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ORIGINAL ARTICLE

Influence of cultivation conditions on the production of a thermostable extracellular lipase from *Amycolatopsis mediterranei* DSM 43304

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Abstract Among several lipase-producing actinomycete strains screened, *Amycolatopsis mediterranei* DSM 43304 was found to produce a thermostable, extracellular lipase. Culture conditions and nutrient source modification studies involving carbon sources, nitrogen sources, incubation temperature and medium pH were carried out. Lipase activity of 1.37 ± 0.103 IU/ml of culture medium was obtained in 96 h at 28°C and pH 7.5 using linseed oil and fructose as carbon sources and a combination of phytone peptone and yeast extract (5:1) as nitrogen sources. Under optimal culture conditions, the lipase activity was enhanced 12-fold with a twofold increase in lipase specific activity. The lipase showed maximum activity at 60°C and pH 8.0. The enzyme was stable between pH 5.0 and 9.0 and temperatures up to 60°C. Lipase activity was significantly enhanced by Fe^{3+} and strongly inhibited by Hg^{2+}. Li^{+}, Mg^{2+} and PMSF significantly reduced lipase activity, whereas other metal ions and effectors had no significant effect at 0.01 M concentration. *A. mediterranei* DSM 43304 lipase exhibited remarkable stability in the presence of a wide range of organic solvents at 25% (v/v) concentration for 24 h. These features render this novel lipase attractive for potential biotechnological applications in organic synthesis reactions.

Keywords *Amycolatopsis mediterranei* · Screening · Organic solvent-tolerant · Thermostable · Lipase

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Introduction

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that catalyze the hydrolysis of triacylglycerol at the oil–water interface to release glycerol and free fatty acids. They are produced by animals, plants and microorganisms [57, 65]. Microbial lipases recently have attracted considerable attention owing to their biotechnological potential, ranging from the use in laundry detergents to stereospecific biocatalysis [25, 26]. They are finding increasing uses as food and other industrial processing aids; thus, there is growing interest in discovering new sources of these enzymes with appropriate characteristics to suit particular applications [2]. The majority of commercially produced lipases originate from fungi (genera *Rhizomucor*, *Rhizopus* and *Candida*) and bacteria (genera *Pseudomonas* and *Chromobacterium*) [57, 65], and exploration of new applications broadens lipase research toward as yet unexplored microbial sources of lipase [32].

In recent times, the prospect of lipase catalysis in organic solvents with its associated advantages has received widespread attention [16, 22]. Lipases are widely used in biocatalysis due to their ability to catalyze not only the hydrolysis of triacylglycerides in aqueous solutions, but also enantioselective synthetic reactions in organic media. Lipases must be reasonably thermostable and maintain their activity in organic solvents if they are to be used in organic solvents for synthetic reactions [33]. Among the large number of lipases described in the literature, only the enzymes belonging to a narrow range of species have been shown to have adequate stability and biosynthetic capabilities to allow routine use in organic reactions [8]. Currently known microbial lipases do not have the desirable combination of thermostability and stability in both hydrophobic and hydrophilic organic solvents. This has

stimulated the search for new lipases [33]. In view of this, access to a wider range of lipase types with properties suitable for different applications would be beneficial [9].

Actinomycetes are filamentous saprophytic Gram-positive bacteria ubiquitous in soil, with a high GC content genomes [5]. Although actinomycetes were recognized through their exogenous lipolytic activities more than a decade ago [65], lipases from actinomycetes have not been studied as intensively as those from some other bacteria [26]. The available genome sequence databases for actinomycetes predict a large number of genes encoding enzymes of different lipolytic activities. Although these data indicate the potential for the synthesis of a broad range of lipolytic enzymes, only a few have been studied and reported so far [5]. The present work was undertaken in this context. Eighteen actinomycete strains were screened with the objective of finding a lipase with both reasonable thermostability and stability in a broad range of organic solvents. Early in the work, the lipolytic activity produced by *A. mediterranei* DSM 43304 showed highly interesting properties. The present work aimed to improve culture conditions to enhance lipase production from *A. mediterranei* DSM 43304 and to characterize the enzyme to assess its potential for use in biocatalysis.

Materials and methods

Materials

Analytical reagent grade chemicals were purchased from commercial sources at the highest purity. Unless otherwise mentioned, all culture media and chemicals used were from Sigma (Dublin, Ireland). Phytone peptone was obtained from BBL Microbiology Systems (Cockeysville, MD, USA) and Bacto-peptone was obtained from Difco Laboratories (Detroit, MI, USA). Natural oils were purchased from the local retail outlets in Dublin.

Microorganisms

Actinomycete strains were obtained from Divisional Culture Collection, School of Biology, Newcastle University, UK.

Maintenance of microorganisms

Actinomycete strains were grown on GYM agar slants (g/l: glucose 4.0 g; yeast extract 4.0 g; malt extract 10 g; $CaCO_3$ 2.0 g; agar 12.0 g; pH 7.2). The working stock cultures were maintained and stored on GYM slants at 4°C.

Culture conditions

The composition (g/l) of basal medium used was $NaNO_3$ 0.5 g, KCl 0.5 g, $MgSO_4 7H_2O$ 0.5 g, KH_2PO_4 2.0 g, yeast extract 1.0 g and Bacto-peptone 5.0 g. The pH was adjusted to 7.2 with 1 M NaOH or 1 M HCl. Then, 1.0% (v/v) olive oil was added. Media were sterilized for 15 min at 121°C at 15 psi. Submerged microbial cultures were incubated in 250 ml Erlenmeyer flasks containing 50 ml of basal medium with 5 ml inoculum on a rotary shaker (130 rpm) at 28°C.

Rhodamine B agar screening

The primary screening for the detection of lipolytic activity was carried out on rhodamine B agar (RBA) as described by Kouker and Jaeger [29] with some modifications. The growth medium containing 0.9% (w/v) peptone water, 0.25% (w/v) of yeast extract, 2% (w/v) of agar, was adjusted to pH 7.2, autoclaved and cooled to 60°C. Then, filter-sterilized rhodamine B stock solution (1.0 mg/ml) in distilled water was added to a substrate lipoidal emulsion to yield a final concentration of 0.001% (w/v). The substrate lipoidal emulsion consisted of 1.5% (w/v) olive oil with 0.25% (v/v) Tween 80 in distilled water that was sterilized by autoclaving. The resulting mixture of lipoidal emulsion with growth medium (1:10) was vigorously stirred to emulsify for 15 min. The medium was allowed to stand for 10 min at 60°C to reduce foaming before pouring 20 ml of medium into plastic petri plates. Fresh RBA plates were spot inoculated with 72 h-old actinomycete cultures in GYM broth (g/l: glucose 4.0 g; yeast extract 4.0 g; malt extract 10 g; pH 7.2) and incubated at 28°C for 6 days. The plates with visible growth were UV irradiated (350 nm). Lipase production was identified as orange fluorescence under UV light.

Screening in submerged fermentation broth

For screening in submerged cultivation conditions, 50 ml of basal medium in 250 ml Erlenmeyer flasks was inoculated with 5 ml, 72 h-old actinomycete culture in GYM broth and incubated at 28°C on a reciprocal shaker (130 rpm). After 96 h, samples were processed for lipase activity assay. One ml of culture was centrifuged at 10,000*g*, at 4°C, for 10 min to obtain a cell-free supernatant. The clear supernatant was filtered through a 0.2 μm filter before lipase activity assay.

Spectrophotometric *p*-NPP assay

Lipase activity was quantitatively assayed in cell-free supernatants using *p*-nitrophenyl palmitate (*p*-NPP) as

substrate. This assay was performed as described by Winkler and Stuckman [67] with some modifications. A stock solution of *p*-NPP was freshly prepared in 2-propanol at a concentration of 0.3% (w/v). This solution (Solution A) was subjected to 3 min sonication (135 W, 42 kHz, Branson 5510E-MT). Then, 900 µl of 1:20 dilution of the substrate stock solution A in solution B (0.1% (w/v) gum arabic and 0.4% (v/v) Triton X100 in distilled water) with 50 µl of appropriate buffer was preincubated for 2 min at the assay temperature before adding 50 µl of enzyme sample. This mixture was incubated at the assay temperature for 10 min, and the reaction was terminated by the addition of 2 ml of 0.2 M Na_2CO_3 solution. Released *p*-nitrophenol (*p*-NP) was immediately determined by measuring the absorbance at 410 nm in a Unicam UV–VIS spectrophotometer (Model UV2 2000E, Cambridge, UK). Appropriate blanks were used to subtract the absorbance corresponding to the reaction mixture other than that produced by the specific hydrolysis of *p*-NPP. The molar extinction coefficient of *p*-NP ($\varepsilon_{410nm} = 16{,}900\ M^{-1}\ cm^{-1}$) was estimated from the absorbance of standard solutions of *p*-NP. One international unit of lipase activity was expressed as the amount of enzyme liberating 1 µM of *p*-NP per minute under the conditions of the assay.

Biomass concentration analysis

After centrifugation at 10,000*g*, at 10°C, for 10 min and washing the pellet in 0.9% (w/v) NaCl solution, the pellet of 5 ml suspension sample was dried to a constant weight at 80°C for 48 h, and the dry biomass weight was determined gravimetrically.

Statistical analysis

Data were analyzed using analysis of variance. In all these cases, the analyses were conducted using SPSS (version 15.0) using the procedure of general linear model (Tukey test). The level of tested significance was at $P \leq 0.05$.

