



Technological University Dublin
ARROW@TU Dublin

Articles

School of Food Science and Environmental
Health

2010-01-01

Purification and Properties of *Amycolatopsis Mediterranei* DSM 43304 Lipase and Its Potential in Flavour Ester Synthesis

Dharmendra Dheeman

Technological University Dublin, dharmendra.dheeman@student.dit.ie

Gary Henehan

Technological University Dublin, gary.henehan@tudublin.ie

Jesus Maria Frias

Technological University Dublin, Jesus.Frias@tudublin.ie

Follow this and additional works at: <https://arrow.tudublin.ie/schfsehart>

 Part of the [Biochemistry Commons](#)

Recommended Citation

Dheeman, D. S., Henehan, G.T.M. and Frias, J.M. (2011) Purification and properties of *Amycolatopsis mediterranei* DSM 43304 lipase and its potential in flavour ester synthesis. *Bioresource Technologies* 102(3) 3373-3379. doi:10.1016/j.biortech.2010.11.074

This Article is brought to you for free and open access by the School of Food Science and Environmental Health at ARROW@TU Dublin. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@TU Dublin. For more information, please contact yvonne.desmond@tudublin.ie, arrow.admin@tudublin.ie, brian.widdis@tudublin.ie.



This work is licensed under a [Creative Commons Attribution-NonCommercial-Share Alike 3.0 License](#)



1 **Purification and properties of *Amycolatopsis mediterranei* DSM 43304 lipase**
2 **and its potential in flavour ester synthesis**

3

4

5 Dharmendra S. Dheeman, Gary T. M. Henehan*, Jesus M. Frías

6

7

8 School of Food Science and Environmental Health, Dublin Institute of Technology,

9 Cathal Brugha Street, Dublin 1, Ireland

10

11

12

13

14 *Corresponding author: Phone: +353 14024408; Fax: +353 14024459;

15 E-mail addresses: dheeman@gmail.com (D. S. Dheeman); gary.henehan@dit.ie (Gary

16 Henehan); jesus.frias@dit.ie (Jesus Frías)

17

18

19 **Abbreviations:** AML: *A. mediterranei* DSM 43304 lipase

20 *p*-NPP: *p*-Nitrophenyl Palmitate

21

22

23 **Abstract**

24 An extracellular thermostable lipase from *Amycolatopsis mediterranei* DSM 43304
25 has been purified to homogeneity using ammonium sulphate precipitation followed by
26 anion exchange chromatography and hydrophobic interaction chromatography. This
27 protocol resulted in 398 fold purification with 36% final recovery. The purified A.
28 *mediterranei* DSM 43304 lipase (AML) has an apparent molecular mass of 33 kDa. The N-
29 terminal sequence, AANPYERGPDPPTTASIEATR, showed highest similarity to a lipase
30 from *Streptomyces exfoliatus*. The values of K_m^{app} and V_{max}^{app} for *p*-nitrophenyl palmitate (*p*-
31 NPP) under optimal temperature (60°C) and pH (8.0) conditions were 0.10 ± 0.01 mM and
32 2.53 ± 0.06 mmol/min mg, respectively. The purified AML displayed significant activity
33 towards a range of short and long chain triglycerides. It was most active on triolein and a
34 wide range of *p*-nitrophenyl esters, with a preference for an acyl chain length of C8:0.
35 Hydrolysis of glycerol ester bonds occurred non-specifically. The purified AML displayed
36 significant stability in the presence of organic solvents (40% v/v) and catalyzed the
37 synthesis of flavour ester isoamyl acetate in free and immobilized states.

38

39 **Keywords:** Actinomycete lipase; *Amycolatopsis mediterranei*; Purification;
40 Characterization; Ester synthesis

41 **1. Introduction**

42 Lipases are among the most versatile of the enzyme classes and are used in a
43 number of applications in various industries, including the pharmaceutical, food, detergent,
44 cosmetic, oleochemical, fat-processing, leather, textile and paper industries (Gupta et al.
45 2004; Pandey et al. 1999). Current research on lipases, mainly of microbial origin, has
46 increased in volume because of their great commercial potential (Dandavate et al., 2009;
47 Silva et al., 2009). As the applications increase, the availability of lipases possessing
48 satisfactory operating characteristics for specific purposes is a limiting factor. Since many
49 industrial processes operate at temperatures exceeding 45°C, lipases should ideally have
50 catalytic activity and stability around 50°C (Sharma et al., 2002). Thermophilic
51 microorganisms have been the focus of a number of investigations of novel sources of
52 lipases that are stable and optimally functional at high temperatures (Berekaa et al., 2009;
53 Nawani and Kaur, 2007), although in recent years a few mesophilic actinomycetes have
54 also been reported to produce thermoactive lipases (Abramić et al., 1999; Côté and
55 Shareck, 2008; Zhang et al., 2008).

56 Lipases from actinomycetes have not been studied as intensively as those from other
57 bacteria. In a previous paper from this laboratory we reported the presence of a novel lipase
58 in crude extracts of a mesophilic actinomycete *Amycolatopsis mediterranei* DSM 43304
59 (Dheeman et al., 2010). Characterization of this *A. mediterranei* DSM 43304 lipase (AML)
60 activity indicated it had high thermostability and organic solvent stability indicating its
61 potential in organic synthesis. This has led to further interest in purification of AML and
62 investigating its potential in organic synthesis. In the present work we report the
63 purification and characterization of AML and evaluate its potential in the synthesis of an

64 industrially important flavour ester, isoamyl acetate. The main highlight of our study is the
65 potential of purified AML in the synthesis of flavour ester through direct esterification of
66 isoamyl alcohol using acetic acid as an acyl donor.

