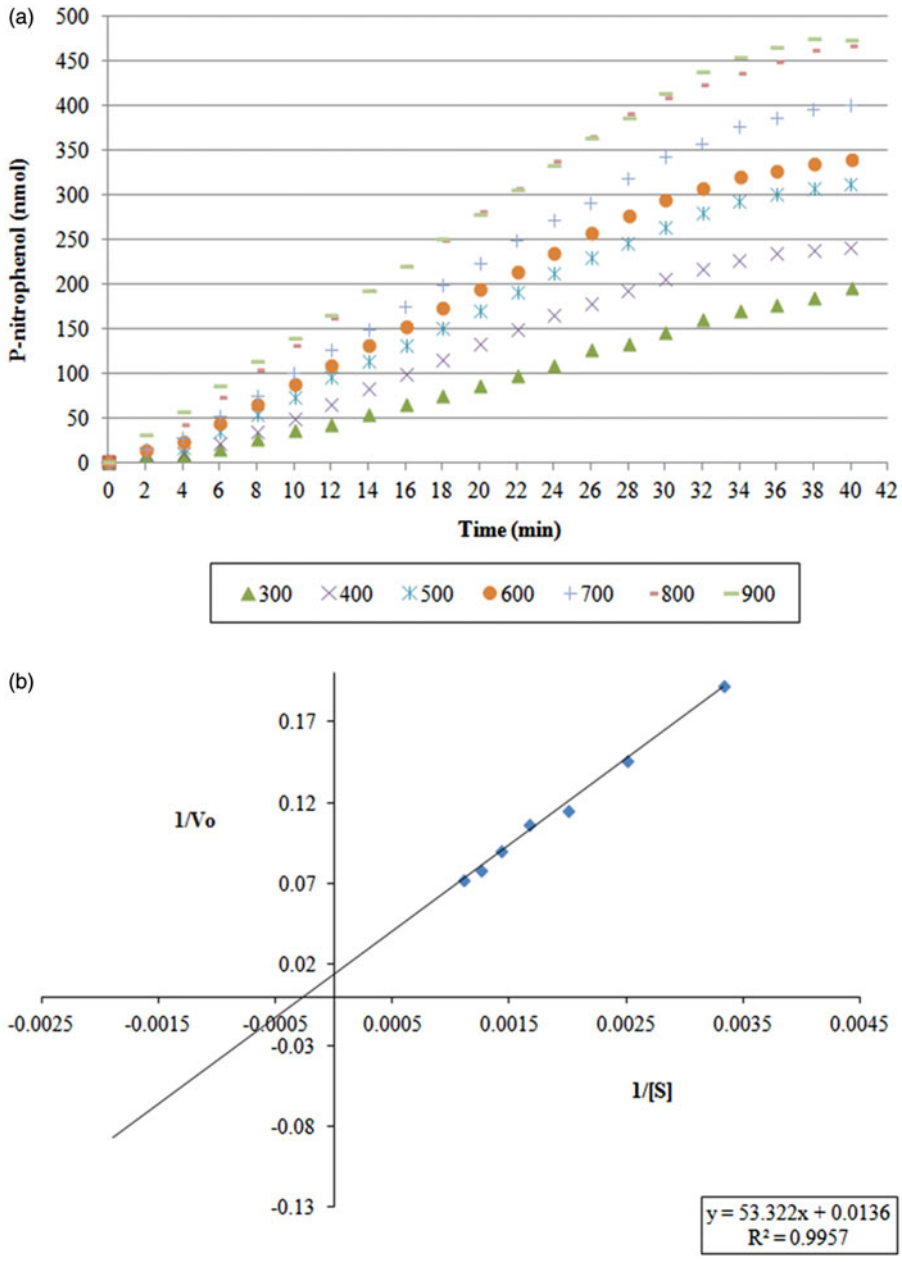

Figure 4. *p*-nitrophenol concentration (nmol) versus time graph obtained by measuring enzyme activity of the purified lipase at various concentrations of *p*-nitrophenyl laurate (pNPL) (300–900 μM) over 40 min.

(Uttatree et al. 2010). The lipase enzyme of *Acinetobacter* sp. reported by Khoramnia et al. (2011) shows increase in the activity in presence of methanol (114.7%), acetonitrile (105.9%), ethanol (153.4%), acetone (133.9%), 2-propanol (135.9%), ethyl acetate (111.4%) and hexane (114.0%).