Time course of lipase production by *A. mediterranei* DSM 43304

Time course of lipase production was studied in the basal medium using shake flask cultures. A 10% (v/v) of 72 h-old inoculum grown in GYM broth was added to 50 ml medium, in a 250 ml Erlenmeyer flask and incubated at 130 rpm on a rotary shaker, at 28°C. Samples were analyzed at 24 h intervals to determine pH, dry biomass and lipase activity in the culture supernatant. The effects of cultivation conditions in shake flask experiments are commonly investigated by subjecting the microorganism to different environmental conditions [68]. In the present work, the effect of initial pH and incubation temperature on the culture was studied using shake flask cultures at different temperatures (20–45°C) and initial values of pH (5.0–9.0). The effect of inoculum size on lipase production was investigated by varying culture inoculum size from 2 to 12% of total volume. All experiments were carried out at least in triplicate.

Nutritional factors affecting lipase production by *A. mediterranei* DSM 43304

The general procedure for cultivation was as follows: 10% (v/v) of 72 h-old inoculum grown in GYM broth was inoculated into 50 ml of culture medium and incubated at 28°C for 96 h. The culture was harvested by centrifugation at 10,000*g*, 4°C for 10 min. The cell-free supernatant was filtered (0.2 µm filter, Millipore) before spectrophotometric determination of lipase activity. Each experiment was carried out in triplicate.

Effect of inducers on lipase production

To determine the effects of substrate related compounds, the olive oil in the basal medium was substituted with natural oils (jojoba, corn, cottonseed, grapeseed, groundnut, linseed, rapeseed, soybean and sunflower) at 1.0% (v/v) concentration.

Effect of surfactants on lipase production

The following detergents were added to the basal medium as lipase inducers, replacing olive oil, at 0.5% (w/v) concentration: Span 40, Span 65, Span 80, Tween 20, Tween 21, Tween 40, Tween 80 and Triton X100.

Effect of carbon source additives on lipase production

In order to elucidate the effect of carbon source additives on lipase production, olive oil in the basal medium was combined with 1.0% (w/v) of the following sugars: arabinose, dextrin, fructose, galactose, glucose, lactose, mannitol, maltose, maltotetraose, mannose, raffinose, rhamnose, sorbitol, sucrose, starch and xylose.

Effect of organic nitrogen sources on lipase production

To assess the effects of organic nitrogen source in combination with yeast extract on lipase production, Bacto-peptone in the basal medium was substituted with phytone peptone, yeast extract, corn steep liquor, beef extract, skim milk, wheat peptone, fish peptone, tryptone, casein hydrolysate, casein and wheat gluten, each at a concentration of 0.5% (w/v). The influence of the best organic nitrogen

source and yeast extract in the basal medium was also tested by varying their concentrations in the basal medium.

Effect of inorganic nitrogen sources on lipase production

In order to assess the effects of inorganic nitrogen sources on lipase production, $NaNO_3$ in the basal medium was substituted with: NH_4Cl, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$, CH_3COONH_4, NH_4NO_3 and urea, each at 0.05% (w/v).

Effect of metal ions on lipase production

Metal ions individually and in combination were tested for their effects on optimal lipase production in basal medium. Mg^{2+}, Ca^{2+} and Fe^{3+} salts were incorporated into the basal medium at 0.05% (w/v) concentration. Also combinations of Mg^{2+} and Ca^{2+}, Ca^{2+} and Fe^{3+}, and Mg^{2+} and Fe^{3+} were added at individual final concentration of 0.025% (w/v) to assess their combined effect on lipase production.

Characterization of *A. mediterranei* DSM 43304 lipase

Gel electrophoresis and activity detection

SDS–Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in 15% (w/v) gels according to Laemmli [30] at room temperature (ATTO AE-6450 mini slab-gel unit, Tokyo, Japan). In order to allow the detection of activity after the electrophoresis, the samples were not heated to 100°C. Calibration for molecular mass was made using the following molecular weight markers: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Zymographic analysis was performed, essentially as described by Prim et al. [51]. After the run, gel was soaked for 30 min in 2.5% Triton X-100 at room temperature, briefly washed in 50 mM phosphate buffer, pH 7.0 and covered by a solution of 100 μM methylumbeliferyl butyrate (MUF-butyrate). This solution had been previously prepared by dissolving 2.46 mg of MUF-butyrate in 1 ml of ethyleneglycol monomethylether to which was then added 100 ml of 50 mM phosphate buffer, pH 7.0. Activity bands became visible in a short time (~1 min) under UV illumination. Following zymogram analysis, the same SDS–PAGE was silver stained to visualize protein bands.

Effect of temperature on activity and stability

Lipase activity was measured at various temperatures (30–80°C) under standard assay conditions. Thermal stability of the enzyme was investigated by preincubating the enzyme at various temperatures (30–80°C) for 3 h. The samples were then assayed for residual lipase activity under standard assay conditions.

Effect of pH on activity and stability

The effect of pH on lipolytic activity was determined in the following buffers (all at 50 mM): HCl–KCl (pH 2.0), sodium citrate (pH 3.0), succinate-NaOH (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris–HCl (pH 8.0 and 9.0) and glycine-NaOH (pH 10.0). The optimum pH obtained was used for the investigation of thermostability and other parameters. These buffers were used to determine pH stability of the crude lipase preparation. A mixture (1:1) of crude enzyme and buffers (0.1 M) was incubated for 24 h at 4°C, and the standard enzyme assay described previously was performed. The residual activities were calculated by comparison with the activity in 50 mM Tris–HCl buffer, pH 8.0, without preincubation.

Effect of organic solvents on lipase stability

The effect of various polar and non-polar organic solvents with different log *P* values on crude lipase stability was investigated. One ml of organic solvent was added to 3.0 ml of cell-free supernatant and incubated at 30°C, while shaking at 200 rpm for 24 h to ensure the continuous mixing of enzyme and the solvents. The enzyme stability was expressed as the remaining activity relative to the control without solvent.

Effect of metal ions and effector molecules on lipase stability

The effect of metal ions and effector molecules on lipase activity was studied at pH 8.0 by incubating the enzyme in presence of 1 mM of metal ions (Ag^{+}, Ba^{2+}, Co^{2+}, Li^{+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+} and Hg^{2+}) and effectors (PMSF, EDTA, SDS, NH_4^{+} and urea). Incubation was carried out at 60°C for 10 min and assayed for lipase activity. Residual lipase activity was calculated as a percentage of that without metal ions/effectors.

Results

Screening of strains for lipase production

Figure 1 shows rhodamine B agar screening for lipase production by actinomycete strains. Except for *Amycolatopsis coloradensis* DSM 44225 and *Streptomyces aureoverticillatus* NRRL B-3326, all actinomycete strains

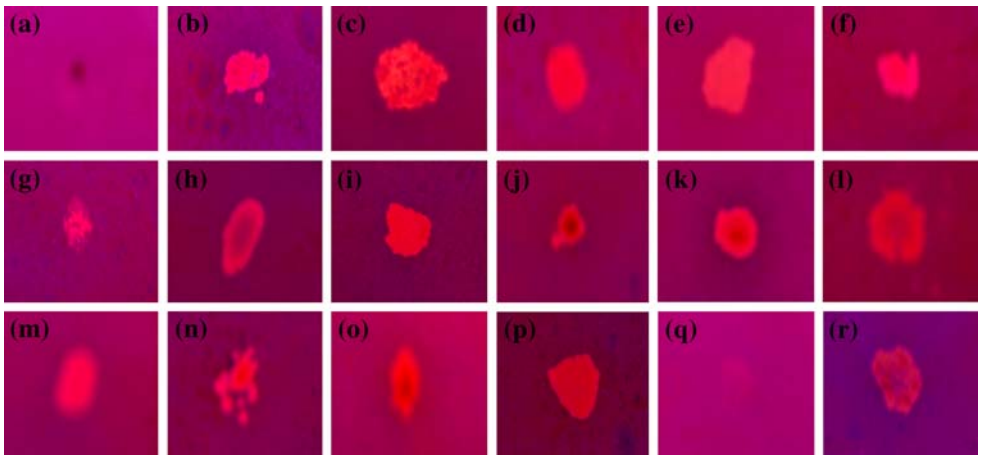

Fig. 1 Screening for lipolytic actinomycete strains on rhodamine B agar. **a** *Amycolatopsis coloradensis* DSM 44225; **b** *A. amakusaensis* NRRL B-3351; **c** *A. fastidiosa* DSM 43855; **d** *A. mediterranei* DSM 43304; **e** *A. rubida* DSM 44637; **f** *A. sulphurea* DSM 46092; **g** *Nocardia araoensis* DSM 44729; **h** *N. higoensis* DSM 44732; **i** *N. kruckzakiae* DSM 44877; **j** *Streptomyces amquistii* NRRL B-1685, **k** *S. griseus* subsp. *griseus* DSM 40236; **l** *S. coelicolor* A3(2); **m** *S. annulatus* NRRL B-2000; **n** *S. arabicus* NRRL B-1733; **o** *S. aurantiogriseus* NRRL B-5416; **p** *S. rochei* DSM 40231; **q** *S. aureoverticillatus* NRRL B-3326; and **r** *S. althioticus* NRRL B-3981

showed brilliant pink-red/orange fluorescence on UV irradiation of RBA plates. Lipase screening in basal medium using shake flask cultures showed the presence of lipolytic activity in cell-free supernatants prepared from actinomycete culture broths (Table 1). Of 18 actinomycetes strains, 7 showed more than 0.05 IU/ml at 96 h. Among these, *A. rubida* DSM 44637, *S. rochei* DSM 40231 and *S. griseus* subsp. *griseus* DSM 40236 produced the highest activities at 0.149 ± 0.017, 0.141 ± 0.004 and 0.116 ± 0.010 IU/ml, respectively. Significant lipolytic activities were also detected in cultures of *A. mediterranei* DSM 43304 (0.086 ± 0.012 IU/ml) and *A. sulphurea* DSM 46092 (0.062 ± 0.008 IU/ml).