67 **2. Materials and methods**

68 **2.1 Chemicals**

69 Analytical reagent grade chemicals were obtained from commercial sources at the
70 purest grade available. Unless otherwise mentioned, all chemicals were purchased from
71 Sigma-Aldrich Ireland Ltd.

72 **2.2 Microorganism and lipase production**

73 *A. mediterranei* DSM 43304 was obtained from the Divisional Culture Collection,
74 School of Biology, Newcastle University, UK. The strain was identified as a lipase
75 producer on olive oil-rhodamine B agar. The lipase was produced in optimized production
76 medium as previously reported (Dheeman et al., 2010).

77 **2.3 Lipase activity assays**

78 **2.3.1 Spectrophotometric assay**

79 Lipase activity was routinely assayed using *p*-nitrophenyl palmitate (*p*-NPP) as
80 substrate according to Winkler and Stuckmann (1979) with some modifications as
81 described previously (Dheeman et al., 2010). The assay was typically run for 10 min at
82 60°C before termination by addition of 2.0 ml of 0.2 M Na₂CO₃. Liberated *p*-nitrophenol
83 (*p*-NP) was determined at 410 nm ($\epsilon_{410\text{ nm}}$: 0.0169/ $\mu\text{mol cm}$) using a UNICAM UV2 2000E
84 UV-VIS Spectrophotometer (Cambridge, UK). Appropriate blanks were used to subtract
85 the absorbance corresponding to the reaction mixture other than that produced by the

86 specific hydrolysis of *p*-NPP. One international unit (IU) of lipase activity was defined as
87 the amount of enzyme needed to liberate 1 μmol of *p*-NP per minute under the assay
88 conditions.

89 **2.3.2 Titrimetric assay**

90 Activity determination was carried out titrimetrically essentially as described by
91 Burkert et al. (2004). Incubations were carried out at 60°C and pH 8.0 for 10 min. The
92 enzymatic reaction was initiated by addition of 1 ml of appropriately diluted enzyme
93 solution to the reaction mixture (5 ml) and stopped by the addition of 15 ml of ethanol.
94 Control was carried out similarly, except that the enzyme solution was added after the
95 addition of ethanol. One IU of lipase activity was defined as the amount of enzyme that
96 caused the release of one μmol of free fatty acid per minute under test conditions. For
97 substrate specificity studies similar method was used but using various substrates.

98 **2.4 Purification of AML**

99 After 96 h, the cells were separated by centrifugation at 10,000 $\times g$, at 4°C, for 10
100 min, and the supernatant was recovered and filtered (0.2 μm filter, Millipore). Unless
101 otherwise mentioned, all purification steps were performed at 4°C. The extracellular lipase
102 was concentrated from the filtrate by stepwise saturation to 40% ammonium sulphate. The
103 precipitate was collected by centrifugation (14,000 $\times g$ for 10 min at 4°C), dissolved in 10
104 mM Tris-HCl buffer, pH 8.0 and dialyzed against the same buffer for 12 h. The dialyzed
105 material was centrifuged (12,000 $\times g$ for 10 min at 4°C) and the supernatant was applied to
106 a Q Sepharose HP column (2.5 \times 10 cm, 30 ml gel). The column was preequilibrated with
107 10 mM Tris-HCl buffer, pH 8.0 (buffer A). Bound protein was eluted with a step gradient
108 of increasing NaCl concentration from 0.2 M to 1.0 M using 3.0 column volumes of buffer

109 A at a flow rate of 108 ml/h. Fractions were collected and analyzed for lipase activity and
110 protein content. Active fractions containing high lipase activity were pooled and
111 concentrated by ultrafiltration using a 10 kDa centricon (Amicon, USA), and applied to a
112 Toyopearl Phenyl-650M column (2.5 × 10 cm, 40 ml gel). The column was preequilibrated
113 with 10 mM Tris buffer, pH 8.0, containing 20% ammonium sulphate (buffer B) at room
114 temperature. Equilibration of Toyopearl Phenyl-650M gel with sample at room temperature
115 allowed 100% of the lipase to be bound. After a three column volume wash with buffer B,
116 the bound protein was eluted with three column volumes of decreasing step gradient of
117 ammonium sulphate from 20-0% and three column volumes of increasing step gradient of
118 isopropanol from 0-30% in buffer A at a flow rate of 125 ml/h. Fractions containing high
119 lipase activity were pooled and tested for purity on SDS-PAGE gels. The gels were stained
120 with silver nitrate and the molecular mass of the purified enzyme was estimated using
121 standard protein markers (BioRad Laboratories, CA, USA).

122 **2.5 Gel electrophoresis and zymography**

123 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5 %
124 (w/v) gels at room temperature (ATTO AE-6450, Tokyo, Japan) as previously reported
125 (Dheeman et al., 2010). For activity staining, zymographic analysis was performed,
126 essentially as described by Prim et al. (2003).

127 **2.6 N-terminal sequence analysis**

128 The purified protein band on SDS gel was transferred to a polyvinylidene difluoride
129 membrane (Immobilon[®]-P PVDF, Millipore) by semidry electroblotting (ATTO Horizblot
130 AE-6677, Tokyo, Japan), and stained with Coomassie Brilliant Blue R 250. Automated

131 Edman protein degradation was performed using a protein sequencer (ABI Procise 491
132 Edman micro sequencer connected to a 140C PTH amino acid analyzer).