Hydrophilic solvents, such as acetone are usually unsuited with enzyme activity because they strip off water from the enzymes, leading to the unfolding of the molecule with the exposure of the inner hydrophobic residues (Tsuzuki et al. 2003). Conversely, enzymes naturally stable in the presence of organic solvents and do not require special stabilization; they will be very useful industrially. The solvent-stable enzyme produced by *Acinetobacter* sp. K5b4 in this study will be very useful as catalysts for reactions in the presence of organic solvents.

3.2.4. The effect of pH on pure lipase activity and stability

The effects of the pH, on the activity of pure lipase produced by *Acinetobacter* sp. K5b4 were studied. The results (Figure 6(a)) showed that the pure lipase was

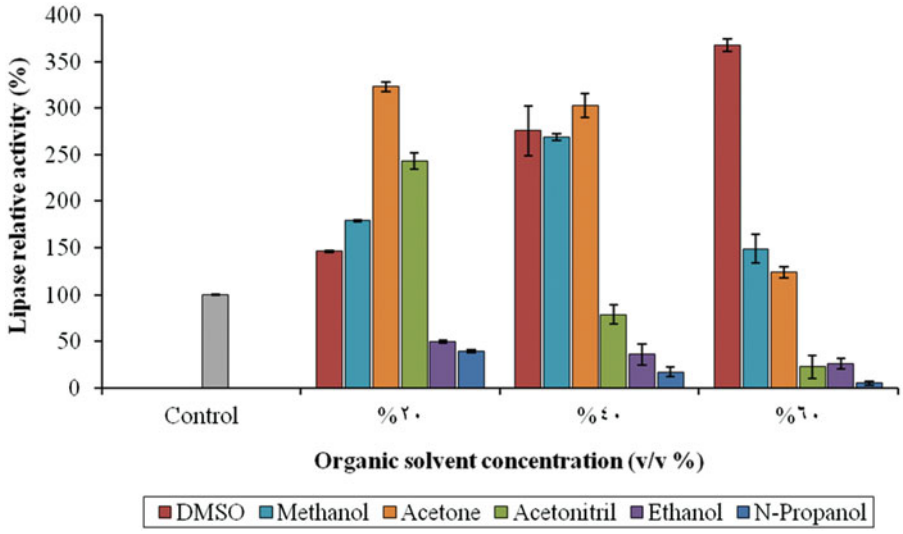

Figure 5. Relative activities was calculated based on the enzyme activity in the control experiment (in absence of organic solvent) being 100%.

