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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 condition 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. In optimal culture conditions the lipase activity was enhanced 12-fold with a 2-fold increase in lipase specific activity. The lipase showed maximum activity at 60 °C and pH 8.0. The enzyme was stable between pH 5.0–9.0 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 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 24h. These features render this novel lipase attractive for potential biotechnological applications in organic synthesis reactions.

Keywords: *Amycolatopsis mediterranei*; Screening; Organic solvent-tolerant; Thermostable; Lipase

45 **1. Introduction**

46 Lipases (triacylglycerol acylhydrolases E.C.3.1.1.3) are serine hydrolases of considerable
47 physiological significance and industrial potential [46]. Interest in lipases has greatly
48 increased in recent years, mainly because they present a broad range of biotechnological
49 applications due to their unique characteristics of substrate specificity, regio-specificity and
50 chiral-selectivity [41]. Many microorganisms, including bacteria, yeast, and fungi, have
51 been shown to secrete lipases during their growth on hydrophobic substrates [25]. Among
52 lipases of various origins, those from bacteria show the highest degree of versatility,
53 reactivity and stability in the catalysis of reactions in organic solvents [19]. Lipase
54 applications range from the hydrolysis of fats in wastewaters to the synthesis of chiral
55 pharmaceutical intermediates [42]. These applications often require harsh reaction
56 conditions *e.g.* high temperatures may be required to either favour stereoselectivity or
57 solubilise high melting point lipids [17]. Biocatalysis with lipases is often carried out in
58 organic solvents to promote synthetic reactions by reducing water activity. Many lipases
59 lose activity in organic solvents, and therefore, there is an ongoing interest in lipases that
60 are solvent-tolerant [7]. Despite the advantages of biocatalysis in organic solvent based
61 systems, the catalytic activities of enzymes in these systems are typically much lower than
62 in aqueous solutions [43]. Currently, since microbial lipases do not have the desirable
63 combination of thermostability and stability in both hydrophobic and hydrophilic organic
64 solvents, the search for new lipases is required [31, 33]. There are two main strategies for
65 obtaining lipases with improved properties either protein engineering of currently known
66 lipases [40] or the search for novel lipolytic activities in previously unexplored

67 microorganisms [11]. As each industrial application requires specific properties of lipases,
68 there is still an active interest in finding novel lipases for specific applications.
69 To date, a large number of lipases from filamentous fungi, yeasts and unicellular bacteria
70 have been extensively studied, both from the biochemical and from genetic point of view
71 [8]. However, despite their high biotechnological potential for the production of secondary
72 metabolites and enzymes, the actinomycetes have not been widely studied for lipase
73 production [1, 30, 50].
74 The present paper deals with the screening of lipase producing actinomycete strains and the
75 culture conditions for optimum enzyme production by a selected strain of *Amycolatopsis*
76 *mediterranei* DSM 43304. Medium composition, initial pH, temperature, and time of
77 incubation were examined for the optimization of lipase production. Lipase characteristics
78 with respect to the optimal temperature and pH for both activity and stability are examined.
79 We also characterize the activity and stability of the lipase in the presence of various
80 organic solvents, metal ions, detergents and inhibitors. To our knowledge, the present work
81 is the first report of an organic solvent-tolerant lipase from the genus *Amycolatopsis*.

82 **2. Materials and methods**

83 **2.1 Materials**

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

89 **2.2 Microorganisms**

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

92 **2.3 Maintenance of microorganisms**

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

96 **2.4 Culture conditions**

97 The composition of basal medium used was (g/l): $NaNO_3$ 0.5 g; KCl 0.5 g; $MgSO_47H_2O$
98 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
99 7.2 with 1 M NaOH or 1 M HCl. Then 1.0% (v/v) olive oil was added. Media were
100 sterilized for 15 min at 121 °C at 15 psi. Submerged microbial cultures were incubated in
101 250 ml Erlenmeyer flasks containing 50 ml of basal medium with 5 ml inoculum on a
102 rotary shaker (130 rpm) at 28 °C.

103 **2.5 Rhodamine B agar screening**

104 The primary screening for the detection of lipolytic activity on solid media was carried out
105 on rhodamine B agar (RBA) as described by Kouker and Jaeger [29] with some
106 modifications. The growth medium containing 0.9% (w/v) peptone water, 0.25 % (w/v) of
107 yeast extract, 2% (w/v) of agar, was adjusted to pH 7.2, autoclaved and cooled to 60 °C.
108 Then, filter sterilized rhodamine B stock solution (1.0 mg/ml) in distilled water was added
109 to a substrate lipoidal emulsion to yield a final concentration of 0.001% (w/v). The
110 substrate lipoidal emulsion consisted of 1.5% (w/v) olive oil with 0.25% (v/v) Tween 80 in
111 distilled water that was sterilized by autoclaving. The resulting mixture of lipoidal emulsion
112 with growth medium (1:10) was vigorously stirred to emulsify for 15 min. The medium

113 was allowed to stand for 10 min at 60 °C to reduce foaming before pouring 20 ml of
114 medium into plastic petri plates. Fresh RBA plates were spot inoculated with 72 h-old
115 actinomycete cultures in GYM broth (g/l: glucose 4.0 g; yeast extract 4.0 g; malt extract 10
116 g; pH 7.2) and incubated at 28 ºC for 6 days. The plates with visible growth were UV
117 irradiated (350 nm). Lipase production was identified as orange fluorescence under UV
118 light.