Effect of culture conditions on lipase production by *A. mediterranei* DSM 43304

The preliminary characterization of extracellular lipases from actinomycete strains in terms of pH optimum, temperature optimum and thermostability identified the lipase from *A. mediterranei* DSM 43304 as the most thermostable (data not shown). Therefore, *A. mediterranei* DSM 43304 was selected for further study, and various culture parameters were investigated to improve lipase production.

Time course of lipase production

The time course of lipase synthesis in basal medium by *A. mediterranei* DSM 43304 was monitored by measurement of lipase activity, dry biomass and pH (Fig. 2). Substantial lipase production commenced at 24 h and reached a maximum at 96 h. Further incubation did not lead to an increase in lipase activity, but a slow decrease in lipase

Table 1 Lipase screening of actinomycete strains in basal medium

Strain	Rhodamine B agar*	Lipase activity (IU/ml)**
Amycolatopsis coloradensis DSM 44225	−	0.046 ± 0.001^{abc}
A. amakusaensis NRRL B-3351	+	0.059 ± 0.004^{bc}
A. fastidiosa DSM 43855	+	0.040 ± 0.002^{abc}
A. mediterranei DSM 43304	+	0.086 ± 0.012^{d}
A. rubida DSM 44637	+	0.149 ± 0.017^{f}
A. sulphurea DSM 46092	+	0.062 ± 0.008^{cd}
Nocardia araoensis DSM 44729	+	0.031 ± 0.002^{a}
N. higoensis DSM 44732	+	0.037 ± 0.001^{abc}
N. kruckzakiae DSM 44877	+	0.033 ± 0.003^{a}
Streptomyces amquistii NRRL B-1685	+	0.046 ± 0.001^{abc}
S. griseus subsp. *griseus* DSM 40236	+	0.116 ± 0.010^{e}
S. coelicolor A3(2)	+	0.033 ± 0.001^{a}
S. annulatus NRRL B-2000	+	0.051 ± 0.002^{abc}
S. arabicus NRRL B-1733	+	0.030 ± 0.001^{a}
S. aurantiogriseus NRRL B-5416	+	0.038 ± 0.006^{abc}
S. rochei DSM 40231	+	0.141 ± 0.004^{ef}
S. aureoverticillatus NRRL B-3326	−	0.034 ± 0.004^{ab}
S. althioticus NRRL B-3981	+	0.037 ± 0.004^{ab}

Data are means ± standard deviations of three determinations. Column data followed by the same superscript letter were not significantly different ($P \leq 0.05$; by Tukey test)

* The RBA plates, after 6 days incubation at 28°C, were exposed to UV (350 nm) to detect fluorescence: orange fluorescence (+); no orange fluorescence (−)

** Lipase activity in cell-free supernatant after 96 h growth in basal medium with 1% (v/v) olive oil as inducer

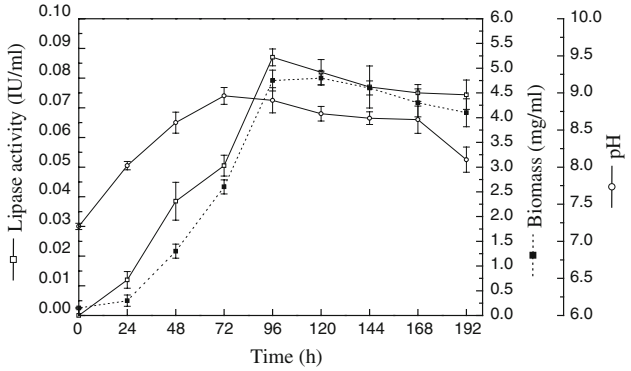

Fig. 2 Time course of lipase production by *A. mediterranei* DSM 43304. Basal medium was seeded with 10% (v/v) inoculum and incubated at 28°C for 192 h at 130 rpm. Samples were withdrawn at 24 h intervals to monitor biomass (*filled square*), pH (*open circle*) and lipase activity (*open square*) in cell-free supernatants

specific activity was observed (data not shown). There was a shift in pH from 7.20 ± 0.02 to 8.02 ± 0.057 during the first 24 h of incubation, rising to pH 8.96 ± 0.11 at 96 h. The maximum enzyme activity was observed when the biomass was the highest at 96–120 h and culture pH was around 8.0–9.0.

Effect of inoculum size on lipase production

In order to determine the effect of inoculum size on lipase production, inoculum in the range of 2–12% (v/v) was employed. Increased enzyme production was observed with the increase in inoculum size and showed maximum enzyme activity (0.105 ± 0.003 IU/ml) and biomass (5.8 ± 0.6 mg/ml) production at 96 h with 10% (v/v) inoculum (Table 2). Further increase in inoculum size resulted in decreased enzyme synthesis.

Table 2 Effect of inoculum level on lipase production

Inoculum volume (%, v/v)	Lipase (IU/ml)	Biomass (mg/ml)	Final pH
2	0.055 ± 0.005^{a}	0.7 ± 0.4^{a}	8.66 ± 0.07
4	0.064 ± 0.002^{ab}	1.0 ± 0.3^{a}	8.82 ± 0.06
6	0.079 ± 0.010^{abc}	1.5 ± 0.1^{a}	8.60 ± 0.08
8	0.085 ± 0.009^{bcd}	4.8 ± 0.4^{b}	8.76 ± 0.04
10	0.105 ± 0.003^{d}	5.8 ± 0.6^{b}	8.77 ± 0.03
12	0.102 ± 0.005^{cd}	5.6 ± 0.7^{b}	8.73 ± 0.04

The shake flask experiments were performed in basal medium for 96 h at 28°C, 130 rpm

Data are means ± standard deviations of three determinations. Column data followed by the same superscript letter were not significantly different ($P \leq 0.05$; by Tukey test)

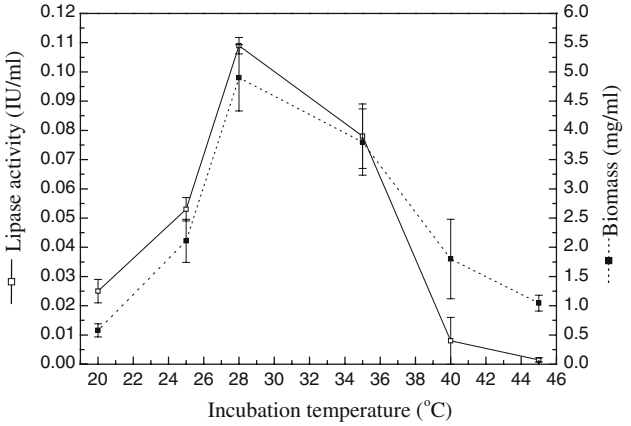

Fig. 3 Effect of incubation temperature on lipase production (*open square*) and biomass (*filled square*) during growth of *A. mediterranei* DSM 43304 in shake flask cultures after 96 h of incubation at temperatures varying from 20 to 45°C

Effect of incubation temperature on lipase production

Submerged fermentation was carried out at 20–45°C with 10% (v/v) inoculum for 96 h to evaluate the effect of incubation temperatures on growth and enzyme production. Maximum enzyme and biomass production was observed in the mesophilic range at 28–35°C (Fig. 3). Incubation at temperatures below 25°C and above 35°C significantly reduced enzyme and biomass production.

Effect of initial medium pH on lipase production

The initial pH of the culture broth was found to be one of the most critical environmental parameters affecting growth and enzyme production by *A. mediterranei* DSM43304. Maximum biomass and lipase production resulted at an initial medium pH of 7.5 (Fig. 4). Lipase

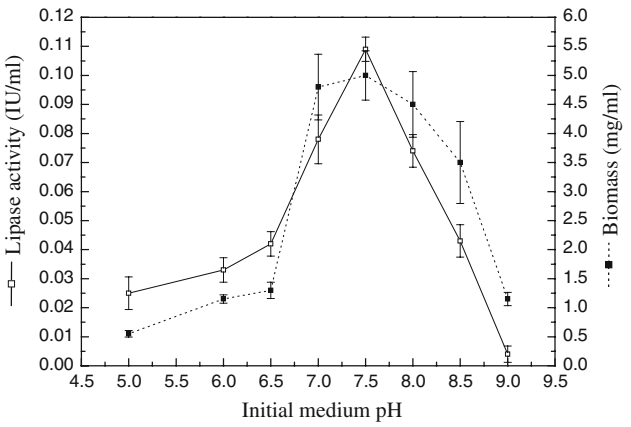

Fig. 4 Effect of initial pH on lipase production (*open square*) and biomass (*filled square*) in shake flask cultures after 96 h of cultivation with initial medium pH varying from 5.0 to 9.0

