



2010-01-01

# Influence of Cultivation Conditions on the Production of a Thermostable Extracellular Lipase from *Amycolatopsis Mediterranei* DSM 43304

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## Recommended Citation

Dheeman D.S., Frias J.M, Henehan, G.T.M. (2010) Influence of Cultivation Conditions on the Production of a Thermostable Extracellular Lipase from *Amycolatopsis Mediterranei* DSM 43304. *Journal of Industrial Microbiology & Biotechnology*, 37, (1),pp. 1-17. doi:10.1007/s10295-009-0643-7 doi:10.1007/s10295-009-0643-7

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

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23 **Abstract**

24 Among several lipase producing actinomycete strains screened, *Amycolatopsis*  
25 *mediterranei* DSM 43304 was found to produce a thermostable, extracellular lipase.  
26 Culture condition and nutrient source modification studies involving carbon sources,  
27 nitrogen sources, incubation temperature and medium pH were carried out. Lipase activity  
28 of  $1.37 \pm 0.103$  IU/ml of culture medium was obtained in 96 h at 28 °C and pH 7.5 using  
29 linseed oil and fructose as carbon sources and a combination of phytone peptone and yeast  
30 extract (5:1) as nitrogen sources. In optimal culture conditions the lipase activity was  
31 enhanced 12-fold with a 2-fold increase in lipase specific activity. The lipase showed  
32 maximum activity at 60 °C and pH 8.0. The enzyme was stable between pH 5.0–9.0 and  
33 temperatures up to 60 °C. Lipase activity was significantly enhanced by  $\text{Fe}^{3+}$  and strongly  
34 inhibited by  $\text{Hg}^{2+}$ .  $\text{Li}^+$ ,  $\text{Mg}^{2+}$  and PMSF significantly reduced lipase activity, whereas other  
35 metal ions had no significant effect at 0.01 M concentration. *A. mediterranei* DSM 43304  
36 lipase exhibited remarkable stability in the presence of a wide range of organic solvents at  
37 25% (v/v) concentration for 24h. These features render this novel lipase attractive for  
38 potential biotechnological applications in organic synthesis reactions.

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40 **Keywords:** *Amycolatopsis mediterranei*; Screening; Organic solvent-tolerant;

41 Thermostable; Lipase

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## 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<sub>3</sub> 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<sub>3</sub> 0.5 g; KCl 0.5 g; MgSO<sub>4</sub>·7H<sub>2</sub>O  
98 0.5 g; KH<sub>2</sub>PO<sub>4</sub> 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 °C 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<sub>2</sub>CO<sub>3</sub> 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 ( $\epsilon_{410\text{nm}} = 16,900 \text{ M}^{-1} \text{ cm}^{-1}$ ) 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  $\mu\text{M}$  of *p*-NP per minute under the  
143 conditions of the assay.

#### 144 **2.8 Biomass concentration analysis**

145 After centrifugation at 10,000  $\times g$ , 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<sub>3</sub> in  
195 the basal medium was substituted with: NH<sub>4</sub>Cl, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>,  
196 CH<sub>3</sub>COONH<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub> 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<sup>2+</sup>, Ca<sup>2+</sup> and Fe<sup>3+</sup> salts were incorporated into the basal  
200 medium at 0.05% (w/v) concentration. Also combinations of Mg<sup>2+</sup> and Ca<sup>2+</sup>, Ca<sup>2+</sup> and Fe<sup>3+</sup>,  
201 and Mg<sup>2+</sup> and Fe<sup>3+</sup> 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 ( $\text{Ag}^+$ ,  $\text{Ba}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Li}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ ,  
228  $\text{Pb}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Hg}^{2+}$ ) and effectors (PMSF, EDTA, SDS,  $\text{NH}_4^+$  and  
229 urea). Incubation was carried out at 60 °C 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  $0.149 \pm 0.017$  IU/ml,  $0.141 \pm 0.004$

241 IU/ml and  $0.116 \pm 0.010$  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  $7.20 \pm 0.02$  to  $8.02 \pm 0.057$  during the first 24 h of incubation, rising to pH  $8.96 \pm$   
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 ( $0.105 \pm 0.003$  IU/ml) and biomass ( $5.8 \pm 0.6$   
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  $20\text{--}45$  °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  $28\text{--}35$  °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 *Pseudomonas 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 mycooides* 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  $0.014 \pm 0.001$   
303 IU/ml to  $0.128 \pm 0.004$  IU/ml. The highest lipase production ( $0.128 \pm 0.004$  IU/ml) and  
304 biomass ( $16.0 \pm 1.4$  mg/ml) was found using linseed oil followed by sunflower oil ( $0.104 \pm$   
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  $0.467 \pm 0.007$  IU/ml with biomass of  $11.2 \pm 0.9$  mg/ml, whereas lactose  
327 produced the highest biomass of  $14.9 \pm 0.4$  mg/ml with lipase activity of  $0.399 \pm 0.021$   
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 ( $0.131 \pm 0.021$  IU/ml) and yeast extract ( $0.104 \pm 0.005$   
346 IU/ml) as organic nitrogen source followed by Bacto-peptone ( $0.071 \pm 0.002$  IU/ml) and  
347 cornsteep liquor ( $0.061 \pm 0.006$  IU/ml) (Table 5). Significant biomass was produced with  
348 beef extract ( $8.4 \pm 0.7$  mg/ml) and tryptone ( $8.1 \pm 0.9$  mg/ml) as nitrogen source but the  
349 lipase activities obtained were low. Wheat gluten gave the lowest lipase ( $0.017 \pm 0.001$   
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  $(\text{NH}_4)_2\text{HPO}_4$  as the best inorganic nitrogen source  
364 producing maximum lipase activity followed by  $\text{NaNO}_3$  (Table 6). In agreement with the  
365 present observation, higher specific activity of *Burkholderia cepacia* lipase was observed in  
366 presence of  $(\text{NH}_4)_2\text{HPO}_4$  as inorganic nitrogen source followed by  $\text{KNO}_3$  [44]. Among the  
367 inorganic nitrogen sources tested for *Pseudomonas* sp. G6,  $\text{NaNO}_3$  supported the best lipase  
368 production [27]. Urea showed the lowest lipase and biomass yield followed by  $\text{NH}_4\text{Cl}$ .  
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 *Pseudomonas* sp. G6 [27] and  $\text{Ca}^{2+}$  was reported to  
377 have strong stimulatory effect on extracellular lipase production by *P. fluorescens* 2D [36].  
378 Similarly,  $\text{Ca}^{2+}$  in presence of  $\text{Mg}^{2+}$  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)  $(\text{NH}_4)_2\text{HPO}_4$ , 0.05% (w/v)  $\text{KCl}$ , 0.05% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2% (w/v)  $\text{KH}_2\text{PO}_4$ ,  
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 °C for 96 h. The lipase activity produced increased from  $0.108 \pm 0.002$   
387 IU/ml ( $3.80 \pm 0.14$  IU/mg) in the basal medium to  $1.372 \pm 0.103$  IU/ml ( $7.27 \pm 0.52$   
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 °C at pH 8.0. It retained 90% of activity at 60 °C after 3 h  
396 incubation and had a half-life of more than 30 min at 70 °C. 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 °C. 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 °C [60] and a  
404 thermostable lipase, most active at 50–60 °C, was purified from *S. rimosus* [1]. Two  
405 different lipases from *S. coelicolor* A3(2) were characterized showing temperature optima  
406 at 20–30 °C (SCO1725) and 45–55 °C (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  $\text{Hg}^{2+}$  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].  $\text{Li}^+$  and  $\text{Mg}^{2+}$  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,  $\text{Ca}^{2+}$  did not cause significant reduction in lipase, even though  $\text{Ca}^{2+}$  has been  
459 reported to stabilize lipolytic activity [9, 20].  $\text{Cu}^{2+}$  has been reported to be a strong inhibitor  
460 of lipase activity [19, 26], but no inhibition was observed with  $\text{Cu}^{2+}$  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  $\text{Cu}^{2+}$  for 30 min [60].  $\text{Cu}^{2+}$  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 extracellular 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.