133 **2.7 The effect of pH and temperature on activity and stability**

134 The effect of pH and temperature on purified AML was investigated by using *p*-
135 NPP as the substrate. The optimal pH of the purified enzyme was determined at 60°C over
136 a pH^{60°C} range of 2-10 at constant molarity (50 mM) in different buffers (glycine-HCl (pH
137 2.0-3.0), citrate-phosphate (pH 3.0-6.0), sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.0
138 and 9.0) and 2-amino-2-methyl-1,3-propanediol (ammediol) buffer (pH 9.0-10.0)) . The pH
139 stability was studied by incubating the purified AML in selected buffers of pH range 2-12
140 for 24 h at 20°C. The residual enzyme activity was measured by spectrophotometric assay
141 at 60°C, pH 8.0. The optimum temperature of the purified enzyme was determined by
142 measuring the enzyme activity at various temperatures (20-80°C) in 50 mM Tris-HCl
143 buffer, pH 8.0. Thermostability was determined by incubating purified lipase in 20 mM
144 Tris-HCl buffer, (pH 8.0) at various temperatures (60-90°C) for 3 h and residual activity
145 was analyzed by spectrophotometric assay at 60°C, pH 8.0.

146 **2.8 Determination of kinetic constants**

147 Initial rates measurements with 0.59 µg of purified AML were performed in 50 mM
148 Tris-HCl buffer, pH 8.0 at 60°C with increasing concentration of *p*-NPP (0.02–0.93 mM).
149 Kinetic constants were obtained by fitting experimental data to the Michaelis-Menten
150 equation using EnzFitter (Biosoft, Cambridge, UK) to obtain estimates of Michaelis
151 constant (K_m^{app}) and maximal velocity (V_{max}^{app}). Turnover number k_{cat}^{app} was calculated using
152 the equation $k_{cat}^{app} = V_{max}^{app} / [E]_T$, where $[E]_T$ is the molar amount of enzyme in the reaction.

153 **2.9 Determination of substrate range**

154 To determine the substrate range of the purified AML, the relative activities were
155 investigated against a series of *p*-nitrophenyl esters (*p*-nitrophenyl acetate (C2:0), *p*-
156 nitrophenyl butyrate (C4:0), *p*-nitrophenyl caproate (C6:0), *p*-nitrophenyl caprylate (C8:0),
157 *p*-nitrophenyl laurate (C12:0), *p*-nitrophenyl myristate (C14:0) and *p*-nitrophenyl palmitate
158 (C16:0)) differing in fatty acyl chain length. AML substrate specificity for triacylglycerides
159 was analyzed using a variety of triacylglyceride substrates including olive oil, corn oil,
160 castor oil, sunflower oil, rape seed oil, linseed oil, cotton seed oil and jojoba oil. Also
161 relative activities of purified AML against a series of triacylglycerol substrates (trioctanoin
162 (C8:0), tripalmitin (C16:0), tristearin (C18:0), triolein (C18:1, *cis*-9), trivaccinin (C18:1,
163 *trans*-9), trilinolein (C18:2, *cis*-9,12) and trilinolenin (C18:3, *cis*-9, 12, 15) differing in
164 chain length and saturation were similarly determined.

165 **2.10 Determination of position specificity**

166 Position specificity of the lipase was examined by thin-layer chromatography of the
167 reaction product obtained by using pure triolein as substrate (Sugihara et al., 1992). A
168 reaction mixture composed of 20 mM triolein, 2 ml of 50 mM phosphate buffer (pH 7.6),
169 and 20 IU of the purified AML were incubated at 30°C for 30 min with magnetic stirring.
170 After incubation, the reaction product was extracted with 8 ml of ethyl ether. Aliquots (10
171 µl) of the ether layer were applied to a Silica Gel 60 plate (Merck KgaA, Darmstadt,
172 Germany) and developed with a 95:4:1 (v/v) mixture of chloroform, acetone, and acetic
173 acid. The spots were visualized using saturated iodine chamber and compared with
174 standards from Sigma.

175 **2.11 Effect of various reagents and organic solvents**

176 The effect of various detergents, oxidizing-reducing agents, chelating agents, free
177 fatty acids, and metal ions (Ag^+ , Co^{2+} , Ni^{2+} , Pb^{2+} , Ca^{2+} , Fe^{3+} , Cu^{2+} , Zn^{2+} , Mg^{2+} and Hg^{2+})
178 on purified AML activity was analyzed by incubating the pure enzyme in 1 mM of these
179 effectors for 1 h at 30°C in 50 mM Tris-HCl buffer (pH 8.0). The effect of urea was
180 assessed at 6.0 M. The effect of group specific reagents (*N*-acetylimidazole (NAI), *N*-
181 bromosuccinimide (NBS), phenylmethyl-sulfonylfluoride (PMSF), diethylpyrocarbonate
182 (DEPC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), iodoacetate (IA),
183 citraconic anhydride (CA), phenylglyoxal (PG)) on AML was determined by incubating the
184 purified enzyme in presence of 5 mM of these reagents for 1 h at 30°C. The effect of
185 various organic solvents (40%, v/v) on AML activity was determined by incubating 1 ml of
186 purified enzyme solution in 1.5 ml of the different organic solvents in airtight vials at 30°C,
187 200 rpm for 24 h. The control used was an enzyme sample without reagent/organic solvent
188 under the same experimental conditions. Residual activity was measured using the
189 spectrophotometric assay at 60°C, pH 8.0.