Table 3 Effect of inducers on lipase production

Inducer	Lipase (IU/ml)	Biomass (mg/ml)	Final pH
Natural oils (1.0%, v/v)			
Control*	0.081 ± 0.004^{f}	6.0 ± 2.8^{ab}	7.90 ± 0.03
Jojoba	0.071 ± 0.003^{ef}	4.0 ± 1.4^{a}	8.84 ± 0.07
Corn	0.052 ± 0.002^{cd}	4.5 ± 3.5^{ab}	8.71 ± 0.04
Cottonseed	0.060 ± 0.007^{de}	4.5 ± 0.7^{ab}	8.70 ± 0.28
Grapeseed	0.046 ± 0.001^{bc}	2.5 ± 0.7^{a}	8.67 ± 0.03
Groundnut	0.037 ± 0.001^{b}	4.5 ± 0.7^{ab}	8.64 ± 0.06
Linseed	0.128 ± 0.004^{h}	16.0 ± 1.4^{b}	7.01 ± 0.01
Rapeseed	0.013 ± 0.004^{a}	2.5 ± 0.7^{a}	8.66 ± 0.18
Soybean	0.014 ± 0.001^{a}	1.5 ± 0.7^{a}	8.70 ± 0.42
Sunflower	0.104 ± 0.004^{g}	11.5 ± 2.1^{bc}	7.81 ± 0.07
Surfactants (0.5%, w/v)			
Control*	0.125 ± 0.006^{e}	5.5 ± 0.1^{d}	7.26 ± 0.03
Span 40	0.031 ± 0.003^{ab}	1.6 ± 0.8^{ab}	8.71 ± 0.03
Span 65	0.034 ± 0.001^{b}	1.6 ± 0.1^{ab}	8.68 ± 0.04
Span 80	0.063 ± 0.002^{d}	4.7 ± 0.8^{cd}	8.10 ± 0.21
Tween 20	0.056 ± 0.003^{cd}	1.5 ± 0.7^{ab}	8.75 ± 0.03
Tween 21	0.040 ± 0.004^{bc}	0.4 ± 0.1^{a}	6.99 ± 0.01
Tween 40	0.053 ± 0.003^{cd}	1.2 ± 0.1^{ab}	8.74 ± 0.03
Tween 80	0.067 ± 0.007^{d}	3.2 ± 0.1^{bc}	8.54 ± 0.03
Triton X-100	0.016 ± 0.002^{a}	0.2 ± 0.1^{a}	7.26 ± 0.06

The shake flask experiments were performed for 96 h at 28°C, 130 rpm. Data are means ± standard deviations of three determinations. Column data followed by the same superscript letter were not significantly different ($P \leq 0.05$; by Tukey test)

* Control refers to the basal medium with 1% (v/v) olive oil as lipase inducer

activity and biomass decreased significantly at alkaline and acidic pH of 9.0 and 5.0, respectively.

Effect of inducers on lipase production

Natural oils as carbon sources had different effects on lipase production. The results (Table 3) indicate that all lipidic sources supported lipase production, ranging from 0.014 ± 0.001 to 0.128 ± 0.004 IU/ml. The highest lipase production (0.128 ± 0.004 IU/ml) and biomass (16.0 ± 1.4 mg/ml) was obtained with linseed oil followed by sunflower oil (0.104 ± 0.004 IU/ml). Among the natural oils tested, rapeseed and soybean oils gave the lowest enzyme activities and biomass. Different surfactants as lipase inducers in the medium did not enhance lipase production as compared to olive oil (Table 3).

Effect of carbon source additives on lipase production

Lipase production and biomass was significantly enhanced with the addition of sugars to the basal medium in most cases (Table 4). Addition of fructose gave the highest activity of 0.467 ± 0.007 IU/ml with biomass of 11.2 ± 0.9 mg/ml, whereas lactose produced the highest biomass of 14.9 ± 0.4 mg/ml with lipase activity of 0.399 ± 0.021 IU/ml. Lipase production increased three-fold with lactose, sorbitol, maltose and xylose as carbon source additives. Arabinose, rhamnose, sucrose and dextrin were found to be comparatively less adequate in enhancing lipase production, whereas the addition of starch significantly decreased lipase production.

Effect of nitrogen sources on lipase production

Lipase production and biomass was found to be highest with phytone peptone (0.131 ± 0.021 IU/ml) and yeast extract (0.104 ± 0.005 IU/ml) as organic nitrogen sources followed by Bacto-peptone (0.071 ± 0.002 IU/ml) and corn steep liquor (0.061 ± 0.006 IU/ml; Table 5). Significant biomass was produced with beef extract (8.4 ± 0.7 mg/ml) and tryptone (8.1 ± 0.9 mg/ml) as nitrogen source, but the lipase activities obtained were low. Wheat gluten gave the lowest lipase (0.017 ± 0.001 IU/ml) yield followed by casein and casein hydrolysate. Phytone peptone with yeast extract was identified as the best nitrogen sources for lipase production by *A. mediterranei* DSM 43304. No significant difference was observed in terms of lipase activities and biomass production by different concentrations of phytone peptone and yeast extract as organic nitrogen source in the basal medium (Table 5). The effect of different inorganic nitrogen sources showed $(NH_4)_2HPO_4$ as the best inorganic nitrogen source producing significant increase in lipase production (Table 6). Significantly reduced lipase activities were obtained with other inorganic nitrogen sources tested with urea showing the greatest decrease in lipase activity and biomass production.

Effect of metal ions on lipase production

The effect of different metal ions and metal ion combinations on lipase production is shown in Table 4. Metal ions did not make any significant difference to lipase activity and biomass production by *A. mediterranei* DSM 43304.

Lipase production in modified medium

The extent of improvement in lipase activity yield by fermentation using the optimal culture parameters was investigated. The modified culture medium for lipase production by *A. mediterranei* DSM 43304 consisted of 1% (v/v) linseed oil, 1% (w/v) fructose, 0.05% (w/v) $(NH_4)_2HPO_4$, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_4 7H_2O$, 0.2% (w/v) KH_2PO_4, 0.5% (w/v) phytone peptone, 0.1% (w/v) yeast extract, initial pH 7.5, and the culture was incubated at 28°C for 96 h. The lipase activity produced increased from 0.108 ± 0.002 IU/ml

Table 4 Effect of sugar additives and metal ions on lipase production

Sugar additive/metal ion	Lipase activity (IU/ml)	Biomass (mg/ml)	Final pH
Sugar additive (1.0%, w/v)			
Contol*	0.132 ± 0.007^{ab}	7.3 ± 0.5^{abc}	8.66 ± 0.01
Arabinose	0.158 ± 0.013^{abc}	4.6 ± 0.3^{a}	8.90 ± 0.08
Dextrin	0.194 ± 0.042^{bcd}	4.7 ± 0.2^{a}	8.57 ± 0.07
Fructose	0.467 ± 0.007^{j}	11.2 ± 0.9^{gh}	8.65 ± 0.04
Galactose	0.349 ± 0.007^{fghi}	12.7 ± 0.2^{hi}	8.81 ± 0.03
Glucose	0.317 ± 0.019^{efgh}	10.7 ± 0.1^{defgh}	8.69 ± 0.03
Lactose	0.399 ± 0.021^{hij}	14.9 ± 0.4^{i}	8.72 ± 0.03
Mannitol	0.343 ± 0.035^{fghi}	10.9 ± 0.6^{fgh}	8.64 ± 0.03
Maltose	0.424 ± 0.071^{ij}	8.2 ± 0.4^{bcdef}	8.79 ± 0.01
Maltotetraose	0.237 ± 0.007^{cde}	8.7 ± 0.6^{cdef}	8.49 ± 0.03
Mannose	0.266 ± 0.003^{defg}	8.2 ± 0.2^{bcde}	8.64 ± 0.03
Raffinose	0.350 ± 0.007^{ghi}	8.1 ± 0.5^{bcd}	8.71 ± 0.03
Rhamnose	0.211 ± 0.014^{bcd}	10.6 ± 0.2^{efgh}	8.73 ± 0.06
Sorbitol	0.410 ± 0.006^{hij}	9.7 ± 1.3^{cdefg}	8.66 ± 0.04
Sucrose	0.253 ± 0.014^{cdef}	10.2 ± 1.8^{defgh}	8.88 ± 0.03
Starch	0.078 ± 0.007^{a}	5.7 ± 0.1^{ab}	8.76 ± 0.03
Xylose	0.417 ± 0.008^{ij}	9.8 ± 0.5^{cdefg}	8.78 ± 0.04
Metal ions (%, w/v)			
Control**	0.096 ± 0.009^{a}	6.1 ± 0.4^{a}	8.70 ± 0.10
Fe^{3+} (0.05%)	0.109 ± 0.005^{a}	6.9 ± 0.5^{a}	8.62 ± 0.07
Ca^{2+} (0.05%)	0.102 ± 0.001^{a}	5.8 ± 0.4^{a}	8.75 ± 0.06
Mg^{2+} (0.025%) + Fe^{3+} (0.025%)	0.097 ± 0.003^{a}	4.8 ± 0.6^{a}	8.60 ± 0.07
Fe^{3+} (0.025%) + Ca^{2+} (0.025%)	0.104 ± 0.001^{a}	6.1 ± 1.8^{a}	8.82 ± 0.04
Mg^{2+} (0.025%) + Ca^{2+} (0.025%)	0.092 ± 0.004^{a}	5.8 ± 0.3^{a}	8.76 ± 0.03

The shake flask experiments were performed for 96 h at 28°C, 130 rpm. Data are means ± standard deviations of three determinations. Column data followed by the same superscript letter were not significantly different ($P \leq 0.05$; by Tukey test)

* Control refers to the basal medium in the absence of any sugar additive

** Control with 0.05% (w/v) of Mg^{2+} in the basal medium

(3.80 ± 0.14 IU/mg) in the basal medium to 1.372 ± 0.103 IU/ml (7.27 ± 0.52 IU/mg) in the modified medium. Thus, a 12-fold increase in lipase activity yield and twofold increase in lipase specific activity were achieved by employing the newly formulated production medium.