119 **2.6 Screening in submerged fermentation broth**

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

126 **2.7 Spectrophotometric *p*-NPP assay**

127 Lipase activity was quantitatively assayed in cell free supernatant using *p*-nitrophenyl
128 palmitate (*p*-NPP) as substrate. This assay was performed as described by Winkler and
129 Stuckman [55] with some modifications. A stock solution of *p*-NPP was freshly prepared in
130 2-propanol at a concentration of 0.3% (w/v). This solution (Solution A) was subjected to 3
131 min sonication (135W, 42 kHz, Branson 5510E-MT). Then, 900 µl of 1:20 dilution of the
132 substrate stock solution A in solution B (0.1% (w/v) gum arabic, 0.4% (v/v) Triton X100 in
133 distilled water) with 50 µl of appropriate buffer were preincubated for 2 min at the assay
134 temperature before adding 50 µl of enzyme sample. This mixture was incubated at the
135 assay temperature for 10 min, and the reaction was terminated by addition of 2 ml of 0.2 M

136 Na_2CO_3 solution. Released *p*-nitrophenol (*p*-NP) was immediately determined by
137 measuring the absorbance at 410 nm in a Unicam UV-VIS spectrophotometer (Model UV2
138 2000E, Cambridge, UK). Appropriate blanks were used to subtract the absorbance
139 corresponding to the reaction mixture other than that produced by the specific hydrolysis of
140 *p*-NPP. The molar extinction coefficient of *p*-NP (ε_{410nm} = 16,900 M^{-1} cm^{-1}) was estimated
141 from the absorbance of standard solutions of *p*-NP. One international unit of lipase activity
142 was expressed as the amount of enzyme liberating 1 μM of *p*-NP per minute under the
143 conditions of the assay.

144 **2.8 Biomass concentration analysis**

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

148 **2.9 Statistical analysis**

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

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

153 Time course of lipase production was studied in the basal medium using shake flask
154 cultures. A 10% (v/v) of 72 h-old inoculum grown in GYM broth was added to 50 ml
155 medium, in a 250 ml Erlenmeyer flask and incubated at 130 rpm on a rotary shaker, at 28
156 °C. Samples were analyzed at 24 h intervals to determine pH, dry biomass and lipase
157 activity in the culture supernatant. The effects of the cultivating conditions in shake flask
158 experiments are commonly investigated by subjecting the microorganism to different

159 environmental conditions [56]. In the present work the effect of initial pH and incubation
160 temperature on the culture was studied using shake flask cultures at different temperatures
161 (20–45 °C) and initial values of pH (5.0–9.0). The effect of inoculum size on lipase
162 production was investigated by varying culture inoculum size from 2% to 12% of total
163 volume. All experiments were carried out at least in triplicate.

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

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

170 **4.1 Effect of inducers on lipase production**

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

174 **4.2 Effect of surfactants on lipase production**

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

178 **4.3 Effect of carbon source additives on lipase production**

179 In order to elucidate the effect of carbon source additives on lipase production, olive oil in
180 the basal medium was combined with 1.0% (w/v) of the following sugars: arabinose,

181 dextrin, fructose, galactose, glucose, lactose, mannitol, maltose, maltotetraose, mannose,
182 raffinose, rhamnose, sorbitol, sucrose, starch and xylose.

183 **4.4 Effect of organic nitrogen sources on lipase production**

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

189 **4.5 Effect of phytone peptone and yeast extract on lipase production**

190 Phytone peptone with yeast extract was the best nitrogen source for *A. mediterranei* DSM
191 43304 lipase production. The influence of the amount of phytone peptone and yeast extract
192 in basal medium was tested by varying their concentrations in the basal medium.

193 **4.6 Effect of inorganic nitrogen sources on lipase production**

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

197 **4.7 Effect of metal ions on lipase production**

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

203 **5. Characterization of *A. mediterranei* DSM 43304 lipase**

204 **5.1 Effect of temperature activity and stability**

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

209 **5.2 Effect of pH on activity and stability**

210 The effect of pH on lipolytic activity was determined in the following buffers (all at 50
211 mM): HCl–KCl (pH 2.0), sodium citrate (pH 3.0), succinate–NaOH (pH 4.0 and 5.0),
212 sodium phosphate (pH 6.0 and 7.0), Tris–HCl (pH 8.0 and 9.0) and glycine–NaOH (pH
213 10.0). The optimum pH obtained was used for investigation of thermostability and other
214 parameters. These buffers were used to determine pH stability of the crude lipase
215 preparation. A mixture (1:1) of crude enzyme and buffers (0.1 M) was incubated for 24 h at
216 4 °C and the standard enzyme assay described previously was performed. The residual
217 activities were calculated by comparison with the activity in 50 mM Tris–HCl buffer, pH
218 8.0, without pre-incubation.

219 **5.3 Effect of organic solvents on lipase stability**

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

225 **5.4 Effect of metal ions and effector molecules on lipase stability**

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

231 **6. Results and discussion**

232 **6.1 Screening of strains for lipase production**

233 Figure 1 shows rhodamine B agar screening for lipase production by actinomycete strains.
234 Except for *Amycolatopsis coloradensis* DSM 44225 and *Streptomyces aureoverticillatus*
235 NRRL B-3326, all actinomycete strains showed brilliant pink-red/orange fluorescence on
236 UV irradiation of RBA plates. Lipase screening in basal medium using shake flask cultures
237 showed presence of lipolytic activity in cell free supernatants prepared from actinomycete
238 culture broths (Table 1). Of 18 actinomycetes strains, 7 showed more than 0.05 IU/ml at 96
239 h. Among these, *A. rubida* DSM 44637, *S. rochei* DSM 40231 and *S. griseus* subsp.
240 *griseus* DSM 40236 produced the highest activities at 0.149 ± 0.017 IU/ml, 0.141 ± 0.004
241 IU/ml and 0.116 ± 0.010 IU/ml, respectively.
242 Actinomycetes are Gram-positive bacteria with a remarkable genetic repertoire for
243 producing secondary metabolites and enzymes. Despite their high biotechnological
244 potential, the actinomycetes have not been widely investigated for lipase activity [8, 13,
245 50]. Only a few studies have been reported on their esterase and lipolytic activities [6].
246 Large et al. described lipase activity associated with the cells of different *Streptomyces* sp.,
247 which were found to be induced and enhanced by the presence of a lipid substrate in the
248 fermentation medium [30]. Gandolfi et al. carried out carboxylesterase screening of

249 *Streptomyces* strains on solid media using tributyrin, triolein and Tween 60 as substrate and
250 evaluated their cell bound and extracellular hydrolytic activities [18]. Cardenas et al.
251 isolated novel actinomycetes and fungal strains and demonstrated their lipolytic activity by
252 employing screening techniques on solid and liquid media using agar plates supplemented
253 with emulsified olive oil and tributyrin [8]. The present investigation identified a number of
254 actinomycetes producing significant extracellular lipolytic activities.