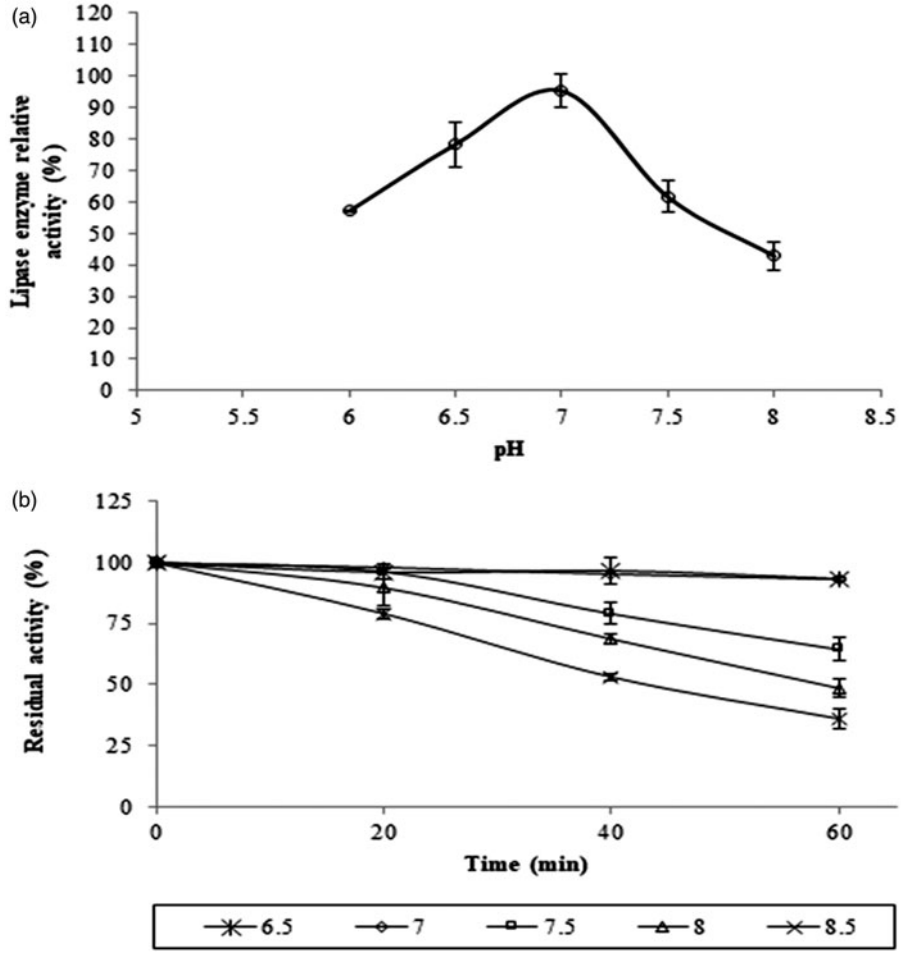

Figure 6. Effect of pH on *Acinetobacter* sp. K5b4 pure lipase enzyme (a) activity and (b) stability. Relative enzyme activity was calculated based on the maximum enzyme activity at the optimum pH being 100%. Residual activity was calculated based on the 0 time activity being 100%.

active at different pH environments ranging from pH 6.0 to pH 8.0. Maximal activity of the enzyme was obtained at pH 7.0. However, a few reports were exists for lipases from *Acinetobacter* strains that showed maximum activity at acidic pH such as the lipase produced by *Acinetobacter* sp. which shows maximum activity at

pH 6.0 (Khoramnia et al. 2011). Enzymes that showed their maximal activity at neutral pH (7.0) include lipase from *Acinetobacter calcoacetius* LP009 and *Acinetobacter* sp. ES-1 (Pratuangdejkul and Dharmsthiti 2000; Lee et al. 2006). However, several *Acinetobacter* lipases were reported with maximal activity at alkaline pH such as the lipase produced by *Acinetobacter radioresistens* CMC-1, *Acinetobacter* sp. KM109, *Acinetobacter* sp. RAG-1, *Acinetobacter lwoffii* 16C-1, *Acinetobacter species* SY-01, *Acinetobacter* sp., *Acinetobacter lwoffii* CR9, *Acinetobacter baumannii* BD5, *Acinetobacter* sp. EH28, *Acinetobacter baylyi*, *Acinetobacter johnsonii* LP28, *Acinetobacter* sp. XMZ-26 and *Acinetobacter venetians* V2 (Hong and Chang 1998; Mitsuhashi et al. 1999; Kim and Park 2002; Snellman et al. 2002; Han et al. 2003; Kasana et al. 2008; Saisubramanian et al. 2008; Park et al. 2009; Ahmed et al. 2010; Uttatree et al. 2010; Wang et al. 2011; Zheng et al. 2011; Kim et al. 2012).

Purified *Acinetobacter* sp. K5b4 lipase was able to retain 100% of the original activity after 1-h incubation at pHs 6.5 and 7.0. On the other hand, the enzyme activity was drastically minimized at pHs 7.5, 8.0 and 8.5 by 64.3, 48.5 and 36%, respectively after 1 h incubation (Figure 6(b)). Generally speaking *Acinetobacter* lipases showed stability at extensive range of pH values such as the lipases produced by *Acinetobacter* sp. KM109 (pH range of 6–8) (Mitsuhashi et al. 1999), *Acinetobacter calcoacetius* LP009 (pH range of 4.0–8.0) (Pratuangdejkul and Dharmsthiti 2000), *Acinetobacter* sp. RAG-1 (pH range of 5.8–9.0) (Snellman et al. 2002), *Acinetobacter species* SY-01 (pH range of 9–11) (Han et al. 2003), *Acinetobacter baylyi* (pH range of 6.0–9.0) (Uttatree et al. 2010), *Acinetobacter johnsonii* LP28 (pH range of 8.0–11.0) (Wang et al. 2011), *Acinetobacter* sp. (pH range of 6–11) (Khoramnia et al. 2011).