190 **2.12 Potential of purified AML in ester synthesis**

191 Isoamyl acetate synthesis was carried out in a stirred reactor with a capacity of 4 ml
192 in *n*-hexane using free (0.1 mg) or immobilized AML and acetic acid as acyl donor. For
193 immobilization, 2 ml of purified enzyme solution (0.1 mg of lipase, equivalent to 78.2 IU,
194 in 10 mM Tris-HCl buffer, pH 8.0) were mixed with 0.5 g of celite and the suspension was
195 stirred for 1 h at 4°C. Then this suspension, containing the pure enzyme immobilized on
196 celite, was dried for 6 h at room temperature (GeneVac EZ-2 Plus, UK). The resulting
197 powder was suspended in 2 ml of *n*-hexane containing 500 mM of isoamyl alcohol. When
198 the reaction temperature reached (40°C), the esterification reaction was initiated by adding

199 300 mM of acetic acid to the reaction mixture. The reaction mixture was incubated at 40°C
200 for 72 h. Control experiments were conducted in parallel without lipase under similar
201 conditions.

202 **2.12.1 Quantification of ester synthesis**

203 Aliquots of the reaction mixture were withdrawn at definite time intervals and
204 extent of esterification monitored by a titration procedure to estimate the decrease in total
205 acid content of the reaction mixture. Titration was carried out with standardized 0.05 N
206 NaOH using phenolphthalein as indicator and ethanol as a quenching agent. The accuracy
207 of the titration method was verified by gas chromatography. Isoamyl acetate concentration
208 was determined using a gas chromatography (Perkin Elmer Autosystem XL GC, USA)
209 equipped with a DB-5 column (30 m length, 0.25 mm i.d., 0.25 µm film thickness) and a
210 flame ionization detector. Nitrogen was used as a carrier gas with a flow rate of 1 ml/min.
211 The temperatures of the column oven, the injection port and the detector were maintained at
212 60, 250 and 200°C, respectively. The conversion percentage calculated by both GC analysis
213 (which showed product formation) and titrimetry (which showed acid consumption) were
214 in good agreement.

215 **3. Results**

216 **3.1 Lipase purification**

217 The isolation of the enzyme from the culture filtrate was achieved by a three-step
218 procedure. The lipase from crude filtrate was precipitated by ammonium sulphate at 40%
219 saturation. The precipitated enzyme was dissolved in a minimum volume of 10 mM Tris-
220 HCl buffer, pH 8.0. The first step of precipitation and dialysis resulted in 92.52% overall
221 yield with specific activity of 3.66 IU/mg. The first chromatographic step of anion

222 exchange (Q Sepharose HP column) separated *p*-NPP hydrolyzing enzyme from a part of
223 contaminating proteins. In addition, column chromatography on Toyopearl Phenyl-650M
224 was required for the isolation of the enzyme, which resulted in a single peak of active
225 protein and in an electrophoretically homogeneous preparation (Fig. 1a). By this
226 purification procedure a 398-fold increase in enzyme specific activity was achieved, with
227 an overall yield of 36 % (Table 1). The pure enzyme preparations were stored at -20°C and
228 were used to study its properties.

229 **3.2 Gel electrophoresis and zymography**

230 SDS-PAGE of purified lipase showed single protein band of a relative molecular
231 mass of 33 kDa. The activity of the band on the gel was detected using MUF-butyrate *in*
232 *situ* enzyme assay after SDS-PAGE and the *p*-NPP hydrolyzing activity coincided with the
233 purified protein ((Fig. 1b).

234 **3.3 N-terminal sequence of AML**

235 N-terminal sequencing of the PVDF transferred band from an electrophoretic gel
236 allowed the identification of 20 amino acid residues: AANPYERGPDPTTASIEATR. This
237 sequence was compared with the sequences of known lipases (Table 2). It exhibited
238 significant similarity (85%) only with the N-terminal sequence of *Streptomyces exfoliatus*
239 lipase (Wei et al. 1998). Also, the first 19 amino acids of AML were found to be identical
240 to 48-66 amino acids of a *putative lipase* identified from an ORF in recently completed
241 genome sequence of *A. mediterranei* U32 (genebank accession no. ADJ49206).

242 **3.4 The effect of pH and temperature on activity and stability**

243 The purified enzyme was most active toward *p*-NPP at pH 8.0. The activity was not
244 much affected at pH 7 and 9 where it showed around 90% of relative activity. The purified

245 enzyme was stable in the pH range 6–9 retaining more than 95% of relative activity after 24
246 h of incubation (data not shown). The purified enzyme exhibited maximum activity toward
247 *p*-NPP at 60°C. Above this temperature sharp inactivation occurred (data not shown).

248 **3.5 Determination of kinetic constants**

249 The kinetic analysis of purified AML performed on standard assay substrate, *p*-NPP
250 at 60°C produced a Lineweaver Burk plot corroborating the Michaelis-Menten behavior of
251 the enzyme with a V_{\max}^{app} of 2.53 ± 0.06 mmol/min mg, K_m^{app} of 0.10 ± 0.01 mM and k_{cat}^{app} of
252 $1467.59 \pm 34.86/s$.