255 **6.2 Effect of culture conditions on lipase production by *A. mediterranei* DSM 43304**

256 The preliminary characterization of extracellular lipases from actinomycete strains in terms
257 of pH optimum, temperature optimum and thermostability identified the lipase from *A.*
258 *mediterranei* DSM 43304 as the most thermostable and analysis by zymography indicated
259 presence of a single lipase in extracellular culture broth (data not shown). Therefore, *A.*
260 *mediterranei* DSM 43304 was selected for further characterization and various culture
261 parameters were studied to improve lipase production.

262 **6.2.1 Time course of lipase production**

263 It has been reported that the lipase synthesis of *S. exfoliatus* M11 and *S. coleicolor* A3(2) is
264 growth phase dependent [47]. Therefore, the time course of lipase synthesis in basal
265 medium by *A. mediterranei* DSM 43304 was monitored by measurement of lipase activity,
266 dry biomass and pH. Figure 2 shows that substantial lipase production commenced at 24 h
267 and reached a maximum at 96 h. Further incubation lead to increase in lipase activity with
268 a slow decrease in the lipase specific activity (data not shown). Loss of lipase specific
269 activity may be due to secretion of other proteins at the late logarithmic phase leading to an
270 apparent decrease in lipase specific activity. Swift et al. reported that once cell densities
271 have reached certain threshold level, generally in the late logarithmic phase, the expression

272 of genes encoding exoproteins and secretion system is induced [49]. There was a shift in
273 pH from 7.20 ± 0.02 to 8.02 ± 0.057 during the first 24 h of incubation, rising to pH 8.96 ±
274 0.11 at 96 h.

275 **6.2.2 Effect of inoculum size on lipase production**

276 Low inoculum density may give insufficient biomass causing reduced product formation,
277 whereas a higher inoculum may produce too much biomass leading to poor product
278 formation [37]. Increased enzyme production was observed with the increase in inoculum
279 size and showed maximum enzyme activity (0.105 ± 0.003 IU/ml) and biomass (5.8 ± 0.6
280 mg/ml) production at 96 h with 10% inoculum (Table 2). Further increases in inoculum size
281 resulted in decreased enzyme synthesis, probably due to nutrient limitation.

282 **6.2.3 Effect of incubation temperature on lipase production**

283 Temperature may affect lipase production [34]. Submerged fermentation was carried out at
284 20–45 °C with 10% inoculum for 96 h to evaluate the effect of incubation temperatures on
285 growth and enzyme production. Maximum enzyme and biomass production was observed
286 in the mesophilic range at 28–35 °C (Figure 3). The optimal temperature determined for
287 lipase production by *A. mediterranei* DSM 43304 (28 °C) is comparable to those of *S.*
288 *erythraea*, *S. clavuligerus* [30] and *Psedomonas aeruginosa* PseA [45].

289 **6.2.4 Effect of initial medium pH on lipase production**

290 The pH of the culture broth was found to be one of the most critical environmental
291 parameters affecting the growth and enzyme production by *A. mediterranei* DSM43304.
292 The results showed maximum biomass and lipase production at initial medium pH of 7.5
293 (Figure 4). Lipase activity dropped significantly at alkaline and acidic pH of 9.0 and 5.0,
294 respectively. The optimum pH (7.5) is close to optimum pH of 7.0 for lipase production by

295 *Candida* sp. [52] but is lower than that of other reported lipase-producing organisms.
296 *Bacillus mycoides* showed optimal lipase production at pH 8.0. [53] whereas, maximal
297 lipase production by *A. terreus* was observed at an initial medium pH of 9.0 [22]. The pH
298 change observed during growth of the organism may affect the enzyme stability in the
299 medium [23].

300 **6.2.5 Effect of inducers on lipase production**

301 Natural oils as carbon sources had different effects on lipase production. The results (Table
302 3) indicate that all lipidic sources supported lipase activity, ranging from 0.014 ± 0.001
303 IU/ml to 0.128 ± 0.004 IU/ml. The highest lipase production (0.128 ± 0.004 IU/ml) and
304 biomass (16.0 ± 1.4 mg/ml) was found using linseed oil followed by sunflower oil (0.104 ±
305 0.004 IU/ml). Soybean oil gave the lowest lipase activities. There are very few examples in
306 literature where linseed oil has been used as a lipid source for lipase production. Linseed oil
307 was tested as one of the oils for lipase production by *Burkholderia cepacia* [44] and in case
308 of *P.camembertii* Thom PG-3 it was found to be the second best inducer for lipase
309 production [51]. Natural oils such as soybean, corn, sunflower, olive, palm and cotton seed
310 oils, amongst others, are cited as inducers of lipase production, comprising at times, the
311 sole source of carbon in the medium [34, 38, 44, 51].
312 Surfactants as lipase inducers in the medium did not enhance lipase production when
313 compared to olive oil (Table 3). Similar effects were reported on lipase production in
314 *Rhizopus* sp. BTNT-2 [3] and *Yarrowia lipolytica* [15]. Surfactants do not always increase
315 lipolytic enzyme production [35] and their effect varies with microorganism, surfactant
316 type and its concentration [15, 52]. However, the addition of surfactant to the culture

317 medium has been shown to increase the secretion of lipolytic enzymes in a number of
318 microorganisms, attributable to alteration of cell permeability leading to increased protein
319 secretion or to surface effects on cell bound enzymes [52]. A wide variety of surfactants
320 like Tweens, Triton, SDS, PEG and gum arabic have been studied by different investigators
321 [15, 35, 52].