Characterization of *A. mediterranei* DSM 43304 lipase

Gel electrophoresis and activity detection

After zymogram analysis of the crude culture medium, there was only one band with lipase activity, suggesting that *A. mediterranei* DSM 43304 secretes only one enzyme with activity against MUF-butyrate into the culture medium. The molecular weight of the protein with activity was estimated as ~33 kDa (Fig. 5). These results strongly suggested the presence of a single enzyme responsible for lipolytic activity in the crude extract, which validates the use of crude medium as enzyme source.

Effect of temperature on activity and stability

The effect of temperature on the activity and stability of lipase is shown in Fig. 6. The lipase from *A. mediterranei* DSM 43304 was found to be highly thermostable with a temperature optimum of 60°C at pH 8.0. It retained 90% of activity at 60°C after 3 h incubation and had a half-life of more than 30 min at 70°C.

Effect of pH on activity and stability

The lipase was found to be quite active over the pH range 5.0–8.0 (Fig. 7). The lipolytic activity was maximum at pH 8.0 and showed retention of 88, 98, 95 and 89% activity at pH 5.0, 6.0, 7.0 and 9.0, respectively. The pH stability profile showed the highest stability at pH 8.0–9.0 with 96% residual activity. The activity was also stable at pH 6.0–7.0 with 94% residual activity. The lipase remained stable even in the pH range 2.0–4.0 with more than 50% residual activity after 24 h.

Effect of organic solvents on lipase stability

The lipase exhibited considerable stability in the presence of hydrophilic organic solvents (log $P < 0.3$) as well as hydrophobic organic solvents (log P 0.85–6.6) with significant activation in most cases (Table 6). After 24 h, the highest activation was in benzene and toluene with residual

Table 5 Effect of nitrogen sources on lipase production

Nitrogen source	Lipase activity (IU/ml)	Biomass (mg/ml)	Final pH
Organic nitrogen source (0.5%, w/v)			
Control*	0.071 ± 0.002^{d}	6.6 ± 1.9^{cd}	8.84 ± 0.03
Phytone peptone	0.131 ± 0.021^{e}	8.8 ± 0.4^{d}	8.72 ± 0.01
Yeast extract	0.104 ± 0.005^{e}	9.1 ± 0.1^{d}	8.69 ± 0.03
Corn steep liquor	0.061 ± 0.006^{cd}	7.6 ± 0.6^{d}	8.77 ± 0.03
Beef extract	0.046 ± 0.004^{bcd}	8.4 ± 0.7^{d}	8.90 ± 0.17
Skim milk	0.042 ± 0.001^{abc}	4.7 ± 0.1^{bc}	8.75 ± 0.17
Wheat peptone	0.030 ± 0.002^{ab}	6.6 ± 0.4^{cd}	8.94 ± 0.03
Fish peptone	0.026 ± 0.006^{ab}	1.0 ± 0.1^{a}	8.63 ± 0.03
Tryptone	0.024 ± 0.002^{ab}	8.1 ± 0.9^{d}	8.64 ± 0.03
Casein hydrolysate	0.023 ± 0.002^{ab}	3.6 ± 0.3^{ab}	8.59 ± 0.01
Casein	0.022 ± 0.001^{ab}	1.0 ± 0.1^{a}	8.82 ± 0.03
Wheat gluten	0.017 ± 0.001^{a}	2.0 ± 0.4^{ab}	8.78 ± 0.04
Different % combination of PP and YE			
0.5% PP + 0.1% YE**	0.096 ± 0.005^{a}	6.7 ± 0.4^{a}	8.67 ± 0.03
0.4% PP + 0.2% YE	0.102 ± 0.001^{a}	7.4 ± 0.3^{a}	8.77 ± 0.04
0.3% PP + 0.3% YE	0.100 ± 0.001^{a}	6.6 ± 0.6^{a}	8.87 ± 0.07
0.2% PP + 0.4% YE	0.104 ± 0.002^{a}	8.9 ± 0.5^{a}	8.74 ± 0.01
0.1% PP + 0.5% YE	0.096 ± 0.002^{a}	6.8 ± 0.8^{a}	8.78 ± 0.10
0.6% PP	0.092 ± 0.007^{a}	6.9 ± 0.7^{a}	8.73 ± 0.01
0.6% YE	0.097 ± 0.001^{a}	6.8 ± 0.6^{a}	8.81 ± 0.03
Inorganic nitrogen source (0.05%, w/v)			
Control***	0.098 ± 0.001^{de}	5.1 ± 0.1^{b}	8.96 ± 0.03
NH_4Cl	0.071 ± 0.001^{ab}	2.5 ± 0.7^{ab}	8.82 ± 0.04
$(NH_4)_2SO_4$	0.078 ± 0.002^{abc}	3.0 ± 1.4^{ab}	9.04 ± 0.06
$(NH_4)_2HPO_4$	0.104 ± 0.003^{e}	5.7 ± 0.5^{b}	8.78 ± 0.03
$NH_4H_2PO_4$	0.090 ± 0.002^{cde}	4.0 ± 1.4^{ab}	9.00 ± 0.03
CH_3COONH_4	0.086 ± 0.001^{bcd}	3.6 ± 0.8^{ab}	9.14 ± 0.06
NH_4NO_3	0.090 ± 0.005^{cde}	4.9 ± 0.5^{ab}	9.02 ± 0.04
Urea	0.062 ± 0.011^{a}	1.5 ± 0.7^{a}	9.13 ± 0.18

The shake flask experiments were performed for 96 h at 28°C, 130 rpm. Data are means ± standard deviations of three determinations. Column data followed by the same superscript letter were not significantly different ($P \leq 0.05$; by Tukey test)

* Control refers to the basal medium with 0.5% (w/v) Bacto-peptone as organic nitrogen source

** Refers to control containing 0.5% (w/v) of phytone peptone (PP) and 0.1% (w/v) of yeast extract (YE) in the basal medium

*** Refers to control containing 0.05% (w/v) of $NaNO_3$ as inorganic nitrogen source in basal medium

activities of 169.1 and 157.2%, respectively. Lipase was significantly activated after 1 h in *p*-xylene and *n*-hexane showing residual activities of 161.9 and 156.7%, respectively. Further incubation for 24 h showed decreased residual activities of 133.7 and 111.7% for *p*-xylene and *n*-hexane, respectively, indicating destabilizing effects of these solvents. The lipase was least stable in pyridine with 49.8% residual activity after 24 h. Similarly, a destabilizing effect was seen with DMSO, *t*-butanol and dodecane.

Effect of metal ions and effectors on lipase stability

The effects of different metal ions and effector molecules on *A. mediterranei* DSM 43304 lipase were assessed (Table 7). The crude lipase was strongly inhibited by Hg^{2+} losing 80% of its activity. Li^{+} and Mg^{2+} also significantly reduced enzyme activity to 79.2 and 81.3%, respectively. In contrast, lipase activity was significantly promoted in presence of Fe^{3+} by 15.2%. The other metal ions tested had no significant effect on the enzyme activity. The lipase activity was unaffected by EDTA, SDS and urea. However, serine hydrolase inhibitor PMSF reduced the enzyme activity by 15%.

Discussion

Actinomycetes are a group of filamentous Gram-positive bacteria with a remarkable genetic repertoire for producing secondary metabolites and enzymes. Despite their high biotechnological interest, the actinomycetes have not been widely investigated for lipase studies, and there is little information available about lipase-producing actinomycetes [8, 12, 65]. During the last few decades, a few reports described screening for lipolytic activities among different

Table 6 Stability of *A. mediterranei* DSM 43304 lipase in organic solvents

Organic solvent	log *P*	Relative activity (%)	
		After 1 h	After 24 h
Control		100.0 ± 1.2	100.0 ± 0.6
DMSO	−1.3	109.4 ± 2.3	89.6 ± 3.7[a]
DMF	−1.04	131.3 ± 6.3[a]	137.6 ± 0.9[a]
Methanol	−0.76	129.5 ± 2.8[a]	136.5 ± 0.6[a]
2-propanol	−0.28	129.5 ± 4.3[a]	112.6 ± 2.5[a]
Ethanol	−0.24	109.6 ± 2.0	115.9 ± 3.4[a]
Acetone	−0.23	131.7 ± 5.2[a]	134.6 ± 2.8[a]
t-Butanol	0.35	96.3 ± 5.8	75.4 ± 3.4[a]
Pyridine	0.64	72.4 ± 3.5[a]	49.8 ± 2.8[a]
Diethyl ether	0.85	116.8 ± 2.6[a]	136.9 ± 1.8[a]
Benzene	2.0	169.7 ± 2.0[a]	169.1 ± 1.2[a]
Chloroform	2.0	113.8 ± 6.3	134.1 ± 2.2[a]
Toluene	2.5	153.3 ± 3.6[a]	157.2 ± 2.8[a]
Carbon tetrachloride	2.64	137.2 ± 3.7[a]	117.6 ± 0.9[a]
Cyclohexene	2.86	138.6 ± 2.3[a]	109.3 ± 2.2
p-Xylene	3.1	161.9 ± 2.0[a]	133.7 ± 2.2[a]
n-Hexane	3.5	156.7 ± 3.2[a]	111.7 ± 4.3[a]
Dodecane	6.6	115.5 ± 3.3[a]	68.0 ± 3.9[a]
Petroleum ether	≈3.0	140.5 ± 2.6[a]	123.7 ± 2.2[a]