3.2.5. The effect of temperature on the pure enzyme activity and stability

The effect of incubation temperature on *Acinetobacter* sp. K5b4 pure lipase activity showed that the enzyme was active at cramped area of temperature values ranging from 24 to 35 °C (Figure 7(a)). Maximum hydrolysis activity of the pure enzyme detected at 27 °C. However, various *Acinetobacter* strains lipases were stated to have optimal temperatures ranged from 15 to 60 °C. These includes *Acinetobacter* sp. KM109 (45 °C) (Mitsuhashi et al. 1999), *Acinetobacter calcoacetius* LP009 (50 °C) (Pratuangdejkul and Dharmsthiti 2000), *Acinetobacter* sp. RAG-1 (55 °C) (Snellman et al. 2002), *Acinetobacter lwoffii* 16C-1 (37 °C) (Kim and Park 2002), *Acinetobacter* species SY-01 (50 °C) (Han et al. 2003), *Acinetobacter* sp. ES-1, (40 °C) (Lee et al. 2006), *Acinetobacter* sp. (37 °C) (Saisubramanian et al. 2008), *Acinetobacter* sp. (40 °C) (Kasana et al. 2008), *Acinetobacter baumannii* BD5 (35 °C) (Park et al. 2009), *Acinetobacter* sp. EH28 (50 °C) (Ahmed et al. 2010), *Acinetobacter baylyi* (60 °C) (Uttatree et al. 2010), *Acinetobacter johnsonii* LP28 (30 °C) (Wang et al. 2011), *Acinetobacter* sp. XMZ-26 (15 °C) (Zheng et al. 2011), *Acinetobacter* sp. (45 °C) (Khoramnia et al. 2011), *Acinetobacter* sp. XMZ-26 (55 °C) (Zheng et al. 2012) and *Acinetobacter venetians* V28 (40 °C) (Kim et al. 2012).

Thermostability test showed that the pure lipase enzyme of *Acinetobacter* sp. K5b4 was unstable over all the studied temperatures. The residual activity of the pure enzyme after 1 h incubation at 30, 35, 40, 45, 50 and 55 °C calculated to be 80.5, 71.6, 65.6, 52.1, 33.3 and 15.6%, respectively (Figure 7(b)). Regarding thermostability, most of the published reports showed that the most lipase enzymes produced by *Acinetobacter* strains either to be thermostable enzymes or cold active lipases. Thermostable enzymes includes the lipases produced by *Acinetobacter* sp. RAG-1 (70 °C) (Snellman et al. 2002), *Acinetobacter calcoacetius* LP009 (45 °C) (Pratuangdejkul and Dharmsthiti 2000), *Acinetobacter* sp. EH28 (50 °C) (Ahmed et al. 2010), *Acinetobacter baylyi* (60–80 °C) (Uttatree et al. 2010), *Acinetobacter johnsonii* LP28 (20–50 °C) (Wang et al. 2011) and *Acinetobacter* sp. (70 °C) (Khoramnia et al. 2011). Lipases produced by *Acinetobacter* strains with medium to low temperature stability were includes *Acinetobacter* sp. KM109 lipase that was stable at or below 35 °C (Mitsuhashi et al. 1999), *Acinetobacter baumannii* BD5 cold adapted lipase that maintained 28% of the original activity at 35 °C and full activity at 0 °C (Park et al. 2009) and the cold adapted lipase of *Acinetobacter* sp. XMZ-26 lipase that remained most of its activity between 5 °C and 35 °C (Zheng et al. 2011).

3.2.6. Effect of metals on the purified enzyme activity

The enzyme activity was enhanced (0–36%) by KCl, $BaCl_2$, $MgCl_2$ and $CaCl_2$ while obviously inhibited (10–20%) by$CoCl_2$, $ZnCl_2$, $MnCl_2$ and $CuCl_2$ (Figure 8). The effect of metal ions on lipase activity of *Acinetobacter* was studied by several researchers. The previous results showed neither perfect agreement nor disagreement with our results. They showed that the effects of these metals on the different *Acinetobacter* lipases are either dependent or