322 **6.2.6 Effect of carbon source additives on lipase production**

323 The effect of of carbon source in the basal medium on lipase production by *A. mediterranei*
324 DSM43304 is shown in Table 4. Lipase production and biomass was significantly enhanced
325 with the addition of sugars to the basal medium in most cases. Fructose gave the highest
326 activity of 0.467 ± 0.007 IU/ml with biomass of 11.2 ± 0.9 mg/ml, whereas lactose
327 produced the highest biomass of 14.9 ± 0.4 mg/ml with lipase activity of 0.399 ± 0.021
328 IU/ml. Lipase production was increased 3-fold with lactose, sorbitol, maltose and xylose as
329 carbon source additives. However, addition of arabinose, dextrin, and sucrose had no
330 significant effect on lipase production and showed significant decrease in biomass. In
331 contrast, addition of rhamnose showed increased biomass with no significant improvement
332 in lipase activity. A range of different carbon sources (i.e. carbohydrates, alcohols, acids,
333 lipids) have been reported to support both growth of lipolytic enzyme producers and
334 lipase/esterase production [4, 22]. Although lipidic carbon sources seem to be generally
335 essential for obtaining a high enzyme yield, some authors have indicated good results in the
336 absence of fats and oils [34], whereas in some cases, a mixture of compounds has been
337 proposed as optimum carbon source [14, 18]. Similar to the present study, fructose as
338 carbon source was found to significantly increase lipase activity in *Issatchenkia orientalis*
339 [11]. Lactose was reported to support high to moderate lipase production in various

340 microorganisms [26], which is similar to the present study where lactose led to a significant
341 increase in lipase production.

342 **6.2.7 Effect of nitrogen sources on lipase production**

343 Nitrogen sources, including organic nitrogen and inorganic nitrogen sources play an
344 important role in the biosynthesis of lipase [51]. Lipase production and biomass were found
345 to be highest with phytone peptone (0.131 ± 0.021 IU/ml) and yeast extract (0.104 ± 0.005
346 IU/ml) as organic nitrogen source followed by Bacto-peptone (0.071 ± 0.002 IU/ml) and
347 cornsteep liquor (0.061 ± 0.006 IU/ml) (Table 5). Significant biomass was produced with
348 beef extract (8.4 ± 0.7 mg/ml) and tryptone (8.1 ± 0.9 mg/ml) as nitrogen source but the
349 lipase activities obtained were low. Wheat gluten gave the lowest lipase (0.017 ± 0.001
350 IU/ml) yield followed by casein and casein hydrolysate. Similar studies were carried out by
351 other investigators to find the best nitrogen source for lipase production [3, 22]. Soybean
352 meal was reported to be the best nitrogen source for lipase production by *P. camembertii*
353 *PG-3* [51]. Higher lipase production was reported using yeast extract as nitrogen source for
354 *Saccharomyces cerevisiae* [48]. Contrary to the present results, casein and corn gluten were
355 the best sources for lipase production by *A. terreus* [22].

356 Phytone peptone with yeast extract was identified as the best nitrogen sources for lipase
357 production by *A. mediterranei* DSM 43304. The effect of different concentrations of
358 phytone peptone and yeast extract on lipase production is shown in Table 5. There was no
359 significant difference in terms of lipase activities and biomass production by different
360 concentrations of phytone peptone and yeast extract in the basal medium. By contrast, a
361 similar study identified a combination of 0.6% tryptone and 0.2% yeast extract as the best
362 nitrogen source for lipase production by *Bacillus* sp. strain 42 [16]. The effect of different

363 inorganic nitrogen sources showed $(NH_4)_2HPO_4$ as the best inorganic nitrogen source
364 producing maximum lipase activity followed by $NaNO_3$ (Table 6). In agreement with the
365 present observation, higher specific activity of *Burkholderia cepacia* lipase was observed in
366 presence of $(NH4)_2HPO_4$ as inorganic nitrogen source followed by KNO_3 [44]. Among the
367 inorganic nitrogen sources tested for *Psedomonas* sp. G6, $NaNO_3$ supported the best lipase
368 production [27]. Urea showed the lowest lipase and biomass yield followed by NH_4Cl.
369 Urea was also reported to be inhibitory for lipase synthesis by *P. camembertii* Thom PG-3
370 [51].

371 **6.2.8 Effect of metal ions on lipase production**

372 Besides physical and nutritional parameters, metal ions may play an important role in lipase
373 production [39]. The effect of different metal ions and metal ion combinations on lipase
374 production is shown in Table 4. Metal ions did not make any significant difference to lipase
375 activity and biomass production. Contrary to the present results, iron was reported to be
376 critical for the production of lipase by *Psedomonas* sp. G6 [27] and Ca^{2+} was reported to
377 have strong stimulatory effect on extracellular lipase production by *P. fluorescens* 2D [36].
378 Similarly, Ca^{2+} in presence of Mg^{2+} was reported to produce a significant increase in lipase
379 production by *Burkholderia cepacia* [44].

380 **6.2.9 Lipase production in modified medium**

381 The extent of improvement in lipase activity yield by fermentation using the optimal
382 culture parameters was investigated. The modified culture medium for lipase production by
383 *A mediterranei* DSM 43304 consisted of 1% (v/v) linseed oil, 1% (w/v) fructose, 0.05%
384 (w/v) $(NH_4)_2HPO_4$, 0.05% (w/v) KCl, 0.05% (w/v) $MgSO_47H_2O$, 0.2% (w/v) KH_2PO_4,
385 0.5% (w/v) phytone peptone, 0.1% (w/v) yeast extract, initial pH 7.5 and the culture was

386 incubated at 28 ºC for 96 h. The lipase activity produced increased from 0.108 ± 0.002
387 IU/ml (3.80 ± 0.14 IU/mg) in the basal medium to 1.372 ± 0.103 IU/ml (7.27 ± 0.52
388 IU/mg) in the modified medium. Thus, a 12-fold increase in lipase activity yield and 2-fold
389 increase in lipase specific activity was achieved by employing newly formulated production
390 medium.