## 489 **Acknowledgements**

490 This work was supported by ABBEST Research Scholarship from Dublin Institute of  
491 Technology (PB 03557/2007). The authors want to express their thanks to Prof. Michael  
492 Goodfellow and Dr. Amanda L. Jones, School of Biology, Newcastle University, UK for  
493 providing the strains for this study.

## 494 **References:**

- 495 1. Abramic M, Lescic I, Korica T, Vitale L, Saenger W, Pigac J (1999) Purification  
496 and properties of extracellular lipase from *Streptomyces rimosus*. *Enzym Microb*  
497 *Technol* 25:522–529

- 498 2. Andersson RE, Hedlund GB, Jensson V (1979) Thermal inactivation of a heat  
499 resistant lipase produced by the psychrotrophic bacterium *Pseudomonas*  
500 *fluorescens*. J Dairy Sci 62:361–367
- 501 3. Bapiraju KVVSN, Sujatha P, Ellaiah P, Ramana T (2005) Sequential parametric  
502 optimisation of lipase production by a mutant strain *Rhizopus* sp, BTNT-2. J Basic  
503 Microbiol 45:257–273
- 504 4. Benjamin S, Pandey A (1996) Optimization of liquid media for lipase production  
505 by *Candida rugosa*. Bioresour Technol 55:167–170
- 506 5. Bielen A, Cetkovic H, Long PF, Schwab H, Abramic M, Vujaklija D (2009) The  
507 SGNH-hydrolase of *Streptomyces coelicolor* has (aryl)esterase and a true lipase  
508 activity. Biochimie 91:390–400
- 509 6. Bormann C, Nikoleit K, Potgeter M, Tesch C, Sommer P, Goetz F (1993)  
510 Investigation of lipolytic enzymes from *Streptomyces*. In: DECHEMA-  
511 monographs. VCH, Weinheim, Germany, pp 237–247
- 512 7. Boutaiba S, Bhatnagar T, Hacene H, Mitchell DA, Baratti JC (2006) Preliminary  
513 characterisation of a lipolytic activity from an extremely halophilic archaeon,  
514 *Natronococcus* sp. J Mol Catal B: Enzym 41:21–26
- 515 8. Cardenas F, Castro MS, Sanchez-Montero JM, Sinisterra JV, Valmaseda M, Elson  
516 SW, Alvarez E (2001) Novel microbial lipases: catalytic activity in reactions in  
517 organic media. Enzym Microb Technol 28:145–154
- 518 9. Castro-Ochoa LD, Rodriguez-Gomez C, Valerio-Alfaro G, Ros RO (2005)  
519 Screening, purification and characterization of the thermoalkalophilic lipase



- 520 produced by *Bacillus thermoleovorans* CCR11. *Enzyme Microb Technol* 37:648–  
521 654
- 522 10. Clark DS (2004) Characteristics of nearly dry enzymes in organic solvents:  
523 implications for biocatalysis in the absence of water. *Philos T Roy Soc B*  
524 359:1299-1307
- 525 11. Costas M, Deive FJ, Longo MA (2004) Lipolytic activity in submerged cultures  
526 of *Issatchenkia orientalis*. *Process Biochem* 39:2109–2114
- 527 12. Cote A, Shareck F (2008) Cloning, purification and characterization of two lipases  
528 from *Streptomyces coelicolor* A3(2). *Enzym Microb Technol* 42:381–388
- 529 13. Cruz H, Perez C, Wellington E, Castro C, Servin-Gonzalez L (1994) Sequence of  
530 the *Streptomyces albus* G lipase-encoding gene reveals the presence of a  
531 prokaryotic lipase family. *Gene* 144:141–142
- 532 14. Dalmau E, Montesinos JL, Lotti M, Casas C (2000) Effect of different carbon  
533 sources on lipase production by *Candida rugosa*. *Enzyme Microb Technol*  
534 26:657–663
- 535 15. Dominguez A, Deive FJ, Sanroman MA, Longo MA (2003) Effect of lipids and  
536 surfactants on extracellular lipase production by *Yarrowia lipolytica*. *J Chem*  
537 *Technol Biotechnol* 78:1166–1170
- 538 16. Eltaweel MA, Rahman RNZRA, Salleh AB, Basri M (2005) An organic solvent-  
539 stable lipase from *Bacillus* sp. strain 42. *Ann Microbiol* 55:187–192
- 540 17. Ema T (2004) Rational strategies for highly enantioselective lipase-catalyzed  
541 kinetic resolutions of very bulky chiral compounds: Substrate design and high-  
542 temperature biocatalysis. *Tetrahedron: Asymmetry* 15:2765–2770