Lipase preparation was incubated in each organic solvent (25%) at 30°C for 1 and 24 h

Values represent the mean of three replicates

[a] Significantly different ($P \leq 0.05$; by Tukey test) with respect to the control

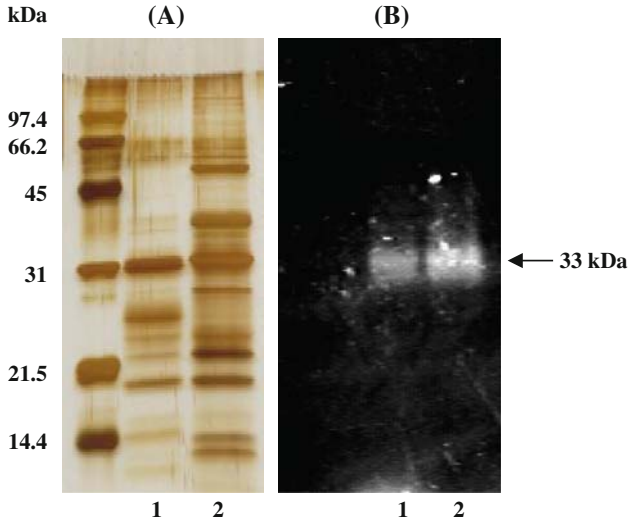

Fig. 5 Zymogram analysis performed on SDS–PAGE gel. SDS–PAGE of crude extracellular broth, analyzed for lipolytic activity using MUF-butyrate (**b**) and subsequently silver stained (**a**) to determine the molecular mass of active protein. The samples loaded correspond to crude broth (*1*) and crude broth after precipitation with ammonium sulfate (*2*)

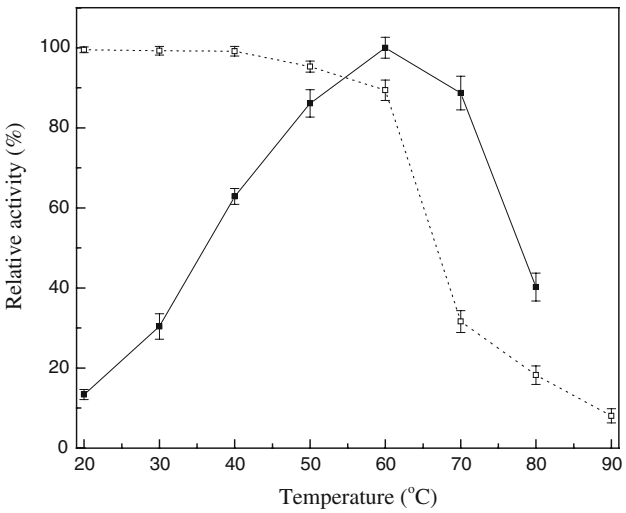

Fig. 6 Effect of temperature on *A. mediterranei* DSM 43304 lipase activity (*filled square*) and stability (*open square*). Assay conditions: 30–90°C, 50 mM Tris–HCl buffer pH 8.0. Thermal stability of the enzyme was studied by incubating the enzyme at various temperatures (30, 40, 50, 60, 70, 80 and 90°C) for 3 h. Residual activity (%) at each temperature was calculated relative to that at 0 h as 100%

actinomycetes. Bormann et al. reported that 51% of 243 streptomycetes investigated showed lipolytic activity through screening using plate and well techniques [6]. Sztajer et al. investigated lipase production in 15 different *Streptomyces* strains and found lipolytic activity in only few strains tested [65]. More recently, Cardenas et al. isolated novel actinomycetes and fungal strains and demonstrated their lipolytic activity on agar plates supplemented with emulsified olive oil and tributyrin [8].

In the present study, most of the actinomycete strains tested showed detectable levels of fluorescence on rhodamine B agar, indicating the presence of lipases acting on long chain triglycerides (Fig. 1). The use of solid media supplemented with emulsified triglycerides is a standard methodology for the selection of lipase-producing microorganisms [61], but they are not suited to real production of enzymes and do not clearly distinguish between cell-bound and extracellular activities. Therefore, the actinomycete strains were cultured in liquid medium using olive oil as lipase inducer. Lipase activities associated with the cells of different *Streptomyces* sp. were induced and enhanced by the presence of a lipid substrate in the fermentation medium [31]. In the present investigation, all actinomycete strains showed the presence of lipase activity when their culture supernatants were tested with *p*-NPP, a chromogenic substrate specific for lipases (Table 1). Similarly, a high percentage of actinomycete strains were reported to produce lipase when grown in liquid culture using olive oil as lipase inducer [8]. The difference observed in the lipolytic activities could be due to the difference in the level of lipase

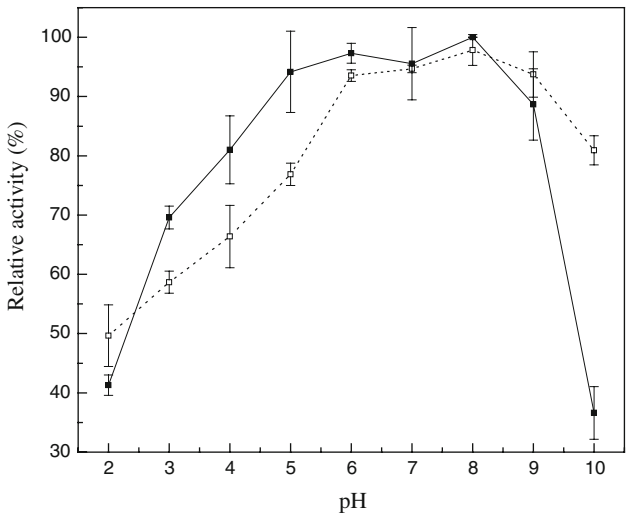

Fig. 7 Effect of pH on *A. mediterranei* DSM 43304 lipase activity (*filled square*) and stability (*open square*). For stability studies, residual activities were measured after 24 h incubation at 4°C in the presence of different buffers: HCl–KCl (pH 2.0), sodium citrate (pH 3.0), succinate–NaOH (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris–HCl (pH 8.0 and 9.0) and glycine–NaOH (pH 10.0), all buffers at 50 mM concentration. Assay conditions: 60°C, Tris–HCl buffer, pH 8.0, 50 mM. The activities were compared to the activity determined in 50 mM Tris–HCl buffer, pH 8.0 without preincubation

Table 7 Effect of metal ions/effectors on stability of *A. mediterranei* DSM 4334 lipase

Metal ion/effector (1 mM)	Relative activity (%)
Control	100.0 ± 1.0
Ag^{+}	88.2 ± 5.5
Ba^{2+}	99.1 ± 1.0
Ca^{2+}	91.1 ± 4.5
Co^{2+}	91.1 ± 0.6
Cu^{2+}	100.2 ± 5.2
Fe^{3+}	115.2 ± 7.1[a]
Hg^{2+}	20.0 ± 3.2[a]
Li^{+}	79.2 ± 2.6[a]
Mg^{2+}	81.3 ± 3.5[a]
Mn^{2+}	112.9 ± 5.2
Ni^{2+}	95.2 ± 1.9
Pb^{2+}	97.0 ± 0.6
Zn^{2+}	95.9 ± 1.6
NH_4^{+}	103.8 ± 1.8
EDTA	103.6 ± 1.6
SDS	100.0 ± 2.9
Urea	88.1 ± 2.9
PMSF	85.3 ± 3.2[a]

The lipase preparation was incubated in the presence of various compounds at 60°C for 10 min

Values represent the mean of three replicates

[a] Significantly different ($P \leq 0.05$; by Tukey test) with respect to the control

production in the liquid medium or different substrate specificities of the lipases from these strains.

The present study identified five different actinomycete strains producing significant extracellular lipolytic activities. Among these, *A. mediterranei* DSM 43304 was selected on the basis of the high thermostability of its lipolytic activity. The lipase from *A. mediterranei* DSM 43304 was rapidly produced during the first 48 h of culture and continued increasing up to 96 h before showing a slow decrease in lipase activity (Fig. 2). This profile is similar to those described for other lipase-producing actinomycetes. However, the time course for optimum lipase production (96 h) in the present study was less than other lipase-producing actinomycetes reported [7]. The decrease in lipase production after long fermentation times could be due to inactivation of the enzyme by extracellular proteases, as observed for other lipase-producing microorganisms [56]. Maximum lipase specific activity was observed at 72 h, which decreased on further incubation (data not shown). This loss could be due to secretion of other proteins at the late logarithmic phase leading to an apparent decrease in lipase specific activity. Swift et al. reported that once cell densities have reached a certain threshold level, generally in the late logarithmic phase, the expression of genes encoding exoproteins and secretion system is induced [62]. It has been reported that the lipase synthesis in *S. exfoliatus* M11 and *S. coelicolor* A3(2) is growth phase dependent [5, 58]. The biomass showed a substantial increase after 24 h and was highest at 96 h and stayed stable up to 120 h before showing a decrease. The lipase production was accompanied by an increase in the medium pH for all cultures. The pH of the culture broth became alkaline reaching to 8.9 at 96 h when the volumetric lipase activity was the highest and stayed stable between 8.6 and 8.7 for 168 h. These trends of stable biomass and pH when lipase production was highest are similar to those observed for *S. clavuligerus* [31].