391 **7. Characterization of lipase**

392 **7.1 Effect of temperature on activity and stability**

393 The effect of temperature on the activity and stability of lipase is shown in Figure 5. The
394 lipase from *A. mediterranei* DSM 43304 was found to be quite thermostable with a
395 temperature optimum of 60 ºC at pH 8.0. It retained 90% of activity at 60 ºC after 3 h
396 incubation and had a half-life of more than 30 min at 70 ºC. High thermal stability of
397 lipolytic activity detected in this study may be useful for several industrial applications.
398 Bacterial lipases generally have temperature optima in the range 30–60 ºC. However,
399 reports exist of bacterial lipases with optima both in lower and higher ranges [24]. Thermal
400 stability of a lipase is obviously related to its tertiary structure and is influenced by
401 environmental factors such as pH and the presence of metal ions [62]. A few thermostable
402 lipases from actinomycetes have been reported. *S. fradiae* var. k11 was reported to produce
403 a proteolysis-resistant lipase having a temperature optimum of 55 ºC [60] and a
404 thermostable lipase, most active at 50–60 ºC, was purified from *S. rimosus* [1]. Two
405 different lipases from *S. coelicolor* A3(2) were characterized showing temperature optima
406 at 20–30 ºC (SCO1725) and 45–55 ºC (SCO7513) [12].

407 **7.2 Effect of pH on activity and stability**

408 Figure 6 shows the effect of pH on lipolytic activity and stability. The lipase was found to
409 be active over the pH range 5.0–8.0. Maximal lipolytic activity was at pH 8.0 and showed
410 retention of 88%, 98%, 95% and 89% activity at pH 5.0, 6.0, 7.0 and 9.0, respectively. The
411 pH stability profile showed the highest stability at pH 8.0 and 9.0 with 96% residual
412 activity. The activity was also stable at pH 6.0 and 7.0 showing 94% residual activity after
413 24 h. The stability of the enzyme in acidic and alkaline pH suggests its usefulness in
414 industrial applications. Generally, lipases of bacterial origin have neutral or alkaline pH
415 optima with the exception of *P. fluorescence* SIK W1 lipase which has an acidic pH
416 optimum of 4.8 [2]. Most of the lipases characterized from actinomycetes so far show
417 alkaline pH optima. *S. rimosus* lipase was reported to show a pH optimum range of 9.0–
418 10.0 [1] and *S. fradiae* var. k11 lipase was most active at pH 9.8 [60]. Similarly, *S.*
419 *coelicolor* A3(2) lipases showed pH optima between 7.5–10.0 [12].

420 **7.3 Effect of organic solvents on lipase stability**

421 The effect of various organic solvents on the stability of *A. mediterranei* DSM 43304 lipase
422 is shown in Table 6. The lipase exhibited considerable stability in the presence of polar
423 solvents (log$P$ < 0.3) as well as non-polar hydrophobic solvents (log$P$ 0.85–6.6) with
424 significant activation observed in most cases. The highest degree of activation was in
425 benzene and toluene. Lipase was significantly activated after 1 h in *p*-xylene and *n*-hexane
426 showing a 61.9% and 56.7% increase in activity, respectively. Further incubation for 24 h
427 led to 28.2% and 45% decrease in activation indicating destabilizing effects of these
428 solvents. The lipase was least stable in pyridine and showed 49.8% residual activity after 24
429 h. Similarly, a destabilizing effect was seen with *t*-butanol, dodecane and DMSO. It has
430 been shown that many enzymes retain activity in organic solvents [59] and have interesting

431 catalytic properties such as higher thermostability and altered stereoselectivity [54]. Despite
432 many advantages of enzymatic reactions in organic solvents, in most cases the catalytic
433 activity in organic solvents is orders of magnitude lower than in aqueous systems [10]
434 because of diffusional limitations, changes in protein flexibility, or destabilization of the
435 enzyme [28]. *A. mediterranei* DSM 43304 lipase was activated and stable in DMF,
436 methanol, ethanol, 2-propanol and acetone. Though solvent stability has been reported for a
437 few actinomycete lipases [5, 8, 32], the high stability and indeed, activation in polar
438 solvents like methanol and ethanol has rarely been observed [8]. *S. rimosus* lipase was
439 reported to show stimulation of activity upon addition of 2.5% (v/v) 1,4-dioxane, THF,
440 acetone and DMF [32]. Similarly, *S. coelicolor* lipase was found to be stable with different
441 water-miscible solvents and showed 50% increase in activity in acetone after 18 h and
442 similar to the present study, significant loss of activity was observed with DMSO, while in
443 contrast, DMF led to almost complete loss of activity [5]. It is reported that polar solvents
444 strip off the essential water molecules from the active site of enzymes [21]. For this reason,
445 use of polar solvents is avoided and hydrophobic solvents are more often employed in non-
446 aqueous enzymology [20]. The polar solvent tolerant lipases therefore appear promising for
447 catalysis in low water media. This property is a novel attribute of *A. mediterranei* DSM
448 43304 lipase, which has been reported in few cases [5, 32, 61]. The high stability and
449 activation of *A. mediterranei* DSM 43304 lipase in a wide range of polar and non-polar
450 organic solvents may make it useful for practical applications in synthetic
451 biotransformation reactions.