- 543 18. Gandolfi R, Marinelli F, Lazzarini A, Molinari F (2000) Cell-bound and  
544 extracellular carboxylesterases from *Streptomyces*: hydrolytic and synthetic  
545 activities. J App Microb 89: 870–875
- 546 19. Gao XG, Cao SG, Zhang KC (2000) Production, properties and application to  
547 nonaqueous enzymatic catalysis of lipase from a newly isolated *Pseudomonas*  
548 strain. Enzym Microb Technol 27:74–82
- 549 20. Gaur R, Gupta A, Khare SK (2008) Purification and characterization of lipase  
550 from solvent tolerant *Pseudomonas aeruginosa* PseA. Process Biochem 43:1040–  
551 1046
- 552 21. Gorman LAS, Dordick JS (1992) Organic solvents strip water off enzymes.  
553 Biotechnol Bioeng 39:392–397
- 554 22. Gulati R, Saxena RK, Gupta R, Yadav RP, Davidson WS (1999) Parametric  
555 optimisation of *Aspergillus terreus* lipase production and its potential in ester  
556 synthesis. Process Biochem 35:459–464
- 557 23. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial  $\alpha$ -  
558 amylases: a biotechnological perspective. Process Biochem 38:1599–1616
- 559 24. Gupta R, Gupta N, Rathi P (2004) Bacterial lipases: an overview of production,  
560 purification and biochemical properties. Appl Microbiol Biotechnol 64:763–781.
- 561 25. Haba E, Bresco D, Ferrer C, Margues A, Busquets M, Manresa A (2000) Isolation  
562 of lipase-secreting bacteria by deploying used frying oil as selective substrate.  
563 Enzym Microb Technol 26:40–44

- 564 26. Kamini NR, Fujii T, Kurosu T, Iefuji H (2000) Production, purification and  
565 characterization of an extracellular lipase from *Cryptococcus* sp. S-2. Process  
566 Biochem 36:317–324
- 567 27. Kanwar L, Gogoi BK, Goswami P (2002) Production of a *Pseudomonas* lipase in  
568 *n*-alkane substrate and its isolation using an improved ammonium sulphate  
569 precipitation technique. Bioresour Technol 84:207–211
- 570 28. Klibanov AM (1997) Why are enzymes less active in organic solvents than in  
571 water? Trends Biotechnol 15:97–101
- 572 29. Kouker G, Jaeger KE (1987) Specific and sensitive plate assay for bacterial  
573 lipases. Appl Environ Microbiol 53:211–213
- 574 30. Large KP, Mirjalili N, Osborne M, Peacock LM, Zormpaidis V, Walsh M,  
575 Cavanagh ME, Leadlay PF, Ison AP (1999) Lipase activity in *Streptomyces*.  
576 Enzym Microb Technol 25:569–575
- 577 31. Lee M-H, Lee CH, Oh TK, Song JK, Yoon JH (2006) Isolation and  
578 characterization of a novel lipase from a metagenomic library of tidal flat  
579 sediments: evidence for a new family of bacterial lipases. Appl Environ Microbiol  
580 72:7406–7409
- 581 32. Lescic I, Vukelic B, Majeric-Elenkov M, Saenger W, Abramic M (2001)  
582 Substrate specificity and effects of water-miscible solvents on the activity and  
583 stability of extracellular lipase from *Streptomyces rimosus*. Enzym Microb  
584 Technol 29: 548–553

- 585 33. Lima VMG, Krieger N, Mitchell DA, Baratti JC, Filippis I, Fontana JD (2004)  
586 Evaluation of the potential for use in biocatalysis of a lipase from a wild strain of  
587 *Bacillus megaterium*. J Mol Catal B: Enzym 31:53–61
- 588 34. Lin ES, Ko HC (2005) Glucose stimulates production of the alkaline-thermostable  
589 lipase of the edible basidiomycete *Antrodia cinnamomea*. Enzyme Microb  
590 Technol 37:261–265
- 591 35. Lin SF, Chiou CM, Tsai YC (1995) Effect of Triton X-100 on alkaline lipase  
592 production by *Pseudomonas pseudoalkaligenes* F-111. Biotechnol Lett 17:959–  
593 962
- 594 36. Makhzoum A, Knapp JS, Owusu RK (1995) Factor affecting growth and lipase  
595 production by *Pseudomonas fluorescens* 2D. Food Microbiol 12:277–290
- 596 37. Mudgetti RE (1986) In: Demain AL, Solmen NA (ed) Manual of industrial  
597 biotechnology. Washington, DC: American Society for Microbiology, pp 66–83
- 598 38. Muralidhar RV, Chirumamila RR, Marchant R, Nigam P (2001) A response  
599 surface approach for the comparison of lipase production from *Candida*  
600 *cylindracea* using two different carbon sources. Biochem Eng J 9:17–23
- 601 39. Obido FJC, Okereke UO, Oyeka CA (1995) Influence of culture conditions on the  
602 production of lipase of *Hendersonula toruloidea*. Bioresour Technol 54:81–83.
- 603 40. Ogino H, Ishikawa H (2001) Enzymes which are stable in the presence of organic  
604 solvents. J Biosci Bioeng 91:109–16
- 605 41. Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol VT (1999) The  
606 realm of microbial lipases in biotechnology. Biotechnol Appl Biochem 29:119–  
607 131