253 **3.6 Substrate range**

254 The enzyme substrate range was studied with *p*-nitrophenyl esters of varying fatty
255 acyl chain lengths. The highest hydrolysis rates were obtained with *p*-NP caprylate (C8:0)
256 followed by *p*-NP caproate (C6:0), indicating the enzyme's preference for medium-size
257 acyl chain lengths (Fig. 2a). Relative activity for each substrate is expressed as a percentage
258 of that for *p*-NP caprylate (C:8). The substrate preferences of AML were characterized
259 with various oil and triacylglycerol substrates. As shown in Figs. 2b and 2c, relative
260 activity for each substrate is expressed as the percentage of that for olive oil. AML showed
261 relatively high activity using various emulsified oils especially for olive oil. Among the
262 substrates tested, AML showed a distinct preference for long, unsaturated fatty acyl chains.
263 The relative activities for substrates with *cis*-9 unsaturation (C18:1, *cis*-9; C18:2, *cis*-9, 12;
264 C18:3, *cis*-9, 12, 15) are higher than the relative activity on the saturated triacylglycerols
265 (C8:0, C16:0, C18:0).

266 **3.7 Position specificity**

267 In order to determine the position specificity (regio-selectivity) of purified AML,
268 thin-layer chromatography of AML catalyzed hydrolysis products of pure triolein was
269 performed (Fig. 3). After 30 min at 30°C, the products of hydrolytic action of purified
270 AML on triolein were oleic acid (major product), 1,3-dioleoylglycerol (1,3-DO), 1,2(2,3)-
271 dioleoylglycerol (1,2(2,3)-DO) and 1(2)-monooleoylglycerol (1(2)-MO) (minor products).
272 From observation of reaction products, AML did not discriminate between *sn*-1 and *sn*-2
273 positions of triolein.

274 **3.8 Effect of various reagents and organic solvents**

275 Various compounds were studied for their effect on purified AML activity (Table
276 3). AML proved to be insensitive to the chelating agents, ethylenediaminetetraacetic acid
277 (EDTA) and sodium citrate. It showed relative insensitivity to SDS, but incubation with 1
278 mM digitonin and sodium deoxycholate caused pronounced activation of the enzyme by
279 42.5% and 141.3%, respectively. The enzyme was activated in 1 mM 1,4-dithiothreitol, β -
280 mercaptoethanol and ascorbic acid by 47.6%, 36.4% and 24.4%, respectively. The
281 incubation with 1 mM of different chain length fatty acids had little effect on the enzyme
282 activity. Significant stability was observed toward metal ions except Hg^{2+} , which showed
283 the highest reduction in AML activity by 83.3% (data not shown). AML was not inhibited
284 by NAI, CA, IA and PG suggesting the non-involvement of tyrosine, lysine, cysteine and
285 arginine residues in catalysis. Strong inhibition of enzyme by PMSF, EDAC, DEPC and
286 NBS was observed which indicated the significant involvement of serine, carboxylate,
287 histidine and tryptophan for catalytic activity (Table 4). Purified AML was stable in the
288 presence of water-miscible solvents (dimethylformamide, methanol, ethanol and 2-
289 propanol) as well as water-immiscible solvents (*n*-hexane, *p*-xylene, cyclohexene and

290 toluene). In most cases the enzyme was significantly activated, with residual activities
291 greater than 100% (data not shown).

292 **3.9 Potential of purified AML in ester synthesis**

293 Purified AML, free and celite-immobilized, was used to catalyze the esterification
294 of isoamyl alcohol to isoamyl acetate in *n*-hexane using acetic acid as an acyl donor. AML
295 exhibited significant potential for synthesis of isoamyl acetate. After 72 h of reaction a
296 yield of isoamyl acetate of 34.4% and 16.2%, with respect to the initial acetic acid, was
297 obtained using immobilized and free AML, respectively (Fig. 4).

298 **4. Discussion**

299 The number of commercially available lipases has increased considerably in recent
300 decades, along with the demand for these biocatalysts. The characterization of new lipolytic
301 enzymes, the development of new purification procedures and the increased number of
302 studies on the subject, mainly on lipases of microbial origin, are all factors that contribute
303 to the novel biotechnological applications of these enzymes (Silva et al., 2009). Lipolytic
304 enzymes are subdivided into different groups including carboxylesterases, lipases and sterol
305 esterases. Some of these enzymes show very wide substrate specificity and it is not always
306 possible to identify the group to which they belong (Calero-Rueda et al., 2002). The
307 purified AML showed activity towards different esters including *p*-NPB (a generic
308 substrate for esterase activity), *p*-NPP (a generic substrate for lipase activity) and triolein (a
309 substrate for detection of true lipase activity). Purified AML seemed to exhibit both an
310 esterase and a true lipase activity as previously reported in the case of *Streptomyces*
311 *coelicolor* hydrolase (Bielen et al., 2009) and *Streptomyces cinnamomeus* Tü89 lipase
312 (Sommer et al., 1997).

313 In the present investigation, electrophoretically homogeneous AML was purified
314 using ammonium sulphate precipitation followed by anion exchange and hydrophobic
315 interaction chromatography. Hydrophobic interaction chromatography has been used for
316 purification of many lipases since these enzymes are hydrophobic and display strong
317 interaction with hydrophobic supports (Sharma et al., 2001). Queiroz et al. (1995) used
318 20% of the ammonium sulphate in the eluent and observed total retention of lipase on a
319 hydrophobic column. We also observed 100% retention of the enzyme on Toyopearl
320 Phenyl-650M column in presence of 20% ammonium sulphate. Isopropanol at 30% (v/v)
321 was required to elute the homogeneous AML from the hydrophobic interaction column
322 with a final yield of 36%, which is a higher yield compared to the only reported purification
323 of a native actinomycete lipase (Abramić et al., 1999). Similar solvent conditions were
324 employed for the elution of bacterial lipases (Kordel et al., 1991; Zhang et al., 2002). In
325 aqueous solutions (including buffers and salts), the purified AML formed aggregates. Gel
326 filtration chromatography of AML at low protein concentration showed elution in the void
327 volume, indicating that AML formed active molecular aggregates (data not shown). The
328 aggregation tendency of lipolytic enzymes is well documented in the literature (Castro-
329 Ochoa et al., 2005; Lima et al., 2004). The presence of aggregates has been reported for
330 other enzymes with lipase activity, and may be explained by the strong hydrophobic
331 character of these enzymes (Castro-Ochoa et al., 2005).