452 **7.4 Effect of metal ions and effectors on lipase stability**

453 The effect of different metal salts and effector molecules on *A. mediterranei* DSM 43304
454 lipase is shown in Table 7. The crude lipase was strongly inhibited by Hg^{2+} losing 80% of
455 activity in 10 min suggesting it is able to alter enzyme conformation as has been reported
456 for other lipases [9]. Li^{+} and Mg^{2+} also significantly reduced enzyme activity. The other
457 metal ions tested did not produce adverse effect on the activity of the enzyme. Compared to
458 the control, Ca^{2+} did not cause significant reduction in lipase, even though Ca^{2+} has been
459 reported to stabilize lipolytic activity [9, 20]. Cu^{2+} has been reported to be a strong inhibitor
460 of lipase activity [19, 26], but no inhibition was observed with Cu^{2+} at a concentration of 1
461 mM under the conditions tested. Similarly, *S. fradiae* var. k11 lipase was reported to retain
462 99.4% lipase activity in the presence of 1 mM Cu^{2+} for 30 min [60]. Cu^{2+} did not
463 significantly reduce the lipolytic activity of SCO1725 and SCO7513 from *S. coelicolor*
464 A3(2) [12]. The lipase activity was unaffected by the metal-chelating agent EDTA
465 indicating that *A. mediterranei* DSM 43304 lipase is probably not a metalloenzyme. The
466 activities of lipases from *S. rimosus* and *S. fradiae* var. k11 have also been reported to be
467 unaffected by EDTA [1, 60]. The effect of a serine inhibitor PMSF, at 1 mM concentration
468 gave a 15% reduction in lipase activity possibly suggesting the presence of a hydrophobic
469 lid hindering access to the catalytic site [12]. The extracellular lipase from *S. rimosus* was
470 found to be marginally affected by 1 mM PMSF showing retention of 90% of residual
471 activity [1]. Similarly, 89–91% residual activity was retained in presence of 1 mM PSMF
472 by *S. coelicolor* A3(2) lipases [12]. The lipase showed 88% retention of activity in presence
473 of 1 mM urea. SDS was found not to affect lipase activity. In agreement with the present
474 study lipase activity was reported to be stable with 79% residual activity in the presence of
475 1 mM SDS by *B. cepacia* lipase [57], whereas *Yarrowia lipolytica* lipase showed

476 significant inhibition of lipase activity with 1 mM SDS [58]. Contrary to the present results,
477 lipase from *B. thermoleovorans* CCR11 was reported to be completely inhibited by 1 mM
478 SDS in 1 h [9].

479 **8. Conclusions**

480 A lipolytic actinomycete strain, *Amycolatopsis mediterranei* DSM 43304 was identified as
481 producing an extracllular organic solvent-tolerant thermostable lipase. A significant 12-fold
482 increase in lipase activity and 2-fold increase in lipase specific activity was achieved by
483 employing the newly formulated culture medium. The extracellular lipase was highly stable
484 in a broad range of polar and non-polar organic solvents, with maximum enhancement of
485 activity in non-polar solvents. The properties of thermostability, stability and enhancement
486 of activity in organic solvents, and stability with a broad range of effector molecules shows
487 that this lipase may be a promising enzyme for applications in a range of *in vitro*
488 biotransformation reactions.

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676 **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}

697 Data are means ± standard deviations of three determinations. Column data followed by the same superscript

698 letter were not significantly different ($p \leq 0.05$; by Tukey test)

699 *The RBA plates, after 6 days incubation at 28 °C, were exposed to UV (350 nm) to detect fluorescence:

700 orange fluorescence (+); no orange fluorescence (–).

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

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711 **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).

739 **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

763 The shake flask experiments were performed for 96 h at 28 ºC, 130 rpm. Data are means ± standard

764 deviations of three determinations. Column data followed by the same superscript letter were not significantly

765 different ($p \leq 0.05$; by Tukey test).

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

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776 **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

804 The shake flask experiments were performed for 96 h at 28 °C, 130 rpm. Data are means ± standard

805 deviations of three determinations. Column data followed by the same superscript letter were not significantly

806 different ($p \leq 0.05$; by Tukey test).

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

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

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816 **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

849 The shake flask experiments were performed for 96 h at 28 °C, 130 rpm. Data are means ± standard deviations of three

850 determinations. Column data followed by the same superscript letter were not significantly different ($p \leq 0.05$; by Tukey test).

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

852 ** 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.

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

854 **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]

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

877 Values represent the mean of three replicates.

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

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902 **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]

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

904 Values represent the mean of three replicates.

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

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953 **Figure captions:**

954 **Figure 1**.

955 Screening for lipolytic actinomycete strains on rhodamine B agar.

956 **a.** *Amycolatopsis coloradensis* DSM 44225; **b.** *A. amakusaensis* NRRL B-3351; **c.** *A.*

957 *fastidiosa* DSM 43855; **d.** *A. mediterranei* DSM 43304; **e.** *A. rubida* DSM 44637; **f.** *A.*

958 *sulphurea* DSM 46092; **g.** *Nocardia araoensis* DSM 44729; **h.** *N. higoensis* DSM 44732; **i.**

959 *N. kruckzakiae* DSM 44877; **j.** *Streptomyces amquistii* NRRL b-1685, **k.** *S. griseus* subsp.

960 *griseus* DSM 40236; **l.** *S. coelicolor* A3(2); **m.** *S. annulatus* NRRL B-2000; **n.** *S. arabicus*

961 NRRL B-1733; **o.** *S. aurantiogriseus* NRRL B-5416; **p.** *S. rochei* DSM 40231; **q.** *S.*

962 *aureoverticillatus* NRRL B-3326; **r.** *S. althioticus* NRRL B-3981

963 **Figure 2**.

964 Time course of lipase production by *A. mediterranei* DSM 43304. Basal medium was

965 seeded with 10% (v/v) inoculum and incubated at 28 °C for 192 h at 130 rpm. Samples

966 were withdrawn at 24 h intervals to monitor biomass (■), pH (○) and lipase activity (□) in

967 cell-free supernatants.

968 **Figure 3**.

969 Effect of incubation temperature on lipase production (□) and biomass (■) during growth of

970 *A. mediterranei* DSM 43304 in shake flask cultures after 96 h of incubation at temperatures

971 varying from 20–45 °C.