- 608 42. Patel RN (2008) Synthesis of chiral pharmaceutical intermediates by biocatalysis.  
609 Coord Chem Rev 252:659–701
- 610 43. Pencreac'h G, Baratti JC (2001) Comparison of hydrolytic activity in water and  
611 heptane for thirty-two commercial lipase preparations. Enzym Microb Technol  
612 28:473–479
- 613 44. Rathi P, Saxena RK, Gupta R (2001) A novel alkaline lipase from *Burkholderia*  
614 *cepacia* for detergent formulation. Process Biochem 37:187–192
- 615 45. Ruchi G, Anshu G, Khare SK (2008) Lipase from solvent tolerant *Pseudomonas*  
616 *aeruginosa* strain: production optimization by response surface methodology and  
617 application. Bioresour Technol 99:4796–4802
- 618 46. Schmid RD, Verger R (1998) Lipases: interfacial enzymes with attractive  
619 applications. Angew Chem Int Ed Engl 37:1608–1633
- 620 47. Servin-Gonzalez L, Castro C, Perez C, Rubio M, Valdez F (1997) bldA-  
621 dependent expression of the *Streptomyces exfoliatus* M11 lipase gene (lipA) is  
622 mediated by the product of a contiguous gene, lipR, encoding a putative  
623 transcriptional activator. J Bacteriol 179:7816–7826
- 624 48. Shirazi SH, Rahman SR, Rahman MM (1998) Production of extracellular lipases  
625 by *Saccharomyces cerevisiae*. World J Microbiol Biotechnol 14:595–597.
- 626 49. Swift S, Throup JP, Williams P, Salmond GP, Stewart GS (1996) Quorum  
627 sensing: a population-density component in the determination of bacterial  
628 phenotype. Trend Biochem Sci 21:214–219
- 629 50. Sztajer H, Maliszewka I, Wieczorek J (1988) Production of exogenous lipases by  
630 bacteria, fungi and actinomycetes. Enzym. Microb. Technol 10:492–497

- 631 51. Tan T, Zhang M, Xu J, Zhang J (2004) Optimization of culture conditions and  
632 properties of lipase from *Penicillium camembertii* Thom PG-3. *Process Biochem*  
633 39:1495–1502
- 634 52. Tan TW, Zhang M, Wang BW, Ying CH, Deng L (2003) Screening of high lipase  
635 producing *Candida* sp. and production of lipase by fermentation. *Process*  
636 *Biochem* 39:459–465
- 637 53. Thomas A, Mathew M, Valsa AK, Mohan S, Manjula R (2003) Optimisation of  
638 growth conditions for the production of extracellular lipase by *Bacillus mycoides*  
639 *Ind J Microbiol* 43:67–69
- 640 54. Wescott CR, Klibanov AM (1994) The solvent dependence of enzyme specificity.  
641 *Biochim Biophys Acta* 1206:1–9
- 642 55. Winkler UK, Stuckman M (1979) Glycogen, hyaluronate and some other  
643 polysaccharides greatly enhance the formation of exolipase by *Serratia*  
644 *marcescens*. *J Bacteriol* 138:663–679
- 645 56. Yang FC, Huang HC, Yang MJ (2003) The influence of environmental conditions  
646 on the mycelial growth of *Antrodia cinnamomea* in submerged cultures. *Enzyme*  
647 *Microb Technol* 33:395–402
- 648 57. Yu L, Xu Y, Yu X (2009) Purification and properties of a highly enantioselective  
649 L-menthyl acetate hydrolase from *Burkholderia cepacia*. *J Mol Catal B Enzym*  
650 57:27–33
- 651 58. Yu MR, Qin SW, Tan TW (2007) Purification and characterization of the  
652 extracellular lipase Lip2 from *Yarrowia lipolytica*. *Process Biochem* 42:384–391

- 653 59. Zaks A, Klivanov AM (1985) Enzyme-catalyzed processes in organic solvents.  
654 Proc Natl Acad Sci U S A 82:3192–3196
- 655 60. Zhang Y, Meg K, Wang Y, Luo H, Yang P, Shi P, Wu N, Fan Y, Li J, Yao B  
656 (2008) A novel proteolysis-resistant lipase from keratinolytic *Streptomyces*  
657 *fradiae* var. k11 Enzym Microb Technol 42:346–352
- 658 61. Zhao L, Xu J, Zhao J, Pan J, Wang Z (2008) Biochemical properties and potential  
659 applications of an organic solvent tolerant lipase isolated from *Serratia*  
660 *marcescens* ECU1010. Process Biochem 43:626–633
- 661 62. Zhu K, Jutila A, Patkar EKJ, Svendsen A, Kinnunen PK (2001) Impact of the  
662 tryptophan residues of *Humicola lanuginosa* lipase on its thermal stability. J  
663 Biochim Biophys Acta 1547:329–338
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- 668
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676 **Table 1.** Lipase screening of actinomycete strains in basal medium:

677	Strain	Rhodamine B agar <sup>*</sup>	Lipase activity <sup>**</sup> (IU/ml)
678			
679	<i>Amycolatopsis coloradensis</i> DSM 44225	–	0.046 ± 0.001 <sup>abc</sup>
680	<i>A. amakusaensis</i> NRRL B-3351	+	0.059 ± 0.004 <sup>bc</sup>
681	<i>A. fastidiosa</i> DSM 43855	+	0.040 ± 0.002 <sup>abc</sup>
682	<i>A. mediterranei</i> DSM 43304	+	0.086 ± 0.012 <sup>d</sup>
683	<i>A. rubida</i> DSM 44637	+	0.149 ± 0.017 <sup>f</sup>
684	<i>A. sulphurea</i> DSM 46092	+	0.062 ± 0.008 <sup>cd</sup>
685	<i>Nocardia araoensis</i> DSM 44729	+	0.031 ± 0.002 <sup>a</sup>
686	<i>N. higoensis</i> DSM 44732	+	0.037 ± 0.001 <sup>abc</sup>
687	<i>N. kruckzakiae</i> DSM 44877	+	0.033 ± 0.003 <sup>a</sup>
688	<i>Streptomyces amquistii</i> NRRL B-1685	+	0.046 ± 0.001 <sup>abc</sup>
689	<i>S. griseus</i> subsp. <i>griseus</i> DSM 40236	+	0.116 ± 0.010 <sup>e</sup>
690	<i>S. coelicolor</i> A3(2)	+	0.033 ± 0.001 <sup>a</sup>
691	<i>S. annulatus</i> NRRL B-2000	+	0.051 ± 0.002 <sup>abc</sup>
692	<i>S. arabicus</i> NRRL B-1733	+	0.030 ± 0.001 <sup>a</sup>
693	<i>S. aurantiogriseus</i> NRRL B-5416	+	0.038 ± 0.006 <sup>abc</sup>
694	<i>S. rochei</i> DSM 40231	+	0.141 ± 0.004 <sup>ef</sup>
695	<i>S. aureoverticillatus</i> NRRL B-3326	–	0.034 ± 0.004 <sup>ab</sup>
696	<i>S. althioticus</i> NRRL B-3981	+	0.037 ± 0.004 <sup>ab</sup>