332 The denatured molecular mass of 33 kDa of AML is in the range reported for other
333 enzymes with lipolytic activity (20–60 kDa) (Gupta et al., 2004). The extracellular lipase
334 reported here is different to the thermophilic lipases characterized from other actinomycete
335 strains, which showed lower molecular mass (23.9–28.5 kDa) and thermostability (Abramić

336 et al., 1999; Côté and Shareck, 2008; Zhang et al., 2008). However, the purified AML
337 showed significant N-terminal sequence homology to *S. exfoliatus* lipase. *S. exfoliatus*
338 lipase is the only lipase from the *Streptomyces* genus whose crystal structure has been
339 determined (Wei et al., 1998). However, its biochemical characterization and chain length
340 specificity has not been reported. Therefore, comparison between these two enzymes is not
341 yet possible.

342 The high activity and stability of AML over a wide pH range (5-9) suggests its
343 usefulness in a range of industrial applications. In different industrial applications
344 thermostability is an important property for applications in processes operating at high
345 temperatures (Nawani and Kaur, 2007; Sharma et al., 2002). Thus, the high activity and
346 stability of AML (50-60°C) makes it potentially useful in biocatalytic processes operating
347 at high temperatures. The purified AML showed low K_m^{app} value (0.10 ± 0.01 mM) and
348 high V_{max}^{app} value (2.53 ± 0.06 mmol/min mg) indicating high affinity between enzyme and
349 substrate and higher catalytic efficiency (Sharma et al., 2001).

350 AML showed highest hydrolytic activity with *p*-NP caprylate, indicating its clear
351 preference for saturated medium acyl chain lengths as previously reported for other
352 bacterial lipases (Abramić et al., 1999; Schmidt-Dannert et al., 1996; Soliman et al., 2007).
353 AML showed relatively high activity using various emulsified oils, particularly olive oil,
354 which could be due to its high content of long, unsaturated fatty acyl chains, such as oleic
355 acid. These results highlight AML could play an important role in applications such as
356 removal of oil spills in the environment (Hasan et al., 2006). It could be used in biodiesel
357 production due to its ability to hydrolyze a wide range of oils (Tan et al., 2010) coupled

358 with stability in polar solvents. Interestingly AML also showed distinct specificity for long,
359 unsaturated fatty acyl chains, which is a very valuable property for enzymatic restructuring
360 by interesterification of fats and oils with unsaturated fatty acids to improve the physical
361 properties of triglycerides for use in food industries (Jackson et al., 1997).

362 In order to determine the position specificity of AML, pure triolein hydrolysis
363 products were analyzed using thin-layer chromatography (Fig. 3). The hydrolysis products
364 by AML were oleic acid (major product), 1,3-DO, 1,2(2,3)-DO and 1(2)-MO (minor
365 products). Spontaneous acyl migration was considered unlikely because of the short
366 reaction time. Like the majority of bacterial lipases (Lešćić et al., 2001; Rahman et al.,
367 2005), AML belongs to the group of nonspecific lipases which are able to hydrolyze both
368 primary and secondary ester bonds in triolein.

369 Many biotechnological processes involve the presence in the reaction media of
370 certain ions that could act as modifiers of the enzyme activity. To test this possibility, the
371 influence of different metal ions and several putative inhibitors or compounds commonly
372 used was assayed on the purified AML. Among the different metal ions tested, only Hg^{2+}
373 strongly inhibited AML activity probably due to the binding of Hg^{2+} to a functional thiol
374 group (Patkar and Björkling, 1994). Other commonly used effector molecules were tested
375 to evaluate their capacity to inhibit or activate the purified AML (Table 3). Urea causes
376 disaggregation of the enzymes and does not produce a significant inhibition of activity at
377 concentrations below 6.0 M (Bofill et al., 2010). AML was only marginally affected in 6.0
378 M urea. The pure enzyme was significantly stable in presence of SDS as reported earlier
379 (Soliman et al., 2007; Yu et al., 2009). Detergents, digitonin and sodium deoxycholate,
380 markedly activated the enzyme which is in contrast with their effect on a thermostable

381 lipase from *Burkholderia cepacia* ATCC 25416 (Wang et al., 2009). Besides direct
382 activation or inactivation, detergents may alter the hydrophobicity of the enzyme; affect
383 micelle formation and the ratio of free-to-micellar substrate (Helistö and Korpela, 1998).
384 Reducing agents often cause inhibition of lipase activity (Sharma et al., 2002; Soliman et
385 al., 2007). In contrast, AML was strongly activated by these reducing reagents. A similar
386 activating effect of reducing agents was observed for *G. thermoleovorans* YN lipase
387 (Soliman et al., 2007). The presence of fatty acids caused slight inhibition; this is consistent
388 with a competition for the catalytic centre. Such inhibition is in agreement with results
389 obtained for other lipases (Ruiz et al., 2004).