972 **Figure 4**.

973 Effect of initial pH on lipase production (□) and biomass (■) in shake flask cultures after 96

974 h of cultivation with initial medium pH varying from 5.0 to 9.0.

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976 **Figure 5**.

977 Effect of temperature on *A. mediterranei* DSM 43304 lipase activity (■) and stability (□).
978 Assay conditions: 30–90 °C, 50 mM Tris–HCl buffer pH 8.0. Thermal stability of the
979 enzyme was studied by incubating the enzyme at various temperatures (30, 40, 50, 60, 70,
980 80 and 90 °C) for 3 h. Residual activity (%) at each temperature was calculated relative to
981 that at 0 h as 100%.

982 **Figure 6**.

983 Effect of pH on *A. mediterranei* DSM 43304 lipase activity (■) and stability (□).
984 For stability studies, residual activities were measured after 24 h incubation at 4 ºC in the
985 presence of different buffers: HCl–KCl (pH 2.0), sodium citrate (pH 3.0), succinate–NaOH
986 (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), Tris–HCl (pH 8.0 and 9.0) and
987 glycine–NaOH (pH 10.0), all buffers at 50 mM concentration. Assay conditions: 60 °C,
988 Tris–HCl buffer, pH 8.0, 50 mM. The activities were compared to the activity determined
989 in 50 mM Tris–HCl buffer, pH 8.0 without pre-incubation.

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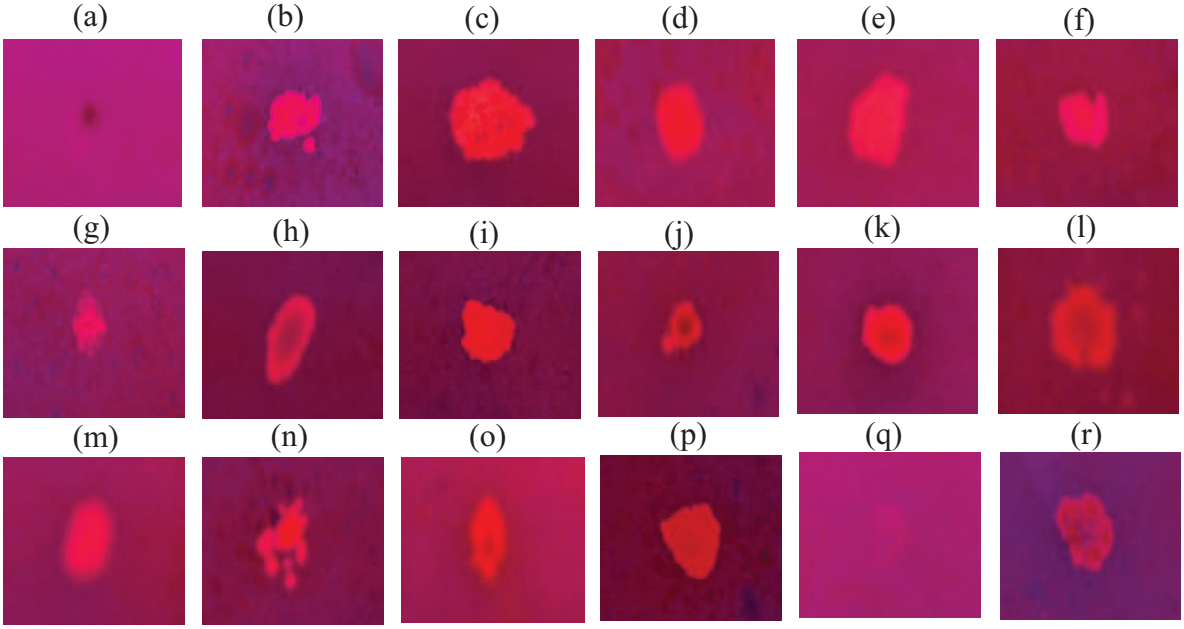
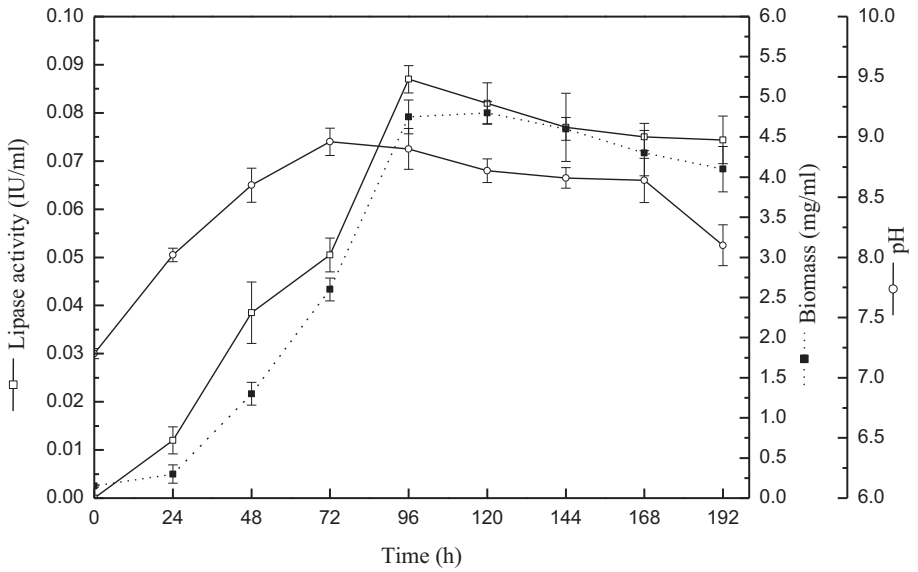
Figure 1.