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 <sup>\*</sup>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 <sup>\*\*</sup>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:

712	Inoculum volume (% v/v)	Lipase (IU/ml)	Biomass (mg/ml)	Final pH
713	2	0.055 ± 0.005 <sup>a</sup>	0.7 ± 0.4 <sup>a</sup>	8.66 ± 0.07
714	4	0.064 ± 0.002 <sup>ab</sup>	1.0 ± 0.3 <sup>a</sup>	8.82 ± 0.06
715	6	0.079 ± 0.010 <sup>abc</sup>	1.5 ± 0.1 <sup>a</sup>	8.60 ± 0.08
716	8	0.085 ± 0.009 <sup>bcd</sup>	4.8 ± 0.4 <sup>b</sup>	8.76 ± 0.04
717	10	0.105 ± 0.003 <sup>d</sup>	5.8 ± 0.6 <sup>b</sup>	8.77 ± 0.03
718	12	0.102 ± 0.005 <sup>cd</sup>	5.6 ± 0.7 <sup>b</sup>	8.73 ± 0.04

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

720 \*\*Data are means ± standard deviations of three determinations. Column data followed by the same

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

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739 **Table 3.** Effect of inducers on lipase production:

740	Inducer	Lipase	Biomass	Final pH
741		(IU/ml)	(mg/ml)	
742	<b>Natural oils</b> (1.0%, v/v)			
743	Control*	0.081 ± 0.004 <sup>f</sup>	6.0 ± 2.8 <sup>ab</sup>	7.90 ± 0.03
744	Joboba	0.071 ± 0.003 <sup>cf</sup>	4.0 ± 1.4 <sup>a</sup>	8.84 ± 0.07
745	Corn	0.052 ± 0.002 <sup>cd</sup>	4.5 ± 3.5 <sup>ab</sup>	8.71 ± 0.04
746	Cottonseed	0.060 ± 0.007 <sup>de</sup>	4.5 ± 0.7 <sup>ab</sup>	8.70 ± 0.28
747	Grapeseed	0.046 ± 0.001 <sup>bc</sup>	2.5 ± 0.7 <sup>a</sup>	8.67 ± 0.03
748	Groundnut	0.037 ± 0.001 <sup>b</sup>	4.5 ± 0.7 <sup>ab</sup>	8.64 ± 0.06
749	Linseed	0.128 ± 0.004 <sup>h</sup>	16.0 ± 1.4 <sup>b</sup>	7.01 ± 0.01
750	Rapeseed	0.013 ± 0.004 <sup>a</sup>	2.5 ± 0.7 <sup>a</sup>	8.66 ± 0.18
751	Soybean	0.014 ± 0.001 <sup>a</sup>	1.5 ± 0.7 <sup>a</sup>	8.70 ± 0.42
752	Sunflower	0.104 ± 0.004 <sup>g</sup>	11.5 ± 2.1 <sup>bc</sup>	7.81 ± 0.07
753	<b>Surfactants</b> (0.5 %, w/v)			
754	Control*	0.125 ± 0.006 <sup>c</sup>	5.5 ± 0.1 <sup>d</sup>	7.26 ± 0.03
755	Span 40	0.031 ± 0.003 <sup>ab</sup>	1.6 ± 0.8 <sup>ab</sup>	8.71 ± 0.03
756	Span 65	0.034 ± 0.001 <sup>b</sup>	1.6 ± 0.1 <sup>ab</sup>	8.68 ± 0.04
757	Span 80	0.063 ± 0.002 <sup>d</sup>	4.7 ± 0.8 <sup>cd</sup>	8.10 ± 0.21
758	Tween 20	0.056 ± 0.003 <sup>cd</sup>	1.5 ± 0.7 <sup>ab</sup>	8.75 ± 0.03
759	Tween 21	0.040 ± 0.004 <sup>bc</sup>	0.4 ± 0.1 <sup>a</sup>	6.99 ± 0.01
760	Tween 40	0.053 ± 0.003 <sup>cd</sup>	1.2 ± 0.1 <sup>ab</sup>	8.74 ± 0.03
761	Tween 80	0.067 ± 0.007 <sup>d</sup>	3.2 ± 0.1 <sup>bc</sup>	8.54 ± 0.03
762	Triton X-100	0.016 ± 0.002 <sup>a</sup>	0.2 ± 0.1 <sup>a</sup>	7.26 ± 0.06

763 The shake flask experiments were performed for 96 h at 28 °C, 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:

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

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**Table 5.** Effect of nitrogen sources on lipase production:

	Nitrogen source	Lipase activity (IU/ml)	Biomass (mg/ml)	Final pH
816	<b>Organic nitrogen source (0.5%, w/v)</b>			
817	Control*	0.071 ± 0.002 <sup>d</sup>	6.6 ± 1.9 <sup>cd</sup>	8.84 ± 0.03
818	Phytone peptone	0.131 ± 0.021 <sup>e</sup>	8.8 ± 0.4 <sup>d</sup>	8.72 ± 0.01
819	Yeast extract	0.104 ± 0.005 <sup>e</sup>	9.1 ± 0.1 <sup>d</sup>	8.69 ± 0.03
820	Corn steep liquor	0.061 ± 0.006 <sup>cd</sup>	7.6 ± 0.6 <sup>d</sup>	8.77 ± 0.03
821	Beef extract	0.046 ± 0.004 <sup>bcd</sup>	8.4 ± 0.7 <sup>d</sup>	8.90 ± 0.17
822	Skim milk	0.042 ± 0.001 <sup>abc</sup>	4.7 ± 0.1 <sup>bc</sup>	8.75 ± 0.17
823	Wheat peptone	0.030 ± 0.002 <sup>ab</sup>	6.6 ± 0.4 <sup>cd</sup>	8.94 ± 0.03
824	Fish peptone	0.026 ± 0.006 <sup>ab</sup>	1.0 ± 0.1 <sup>a</sup>	8.63 ± 0.03
825	Tryptone	0.024 ± 0.002 <sup>ab</sup>	8.1 ± 0.9 <sup>d</sup>	8.64 ± 0.03
826	Casein hydrolysate	0.023 ± 0.002 <sup>ab</sup>	3.6 ± 0.3 <sup>ab</sup>	8.59 ± 0.01
827	Casein	0.022 ± 0.001 <sup>ab</sup>	1.0 ± 0.1 <sup>a</sup>	8.82 ± 0.03
828	Wheat gluten	0.017 ± 0.001 <sup>a</sup>	2.0 ± 0.4 <sup>ab</sup>	8.78 ± 0.04
829	<b>Different % combination of PP and YE</b>			
830	0.5% PP + 0.1% YE	0.096 ± 0.005 <sup>a</sup>	6.7 ± 0.4 <sup>a</sup>	8.67 ± 0.03
831	0.4% PP + 0.2% YE	0.102 ± 0.001 <sup>a</sup>	7.4 ± 0.3 <sup>a</sup>	8.77 ± 0.04
832	0.3% PP + 0.3% YE	0.100 ± 0.001 <sup>a</sup>	6.6 ± 0.6 <sup>a</sup>	8.87 ± 0.07
833	0.2% PP + 0.4% YE	0.104 ± 0.002 <sup>a</sup>	8.9 ± 0.5 <sup>a</sup>	8.74 ± 0.01
834	0.1% PP + 0.5% YE	0.096 ± 0.002 <sup>a</sup>	6.8 ± 0.8 <sup>a</sup>	8.78 ± 0.10
835	0.6% PP	0.092 ± 0.007 <sup>a</sup>	6.9 ± 0.7 <sup>a</sup>	8.73 ± 0.01
836	0.6% YE	0.097 ± 0.001 <sup>a</sup>	6.8 ± 0.6 <sup>a</sup>	8.81 ± 0.03
837	<b>Inorganic nitrogen source (0.05%, w/v)</b>			
838	Control***	0.098 ± 0.001 <sup>de</sup>	5.1 ± 0.1 <sup>b</sup>	8.96 ± 0.03
839	NH <sub>4</sub> Cl	0.071 ± 0.001 <sup>ab</sup>	2.5 ± 0.7 <sup>ab</sup>	8.82 ± 0.04
840	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.078 ± 0.002 <sup>abc</sup>	3.0 ± 1.4 <sup>ab</sup>	9.04 ± 0.06
841	(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	0.104 ± 0.003 <sup>e</sup>	5.7 ± 0.5 <sup>b</sup>	8.78 ± 0.03
842	NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	0.090 ± 0.002 <sup>cde</sup>	4.0 ± 1.4 <sup>ab</sup>	9.00 ± 0.03
843	CH <sub>3</sub> COONH <sub>4</sub>	0.086 ± 0.001 <sup>bcd</sup>	3.6 ± 0.8 <sup>ab</sup>	9.14 ± 0.06
844	NH <sub>4</sub> NO <sub>3</sub>	0.090 ± 0.005 <sup>cde</sup>	4.9 ± 0.5 <sup>ab</sup>	9.02 ± 0.04
845	Urea	0.062 ± 0.011 <sup>a</sup>	1.5 ± 0.7 <sup>a</sup>	9.13 ± 0.18
846	The shake flask experiments were performed for 96 h at 28 °C, 130 rpm. Data are means ± standard deviations of three			
847	determinations. Column data followed by the same superscript letter were not significantly different ( $p \leq 0.05$ ; by Tukey test).			
848	* Control refers to the basal medium with 0.5% (w/v) Bacto-peptone as organic nitrogen source.			
849	** 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.			
850	*** Refers to control containing 0.05 % (w/v) of NaNO <sub>3</sub> as inorganic nitrogen source in basal medium.			

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

855	Organic solvent	log <i>P</i>	Relative activity (%)	
856			After 1 h	After 24 h
857	Control		100.0 ± 1.2	100.0 ± 0.6
858	DMSO	− 1.3	109.4 ± 2.3	89.6 ± 3.7 <sup>a</sup>
859	DMF	− 1.04	131.3 ± 6.3 <sup>a</sup>	137.6 ± 0.9 <sup>a</sup>
860	Methanol	− 0.76	129.5 ± 2.8 <sup>a</sup>	136.5 ± 0.6 <sup>a</sup>
861	2-propanol	− 0.28	129.5 ± 4.3 <sup>a</sup>	112.6 ± 2.5 <sup>a</sup>
862	Ethanol	− 0.24	109.6 ± 2.0	115.9 ± 3.4 <sup>a</sup>
863	Acetone	− 0.23	131.7 ± 5.2 <sup>a</sup>	134.6 ± 2.8 <sup>a</sup>
864	<i>t</i> -Butanol	0.35	96.3 ± 5.8	75.4 ± 3.4 <sup>a</sup>
865	Pyridine	0.64	72.4 ± 3.5 <sup>a</sup>	49.8 ± 2.8 <sup>a</sup>
866	Diethyl ether	0.85	116.8 ± 2.6 <sup>a</sup>	136.9 ± 1.8 <sup>a</sup>
867	Benzene	2.0	169.7 ± 2.0 <sup>a</sup>	169.1 ± 1.2 <sup>a</sup>
868	Chloroform	2.0	113.8 ± 6.3	134.1 ± 2.2 <sup>a</sup>
869	Toluene	2.5	153.3 ± 3.6 <sup>a</sup>	157.2 ± 2.8 <sup>a</sup>
870	Carbon tetrachloride	2.64	137.2 ± 3.7 <sup>a</sup>	117.6 ± 0.9 <sup>a</sup>
871	Cyclohexene	2.86	138.6 ± 2.3 <sup>a</sup>	109.3 ± 2.2
872	<i>p</i> -Xylene	3.1	161.9 ± 2.0 <sup>a</sup>	133.7 ± 2.2 <sup>a</sup>
873	<i>n</i> -Hexane	3.5	156.7 ± 3.2 <sup>a</sup>	111.7 ± 4.3 <sup>a</sup>
874	Dodecane	6.6	115.5 ± 3.3 <sup>a</sup>	68.0 ± 3.9 <sup>a</sup>
875	Petroleum ether	≈ 3.0	140.5 ± 2.6 <sup>a</sup>	123.7 ± 2.2 <sup>a</sup>