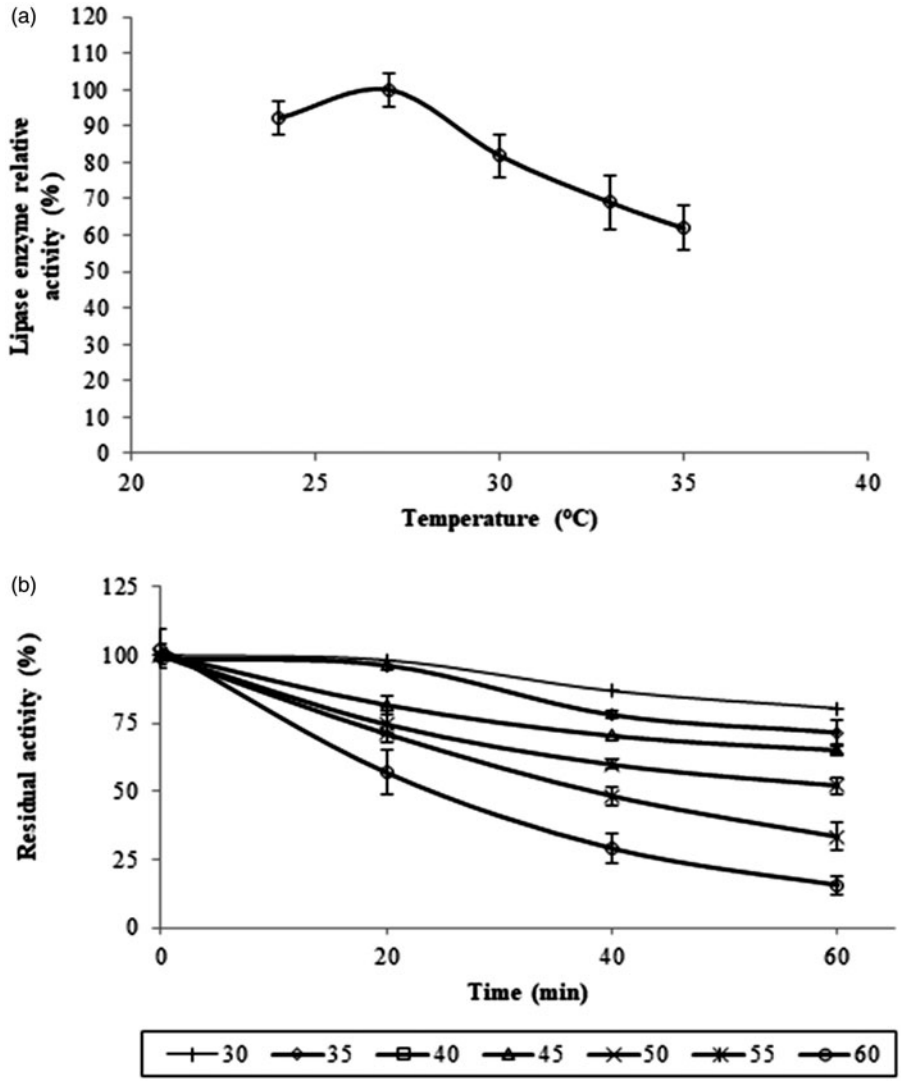

Figure 7. Effect of incubation temperature on *Acinetobacter* sp. K5b4 pure lipaseenzyme (a) activity and (b) stability.Relative enzyme activity was calculated based on the maximum enzyme activity at the optimum temperature being 100%.Residual activity was calculated based on the 0 time activity being 100%.

independent on the enzyme nature and the *Acinetobacter* species studied. For example, *Acinetobacter lwoffii* CR9 lipase was enhanced in the presence of Cu^{+2}, Mo^{+2}, Mg^{+2}, Zn^{+2}, whereas Ca^{+2} had inhibitory effect (Kasana et al. 2008), *Acinetobacter baumannii* BD5 lipase was activated by Ca^{+2}, Mg^{+2} and Mn^{+2}, whereas Zn^{+2} and Cu^{+2} inhibited it (Park et al. 2009), *Acinetobacter baylyi* lipase activity was completely inhibited in the presence of Fe^{2+}, Mn^{2+}, whereas the activity was enhanced by Mg^{2+} and no significant effect was found in the presence of Ca^{2+} and Li^{+} (Uttatree et al. 2010), *Acinetobacter johnsonii* Lp28 enzyme activity was promoted in the presence of Na^{+}, Ca^{2+}, K^{+}, Mg^{+2}. On the other hand, Ba^{+2}, Mn^{+2}, Cr^{+3} and Co^{+2} did not affect the enzyme activity. Whereas, the presence of Al^{+3}, Cu^{+2}, Fe^{+2}, Fe^{+3} and Zn^{+2} were reduced the enzyme activity (Wang et al. 2011), *Acinetobacter* sp. XMZ-26 lipase activity was enhanced by the presence of Ca^{+2}, Mn^{+2} and Ba^{+2} (Zheng et al. 2012).