390 The triad of Ser-His-Asp is observed in catalytic sites of several lipases with
391 carboxylate residue of either aspartic acid or glutamic acid (Schrag et al., 1991). In the
392 present study, chemical modification was employed to determine the amino acids
393 responsible for AML catalysis. The amino acids in the catalytic triad of lipases have
394 previously been determined through chemical modification studies (Hilton and Buckley,
395 1991; Mhetras et al., 2009). The catalytic site in the purified AML involved Ser, His and
396 carboxylate residues. In addition, Trp seemed to play an important role in catalytic activity
397 of AML since the NBS mediated modification resulted in significant inactivation. Trp has
398 been shown to be responsible for interfacial activation (Feng et al., 2002) and may possibly
399 serve the same function in AML catalysis.

400 The stability or enhancement of activity in the presence of organic solvents is
401 generally considered a desirable feature, as it is a prerequisite for synthetic applications in
402 non-aqueous media (Dandavate et al., 2009; Zaks and Klivanov, 1998). AML was
403 remarkably stable with activation in both hydrophilic and hydrophobic organic solvents.

404 Stability of bacterial lipases in hydrophilic solvents with activation is a rare property (Lima
405 et al., 2004), although stability in hydrophilic solvents has been reported in few
406 actinomycete lipases (Bielen et al., 2009; Leščić et al., 2001).

407 Isoamyl acetate is one of the most important flavor and fragrance compounds used
408 in the food, beverage, cosmetics and pharmaceutical industries because of its characteristic
409 banana flavour (Romero et al., 2005). AML exhibited significant potential for the synthesis
410 of isoamyl acetate. Although, the reaction conditions were not optimized, the purified
411 lipase showed considerable esterification capacity (34.4%). Furthermore, lipase
412 immobilized on celite exhibited roughly 2 fold higher esterification for isoamyl acetate
413 synthesis in comparison to free enzyme (Fig. 4). The immobilization of lipases on celite has
414 been previously reported to improve the catalytic activity of enzymes by providing
415 protection against the denaturing effects of organic solvents (Khare and Nakajima, 2000;
416 Salah et al., 2007).

417 **5. Conclusions**

418 In this study, AML was purified to homogeneity with 398 fold purity and a specific
419 activity of 781 IU/mg protein. The characterization study of purified AML showed that it
420 has a number of industrially important characteristics like high thermostability, organic
421 solvent tolerance and specificity towards broad substrate range. All these features make
422 AML, a suitable candidate for application in non aqueous biocatalytic processes such as
423 esterification of primary and secondary alcohols, random interesterification of different oils
424 and fats, oil contaminated biodegradation and biodiesel production. Moreover, purified
425 AML also showed potential in synthesis of industrially important flavor ester, isoamyl

426 acetate. Therefore, future studies should focus on optimization of isoamyl acetate synthesis
427 using AML and examination of related synthetic capabilities.

428 **Acknowledgements**

429 This work was financially supported by DIT ABBEST Research Scholarship (PB
430 03557/2007) to DSD. The authors would like to thank Mr. Kevin Conboy, Centre for
431 Synthesis and Chemical Biology (CSCB), University College Dublin, Dublin, for technical
432 assistance with gas chromatography.

433 **References**

434 Abramić, M., Leščić, I., Korica, T., Vitale, L., Saenger, W., Pigac, J., 1999. Purification
435 and properties of extracellular lipase from *Streptomyces rimosus*. *Enzyme Microb.*
436 *Technol.* 25, 522–529.

437 Berekaa, M.M., Zaghloul, T.I., Abdel-Fattah, Y.R., Saeed, H.M., Sifour, M., 2009.
438 Purification of a novel glycerol-inducible lipase from thermophilic *Geobacillus*
439 *stearothermophilus* strain-5. *World J. Microbiol. Biotechnol.* 25, 287–294.

440 Bielen, A., Cetkovic, H., Long, P.F., Schwab, H., Abramić, M., Vujaklija, D., 2009. The
441 SGNH hydrolase of *Streptomyces coelicolor* has (aryl)esterase and a true lipase
442 activity. *Biochimie* 91, 390–400.

443 Bofill, C., Prim, N., Mormeneo, M., Manresa, A., Javier Pastor, F.I., Diaz, P., 2010.
444 Differential behaviour of *Pseudomonas* sp. 42A2 LipC, a lipase showing greater
445 versatility than its counterpart LipA. *Biochimie* 92, 307–316.

446 Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram
447 quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*
448 72, 248–254.

449 Burkert, J.F.M., Maugeri, F., Rodrigues, M.I., 2004. Optimization of extracellular lipase
450 production by *Geotrichum* sp. using factorial design. *Bioresour. Technol.* 91, 77–
451 84.

452 Calero-Rueda, O., Plou, F.J., Ballesteros, A., Martinez, A.T., Martinez, M.J. 2002.
453 Production, isolation and characterization of a sterol esterase from *Ophiostoma*
454 *piceae*. *Biochim. Biophys. Acta.* 1599, 28–35.

455 Castro-Ochoa, L.D., Rodriguez-Gomez, C., Valerio-Alfaro, G., Ros, R.O. 2005.
456 Screening, purification and characterization of the thermoalkalophilic lipase
457 produced by *Bacillus thermoleovorans* CCR11. *Enzyme Microb. Technol.* 37, 648–
458 654.

459 Côté, A., Shareck, F., 2008. Cloning, purification and characterization of two lipases from
460 *Streptomyces coelicolor* A3(2). *Enzyme Microb. Technol.* 42, 381–388.