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 <sup>a</sup> 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:

903	Metal ion/effector (1 mM)	Relative activity (%)
904	Control	100.0 ± 1.0
905	Ag <sup>+</sup>	88.2 ± 5.5
906	Ba <sup>2+</sup>	99.1 ± 1.0
907	Ca <sup>2+</sup>	91.1 ± 4.5
908	Co <sup>2+</sup>	91.1 ± 0.6
909	Cu <sup>2+</sup>	100.2 ± 5.2
910	Fe <sup>3+</sup>	115.2 ± 7.1 <sup>a</sup>
911	Hg <sup>2+</sup>	20.0 ± 3.2 <sup>a</sup>
912	Li <sup>+</sup>	79.2 ± 2.6 <sup>a</sup>
913	Mg <sup>2+</sup>	81.3 ± 3.5 <sup>a</sup>
914	Mn <sup>2+</sup>	112.9 ± 5.2
915	Ni <sup>2+</sup>	95.2 ± 1.9
916	Pb <sup>2+</sup>	97.0 ± 0.6
917	Zn <sup>2+</sup>	95.9 ± 1.6
918	NH <sub>4</sub> <sup>+</sup>	103.8 ± 1.8
919	EDTA	103.6 ± 1.6
920	SDS	100.0 ± 2.9
921	Urea	88.1 ± 2.9
922	PMSF	85.3 ± 3.2 <sup>a</sup>

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

924 Values represent the mean of three replicates.

925 <sup>a</sup> 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 °C 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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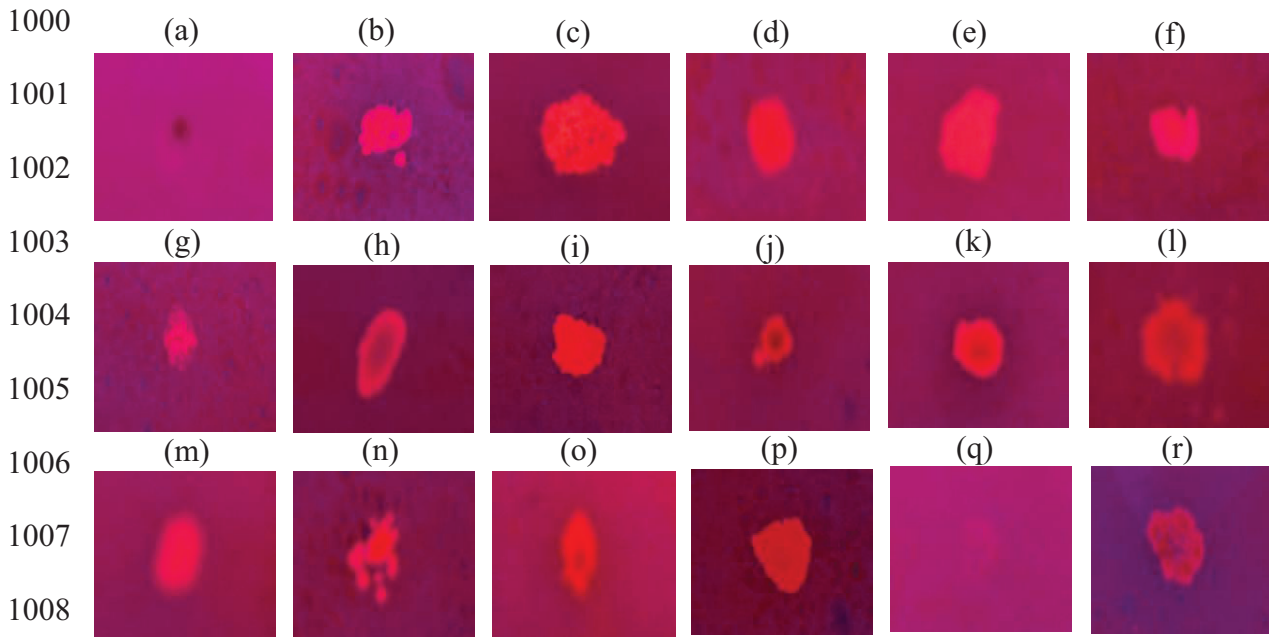
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999 **Figure 1.**



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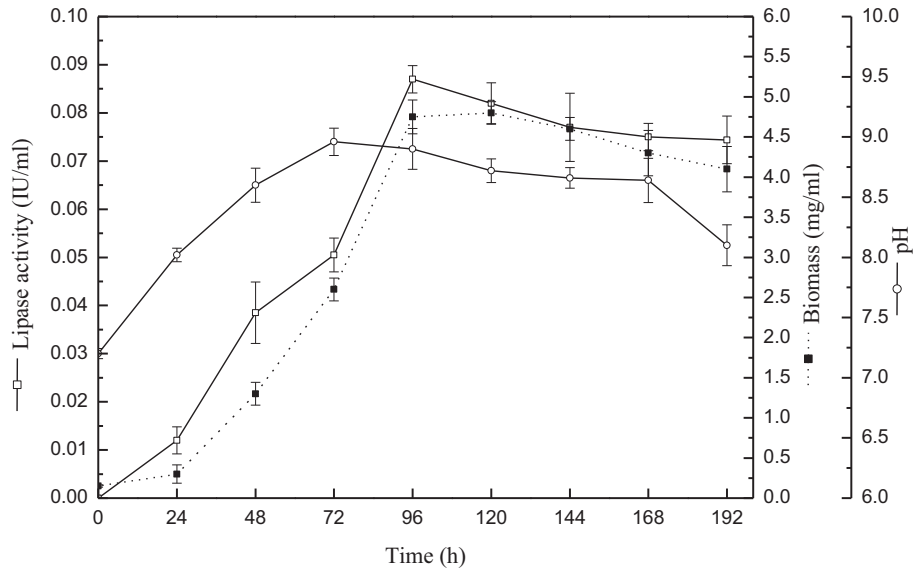
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1022 **Figure 2.**