3.2.7. Effect of various detergents and chemical agents on the purified lipase activity

The effect of the 1.0 and 5 mM of 2-Mercaptoethanol, EDTA and SDS on the lipolytic activity was negligible (Figure 9). At 5.0 mM concentration, the enzyme still resistance to the presence of 2-Mercaptoethanol and EDTA maintained 100% of the activity compared to the control. The resistance of the lipase enzyme to the action of the reducing agents 2-Mercaptoethanol which favor an S–S to –SH interchange suggests that the enzyme is not containing disulfide linkages important for their catalytic activity. In the presence of

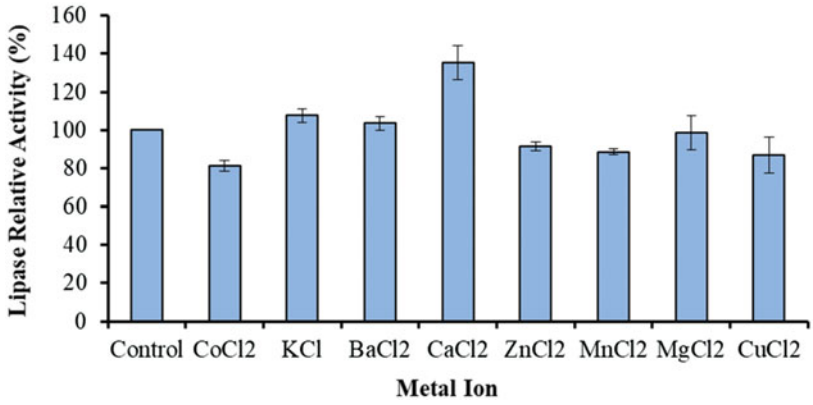

Figure 8. Effects of different metal ions on the hydrolysis activity of *Acinetobacter* sp. K5b4 pure lipase enzyme.

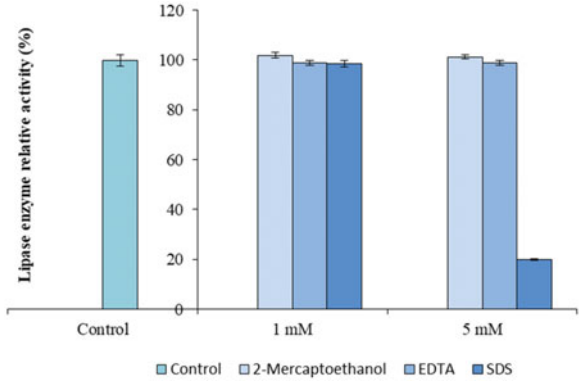

Figure 9. Pure lipase enzyme relative activities in presence of 1 and 5 mM of 2-Mercaptoethanol, EDTA and SDS.

1.0 and 5.0 mM of the metal chelating agent EDTA the enzyme showed high stability which indicating that the enzyme is not a metalloenzyme (Ping et al. 2018). On the other hand, high reduction in the enzyme activity was observed in the presence of 5.0 mM of the anionic surfactant SDS that reached to 80% decrease in the enzyme hydrolysis activity.

4. Conclusion

Biotechnological applications based on enzymatic biotransformation were endlessly growing sector in biotechnology. Various applications of lipases demand a compatible biocatalyst such as organic solvent tolerant enzymes. Water miscible organic solvents are highly denaturing and at the same time are advantageous reaction media. Hydrophilic organic solvent tolerant lipases are an interesting biocatalyst. *Acinetobacter* sp. K5b4 lipase enzyme being remarkably hydrophilic organic solvent tolerant enzyme can be potentially an excellent biocatalyst in organic synthesis in water miscible organic solvents as reaction media. The enzyme was not only able to maintain its activity but also exhibited enhancement in presence of up to 60% (v/v) of methanol, DMSO and acetone. Further application of this enzyme in organic synthesis of fine chemicals and its enantioselectivity is highly recommended in future research.

Disclosure statement

The authors declare no commercial or financial conflict of interest.

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