1022 **Figure 2.**



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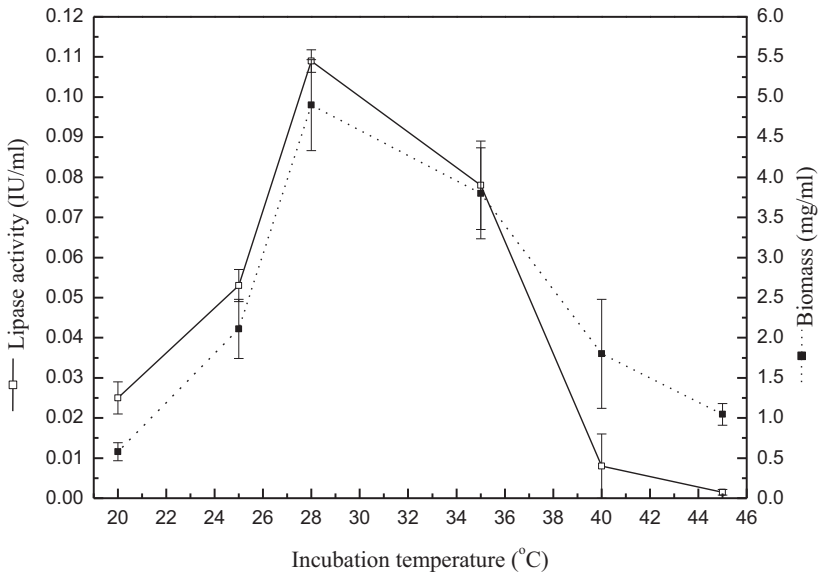
1036 **Figure 3.**



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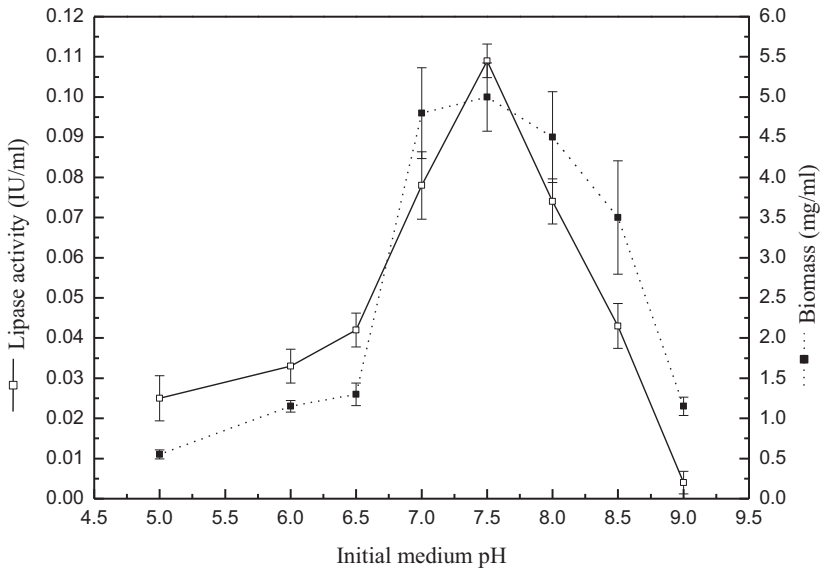
1050 **Figure 4.**



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1064 **Figure 5.**

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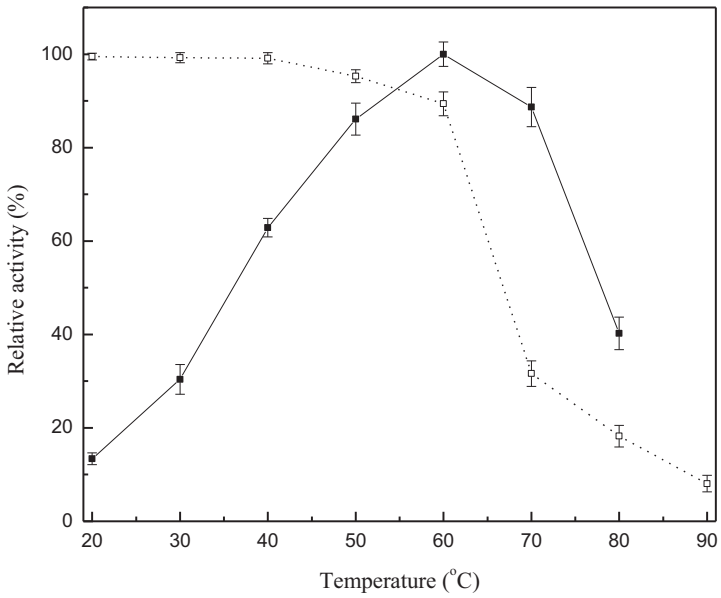

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1078 **Figure 6.**

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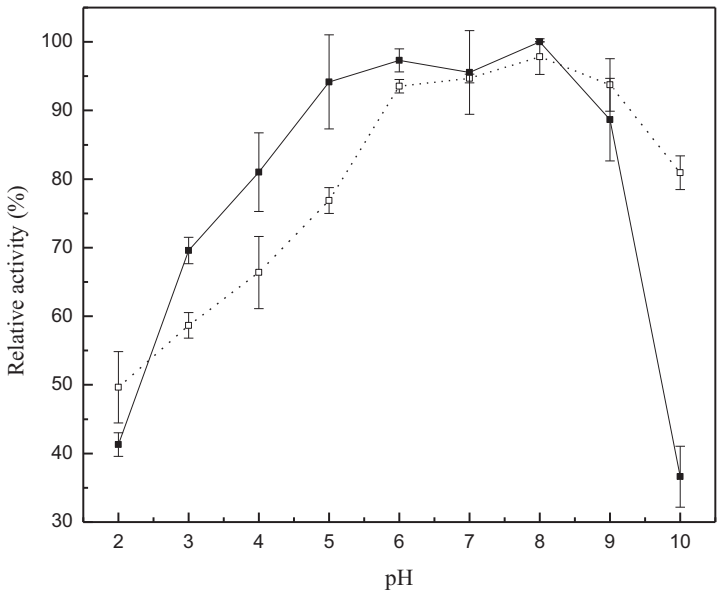

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