It is important to provide an optimum inoculum level in fermentation processes. At a suitable inoculum size, the nutrient and oxygen levels are enough for sufficient growth of bacteria and therefore enhance the lipase production. If the inoculum size is too small, insufficient biomass will lead to reduced levels of secreted lipase. High inoculum size can result in the lack of oxygen and nutrient depletion in the culture media resulting in poor product yield [44]. There was a significant effect of size of inoculum on lipase production (Table 2). Increased lipase production was observed with increase in inoculum size and showed maximum biomass and lipase production at 10% (v/v) inoculum. Further increase in inoculum size resulted in decreased enzyme synthesis, probably due to nutrient limitation.

The incubation temperature may affect lipase production during fermentation process [35]. The results in the present

study demonstrate that temperature control during the submerged culture process was a critical factor significantly affecting growth and lipase production (Fig. 3). The optimal temperature determined for maximum lipase synthesis and biomass production by *A. mediterranei* DSM 43304 (28°C) is comparable to those reported for actinomycetes such as *S. erythraea* and *S. clavuligerus* [31].

The initial pH of the growth medium is important for lipase production [54]. Lipase activity and growth were significantly affected by the initial medium pH. The maximum lipase activity of *A. mediterranei* DSM 43304 was obtained when the initial medium pH was 7.5 (Fig. 4). Most of the actinomycetes reported for lipase production were grown at neutral initial pH for optimal growth and lipase production [7, 31]. However, maximum lipase activity at higher initial pH by various thermophilic *Bacillus* sp. has also been reported [18]. In contrast, Ertugrul et al. reported a moderately acidic pH (6.0) as the optimum initial pH for lipase production by *Bacillus* sp. [15]. The molecular charges and consequently molecular interactions and functions are directly related to medium pH; thus, any change in medium pH affects many biological functions [43]. Therefore, medium pH is very important in nutrient absorption and growth of bacteria, stimulation of enzyme production via signaling pathways and release of extracellular enzymes [50]. Also, the pH change observed during growth of the organism may affect the enzyme stability in the medium [20].

Lipases mostly are inducible enzymes and inducers such as oils are necessary for lipase production [40], although their role in lipase synthesis and stimulation is poorly understood [31]. In some cases, lipases were produced constitutively and showed significant increase in activity on addition of oil to the medium [49]. Similarly, lipase induction in presence of lipid-based carbon sources was reported in actinomycetes [7, 31]. Macris et al. suggested that it was the carbon chain moiety of the fatty acid present in the triacylglycerol that controlled lipase synthesis [38]. Lipase induction appears to be influenced not only by the length of fatty acids in the triacylglycerols, but also by the number of unsaturations. However, the manner by which these compounds influence lipase biosynthesis is not well understood [49]. Natural oils such as soybean, corn, sunflower, olive, palm and cotton seed oils are cited as inducers for lipase production, comprising at times the sole source of carbon in the medium [35, 54, 66]. Submerged cultures of *A. mediterranei* DSM 43304 were carried out in basal medium supplemented with different natural oils. Among the various oils used in the present study, maximum lipase production was achieved using linseed oil followed by sunflower oil and olive oil (Table 3). Fatty acids present in the greatest proportion in these oils are unsaturated fatty acids such as oleic acid ($C_{18:1}$), linoleic ($C_{18:2}$) acid and linoleinic acid ($C_{18::3}$). Triglycerides of linseed oil contain 21% oleic acid, 16% linoleic acid and 53% linoleinic acid, while those of sunflower and olive oil contain 13 and 71% oleic acid and 78 and 10% linoleic acid, respectively. Among the oils showing higher volumetric lipase activity, linseed oil contains much higher % of linoleinic acid, while other oils contain only about 1% linoleinic acid. Better lipase production in *A. mediterranei* DSM 43304 appears therefore, to be correlated with higher content of polyunsaturated fatty acids and shows maximum induction with the higher % of linoleinic acid content in linseed oil. However, significantly reduced activity and biomass production was seen with soybean and rapeseed oils. This may be due to enzyme specificity or to the presence of lipase inhibitors or germicidal agents in rapeseed and soybean oil as reported in the case of Pse-2 strain [65].

Surfactants as substrates for lipase production by *A. mediterranei* DSM 43304 showed significantly reduced lipase activity in the culture medium (Table 3). Similar effects were reported on lipase production in *Rhizopus* sp. BTNT-2 [3] and *Yarrowia lipolytica* [13]. In contrast, the addition of surfactant to the culture medium has been shown to increase the secretion of lipolytic enzymes in a number of microorganisms, attributable to alteration of cell permeability leading to increased protein secretion or to surface effects on cell-bound enzymes [39]. A wide variety of surfactants like Tweens, Triton, SDS, PEG and gum arabic have been studied by different investigators for their effect on lipase production [13, 37, 39]. However, surfactants do not always increase lipase production [37], and their effect varies with microorganism, type of surfactant and its concentration [13, 39].

Although lipid-based carbon sources seem to be generally essential for obtaining a high enzyme yield, some authors reported good results in the absence of fats and oils [35, 36], whereas in some cases, a mixture of compounds were proposed as optimum carbon sources [54]. A range of different carbon sources (i.e. carbohydrates, alcohols, acids, lipids) has been reported to support both growth of lipolytic enzyme producers and lipase/esterase production [4, 19]. Therefore, the suitability of different carbon source additives for lipase production by *A. mediterranei* DSM 43304 was investigated (Table 4). Addition of different sugar additives to the basal medium had a positive effect on growth and lipase production by *A. mediterranei* DSM 43304. Among the sugar additives, fructose showed the highest increase in lipolytic activity whereas, dextrin and arabinose appeared less adequate carbon source additives for lipase production from *A. mediterranei* DSM 43304. A significantly decreased lipolytic enzyme levels were obtained with starch, which is similar to the effect of starch on lipase production in *Issatchenkia orientalis* [10]. Similar

to the present study, glucose used as a sugar additive showed increased lipase production from *Burkholderia cepacia* in the presence of different oils [54]. Also increase in lipase activity was reported in case of a mutant strain (UV-10) of *Aspergillus niger* NCIM 1207 when glucose was added to the production medium in presence of olive oil [39].

Generally, microorganisms provide high yields of lipase when organic nitrogen sources are used. Nitrogen sources play an important role in the biosynthesis of lipase by microorganisms. Inorganic nitrogen sources can be used quickly, while organic nitrogen sources can supply cell growth factors and amino acids needed for cell metabolism and enzyme synthesis [66]. Among the various organic nitrogen sources tested, a significant increase in growth and lipase activity was observed with phytone peptone and yeast extract (Table 5). Similarly, peptone has been shown to support lipase production in the case of *Penicillium citrinum* [64] and *Streptomyces* sp. [63]. Likewise, higher lipase production was reported using yeast extract as a nitrogen source for *Saccharomyces cerevisiae* [59]. In addition, maximum lipase production from *Pseudomonas* sp. KWI-56 [24] and *Candida cylindracea* [45] was obtained in media containing peptone and yeast extract as organic nitrogen sources. Contrary to the present results, casein and corn gluten were the best sources for lipase production by *A. terreus* [19]. No significant effect on lipase production was observed by changing the relative proportion of phytone peptone and yeast extract in the basal medium, indicating that these organic nitrogen sources were equally effective for lipase production by *A. mediterranei* DSM 43304. With regard to the inorganic nitrogen sources, $(NH_4)_2HPO_4$ showed significant increase in lipase production that is in agreement with the higher lipase-specific activity of *B. cepacia* with $(NH_4)_2HPO_4$ as inorganic nitrogen source [54]. Lipase activity and biomass production was significantly reduced by urea. Urea was also reported to be inhibitory for lipase synthesis by *P. camembertii* Thom PG-3 [66]. However, several investigators reported urea as optimal for lipase production in liquid cultures of *C. rugosa* [4, 48].

Besides physical and nutritional parameters, metal ions may play an important role in lipase production. Several authors have reported the stimulatory effects that calcium, magnesium and iron ions have on the lipase production of different organisms [28, 36]. In the present study, metal ions were found to have no significant effect on lipase production (Table 4). In contrast, lipase production by a thermophilic *Bacillus* sp. was reported to increase several fold when magnesium, iron and calcium were incorporated in the production medium [27]. Also, iron was reported to be critical for the production of lipase by *Pseudomonas* sp. G6 [28], and Ca^{2+} had strong stimulatory effect on extracellular lipase production by *P. fluorescens* 2D [41]. Similarly, Ca^{2+} in presence of Mg^{2+} was reported to produce a significant increase in lipase production by *B. cepacia* [54].

The economics of enzyme production using inexpensive raw materials can make enzymatic processes competitive with chemical ones [42]. In most instances, type of carbon substrate and inducer show a profound effect on the yield of microbial lipases during culture and therefore to a great extent influence the final production cost [28]. In the present investigation, due to significantly higher volumetric activity achieved with linseed oil, it was used in the modified fermentation medium. But it is important to note that sunflower oil, a less expensive substrate, could also be used for large-scale production.