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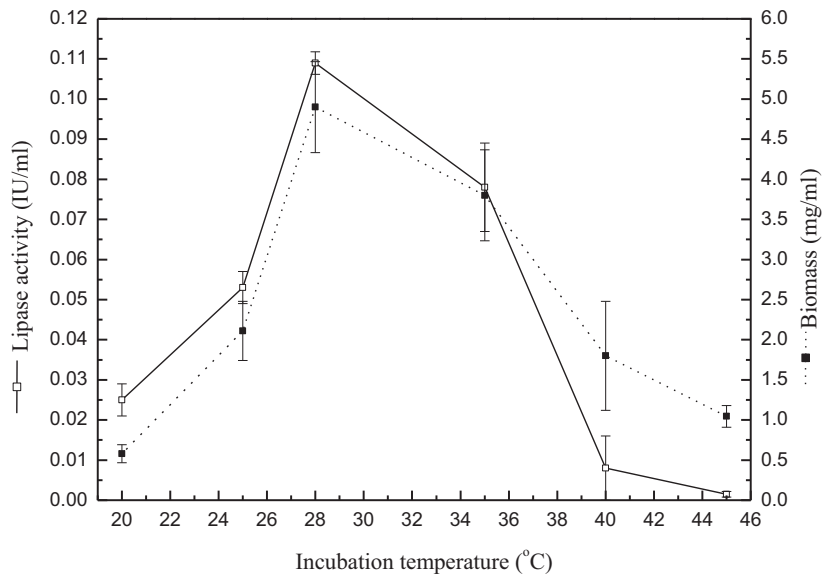
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1036 **Figure 3.**



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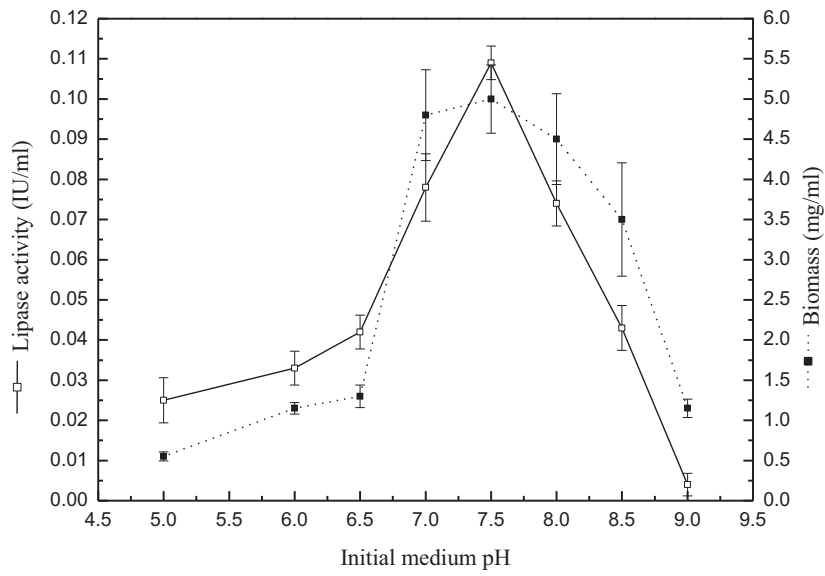
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1050 **Figure 4.**



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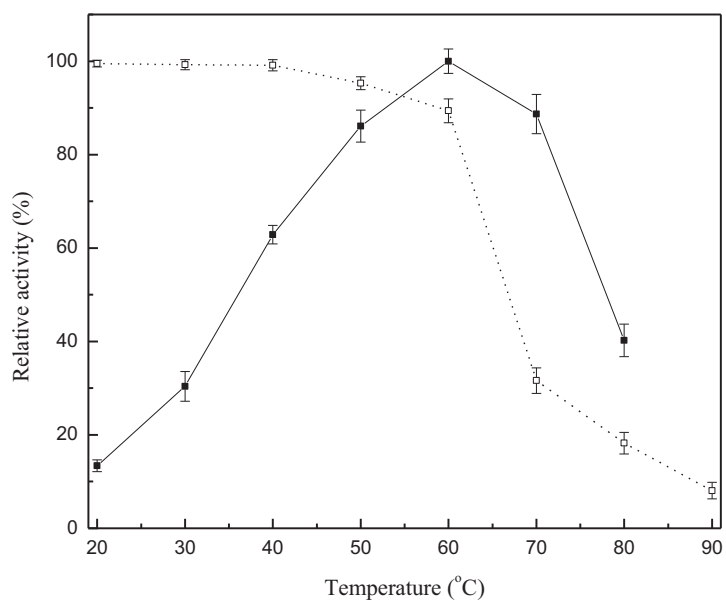
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1064 **Figure 5.**

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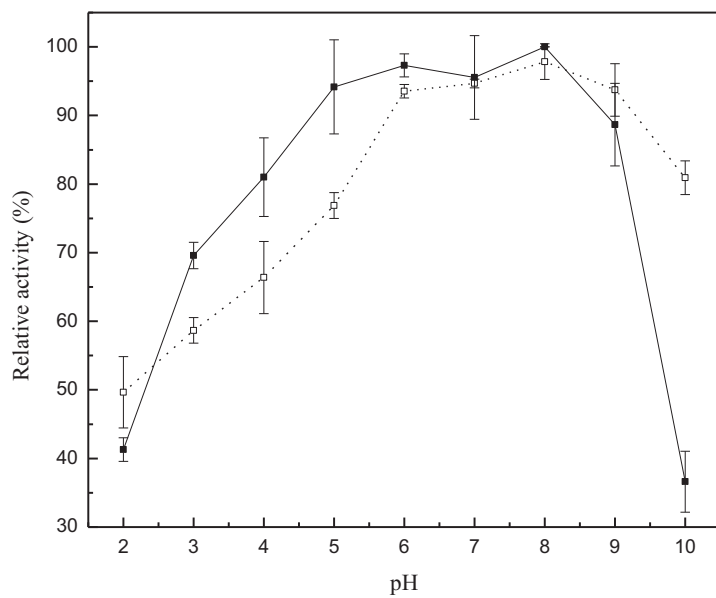
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1078 **Figure 6.**

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