In the current work, a crude extract obtained from the culture medium of *A. mediterranei* DSM 43304 showed lipolytic activity corresponding to a single protein with molecular weight of 33 kDa (Fig. 5), thereby validating the use of crude medium for enzyme characterization. The lipase hydrolyzed *p*-NPP in the temperature range of 20–80°C with a maximum at 60°C (Fig. 6). The lipase was highly stable at 60°C for 3 h with more than 90% activity, suggesting a high thermostability of the protein. Generally, bacterial lipases have temperature optima in the range 30–60°C. However, reports exist of bacterial lipases with optima in both lower and higher ranges [21, 53]. Apart from the species of *Bacillus* and *Pseudomonas* genera, there are only few examples of bacteria reported to produce lipases that are active and stable above 50°C and among fungi and yeast, thermostable lipases are even rarer [33]. Thermostability is a desirable characteristic in lipases for application in different industrial processes operating at high temperatures [27]. Thus, the high activity and stability of the new lipase from *A. mediterranei* DSM 43304 in the temperature range of 50–60°C points to its suitability for applications in biocatalytic processes. Such a high stability in this temperature range has not previously been described for lipases from mesophilic actinomycetes, although a few thermoactive lipases have been reported. A proteolysis-resistant lipase from *S. fradiae* var. k11 was reported having a temperature optimum of 55°C [70], and a lipase (SCO7513) characterized from *S. coelicolor* A3(2) was the most active in the temperature range of 45–55°C [11]. Similarly, *S. rimosus* exhibited optimal activity at 55°C and stability for 30 min at 50°C [1].

The *A. mediterranei* DSM 43304 lipase was significantly active over a broad range of pH with optimum at pH 8.0 (Fig. 7). The activity and the stability shown by this lipase at acidic pH values are not common among the lipases produced by actinomycetes, which in general are reported to be most active at alkaline pH values. The lipase produced by *S rimosus* was found to be optimally active around pH 9.5 and stable over a broad pH range of 4–10

[1]. Similarly, *S. fradiae* k11 lipase showed optimum activity at pH 9.8 and stability over pH 4–10 [70]. Lipases from *S. coelicolor* A3(20) were optimally activity at pH between 8.5 and 9 [11]. *A. mediterranei* DSM 43304 lipase was stable in storage conditions for 24 h over a broad range of pH. However, the enzyme became unstable and showed decreased stability at pH over 9.0 and below 6.0. Similarly, a significant instability was reported in case of *S. coelicolor* A3(2) lipase (SCO1725) and *S. rimosus* lipase at pH over 10, whereas SCO7513 lipase from *S. coelicolor* A3(2) was most stable at pH 6.0 with rapid loss of activity in the pH range 6.5–11 [1, 11]. Thus, the high activity and stability of this lipase in acidic and alkaline conditions suggests its usefulness in a range of industrial applications.

Employing lipases for bioconversions in organic solvents is advantageous from biotechnological point of view; hence, activity and stability in organic solvents are desirable in a lipase [17]. Stability in organic solvents can determine whether the enzyme can be used to catalyze synthetic reactions and also to predict which solvent would be better to perform the reaction [22]. Lipases are diverse in their sensitivity to organic solvents, but there is general agreement that water miscible hydrophilic solvents are more destabilizing than water immiscible hydrophobic solvents [46]. The lipase produced by *A. mediterranei* DSM 43304 was remarkably stable in both hydrophilic and hydrophobic organic solvents (Table 6). Hydrophilic solvents are generally incompatible with enzyme activity because they remove the crucial bound water from the enzyme surface, thereby destabilizing the protein and causing high denaturation rates [47, 60]. Interestingly, the lipase from *A. mediterranei* DSM 43304 presented an opposite behavior and showed activation with solvents of log *P* values less than −0.2. Stability of bacterial and fungal lipases in hydrophilic solvents is rare, and the enhancement of activity by these solvents is even rarer [33], though stability in hydrophilic solvents has been reported in case of few actinomycete lipases [5, 8, 32]. The activation of lipases in the presence of hydrophilic organic solvents can be explained by the disruption of aggregates formed between the enzyme and lipids of fermentation medium or between the enzyme molecules themselves [61]. Indeed, gel exclusion chromatography of the crude extract from *A. mediterranei* DSM 43304 showed that the enzyme was produced in the form of a high-molecular weight aggregate (data not shown). Disruption of these aggregates by the hydrophilic solvents could have led to the enhancement of activity observed. After 24 h, a significant inactivation in presence of DMSO, tert-butanol and dodecane could be due to the nature of the solvent. It is still debated whether solvent polarity (log *P*) is the key factor in enzyme deactivation, since the nature of the solvent and its structure have been shown to greatly influence solvent effect in non-aqueous environment [22].

The activation obtained in the present work with the hydrophobic solvents such as benzene, p-xylene and *n*-hexane have previously been reported for bacterial lipases. The lipase from *B. sphaericus* was reported to be stable and significantly activated in 25% (v/v) *n*-hexane and p-xylene after 30 min incubation [23]. Similarly, S5 lipase from *Pseudomonas* sp. showed stability and activation after 30 min incubation with benzene and *n*-hexane. [52]. However, the high stability and activation obtained in the current work with hydrophobic solvents have not previously been reported for lipolytic enzymes from actinomycete. The activation by hydrophobic solvents could be due to the presence of residues of these solvents transferred into the assay system after the preincubation step. These solvent molecules can interact with hydrophobic amino acid residues present in the lid/flap that covers the catalytic site of the enzyme, thereby keeping the enzyme in a flexible open conformation and consequently increasing the activity [55]. These results indicate that *A. mediterranei* DSM 43304 lipase holds the potential for use in organic synthesis and related applications since an important criterion in the selection of biocatalyst is its ability to be adequately stable under process conditions [16].

The effect of different metal ions on *A mediterranei* DSM 43304 lipase activity and stability (Table 7) showed that the crude lipase was strongly inhibited by Hg^{2+} losing 80% of activity in 10 min suggesting it is able to alter enzyme conformation as has been reported for other lipases [1, 34, 70]. Li^{+} and Mg^{2+} significantly reduced the enzyme activity, whereas *S. fradiae* lipase showed activation in presence of Mg^{2+} and was not affected by Li^{+} [70]. In addition, *S. coelicolor* A3(2) lipases were unaffected in presence of Mg^{2+} [11]. The enzyme was found to be activated in presence of Fe^{3+}, which is similar to the activation effect observed in case of *S. fradiae* var. k11 lipase [70].

The Ca^{2+} ion has been reported to promote and stabilize hydrolytic activity of lipases [17], but no significant influence on *A. mediterranei* DSM 43304 lipase was observed. Similarly, Ca^{2+} ions had no effect on *S. rimosus* lipase [1]; however, *S. fradiae* var. k11 lipase activity was significantly reduced in presence of Ca^{2+} ions [70]. Cu^{2+} has been reported to be a strong inhibitor of lipase activity [34, 52], but no inhibition of *A. mediterranei* DSM 43304 lipase was observed with Cu^{2+} under the test conditions. Similarly, Cu^{2+} had no significant effect on lipases from *S. coelicolor* A3(2), *S. rimosus* and *S. fradaie* var. k11 [1, 11, 70]. The lipase proved to be insensitive to Ag^{+}, which is comparable to the stability of *S rimosus* lipase in presence of Ag^{+} [1]. In contrast, Ag^{+} inhibited *S. fradaie* var. k11 lipase, and a strong inhibition of *S. coelicolor* A3(2) lipases was observed [11, 70]. The lipase activity was unaffected by the

metal-chelating agent EDTA, indicating that *A. mediterranei* DSM 43304 lipase is not dependent on a metal cofactor. The lipases from *S. rimosus* and *S. fradiae* var. k11 have also been reported to be unaffected by EDTA [1, 70].

All known lipases have serine in their active center; nonetheless, some lipases are resistant to inactivation by serine reactive agents [1]. The effect of PMSF at 1 mM concentration gave a 15% reduction in lipase activity possibly suggesting the presence of a hydrophobic lid hindering access to the catalytic site [11]. The extracellular lipase from *S. rimosus* was found to be marginally affected by 1 mM PMSF showing retention of 90% of residual activity [1]. Similarly, 89–91% residual activity was retained in presence of 1 mM PMSF by *S. coelicolor* A3(2) lipases [11].

The urea can affect the enzyme structure by direct interaction with the enzyme or by an indirect action through effects on the structure and properties of the surrounding solvent [14], thereby reducing enzyme stability and activity. However, the enzyme showed no significant effect on the activity in presence of 1 mM urea. Similarly, the enzyme was significantly stable in presence of 1 mM SDS. In agreement with the present study, *B. cepacia* and *P. aurantiogriseum* lipases were reported to be stable with a significant residual activity in SDS [34, 69].

Conclusions

In this work, *Amycolatopsis mediterranei* DSM 43304 was identified as producing an extracellular lipase, and a significant increase in lipase yield from this strain was achieved by improving the culture conditions. Besides being produced at enhanced levels in the newly formulated culture medium, the enzyme exhibited activity and stability at high temperatures and a remarkable stability toward both hydrophilic and hydrophobic organic solvents. Because of the relatively high temperature optimum and pronounced thermal stability, as well as the preservation of activity in a broad pH range, this new extracellular lipase could be of significant biotechnological potential. Thus, these results justify the search for possible applications of this enzyme in biocatalysis in organic media.

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