461 Dandavate, V., Jinjala, J., Keharia, H., Madamwar, D., 2009. Production, partial
462 purification and characterization of organic solvent tolerant lipase from
463 *Burkholderia multivorans* V2 and its application for ester synthesis. *Bioresour.*
464 *Technol.* 100, 3374–3381.

465 Dheeman, D.S., Frias, J.M., Henehan, G.T.M., 2010. Influence of cultivation conditions
466 on the production of a thermostable extracellular lipase from *Amycolatopsis*
467 *mediterranei* DSM 43304. *J. Ind. Microbiol. Biotechnol.* 37, 1–17.

468 Feng, J., Wehbi, H., Roberts, M.F. 2002. Role of tryptophan residues in interfacial
469 binding of phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* 277,
470 19867–19875.

471 Gupta, R., Gupta, N., Rathi, P., 2004. Bacterial lipases: an overview of production,
472 purification and biochemical properties. *Appl. Microbiol. Biotechnol.* 64, 763–781.

473 Hasan, F., Shah, A., Hameed, A., 2006. Industrial application of microbial lipases.
474 *Enzyme Microb. Technol.* 39, 235–251.

475 Helistö, P., Korpela, T., 1998. Effects of detergents on activity of microbial lipases as
476 measured by the nitrophenyl alkanoate esters method. *Enzyme Microb. Technol.* 23,
477 113–117.

478 Hilton, S., Buckley, J.T., 1991. Studies on the reaction mechanism of a microbial
479 lipase/acyltransferase using chemical modification and site-directed mutagenesis. *J.*
480 *Biol. Chem.* 266, 997–1000.

481 Jackson, M.A., King, J.W., List, G.R., Neff, W.E., 1997. Lipase-catalyzed randomization
482 of fats and oils in flowing supercritical carbon dioxide. *J. Am. Oil Chem. Soc.* 74,
483 635–639.

484 Khare, S.K., Nakajima, M., 2000. Immobilization of *Rhizopus japonicus* lipase on celite
485 and its application for enrichment of docosahexaenoic acid in soybean oil. *Food*
486 *Chem.* 68, 153–157.

487 Kordel, M., Hofmann, B., Schomburg, D., Schmid, R.D., 1991. Extracellular lipase of
488 *Pseudomonas* sp. strain ATCC 21808: purification, characterization, crystallization,
489 and preliminary X-ray diffraction data. *J. Bacteriol.* 173, 4836–4841.

490 Lee, D.W., Kim, H.K., Lee, K.W., Kim, B.C., Choe, A.C., Lee, H.S., Kim, D.S., Pyun,
491 Y.R., 2001. Purification and characterization of two distinct thermostable lipases
492 from the gram-positive thermophilic bacterium *Bacillus thermoleovorans* ID-1.
493 *Enzyme Microb. Technol.* 29, 363–371.

494 Leščić, I., Vukelic, B., Majeric-Elenkov, M., Saenger, W., Abramić, M., 2001. Substrate
495 specificity and effects of water-miscible solvents on the activity and stability of
496 extracellular lipase from *Streptomyces rimosus*. *Enzyme Microb. Technol.* 29, 548–
497 553.

498 Lima, V.M.G., Krieger, N., Mitchell, D.A., Baratti, J.C., Filippis, I., Fontana, J.D., 2004.
499 Evaluation of the potential for use in biocatalysis of a lipase from a wild strain of
500 *Bacillus megaterium*. *J. Mol. Catal. B: Enzym.* 31, 53–61.

501 Mhetras, N.C., Bastawde, K.B., Gokhale, D.V., 2009. Purification and characterization of
502 acidic lipase from *Aspergillus niger* NCIM 1207. *Bioresour. Technol.* 100, 1486–
503 1490.

504 Nawani, N., Kaur, J., 2007. Studies on lipolytic isoenzymes from a thermophilic *Bacillus*
505 sp.: production, purification and biochemical characterization. *Enzyme Microb.*
506 *Technol.* 40, 881–887.

507 Pandey, A., Benjamin, S., Soccol, C.R., Nigam, P., Krieger, N., Soccol, V.T., 1999. The
508 realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem.* 29, 119–
509 131.

510 Patkar, S., Björkling, F., 1994. Lipase inhibitors, in: Woolley, P., Petersen, S.B. (Eds.),
511 *Lipases: their structure, biochemistry and application*. Cambridge University Press,
512 pp. 207–224.

513 Prim, N., Sanchez, M., Ruiz, C., Pastor, F.I., Diaz, P., 2003. Use of methylumbeliferyl-
514 derivative substrates for lipase activity characterization. *J. Mol. Catal. B: Enzym.*
515 22, 339–346.

516 Queiroz, J.A., Garcia, F.A.P., Cabral, J.M.S., 1995. Hydrophobic interaction
517 chromatography of *Chromobacterium viscosum* lipase. J. Chromatogr. A 707, 137–
518 142.

519 Rahman, R.N.Z.R.A., Baharum, S.N., Basri, M., Salleh, A.B., 2005. High-yield
520 purification of an organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5.
521 Anal. Biochem. 341, 267–274.

522 Romero, M.D., Calvo, L., Alba, C., Daneshfar, A., Ghaziaskar, H.S. 2005. Enzymatic
523 synthesis of isoamyl acetate with immobilized *Candida antarctica* lipase in n-
524 hexane. Enzyme Microb. Technol. 37, 42–48.

525 Ruiz, C., Falcocchio, S., Xoxi, E., Pastor, F.I.J., Diaz, P., Saso, L., 2004. Activation and
526 inhibition of *Candida rugosa* and *Bacillus*-related lipases by saturated fatty acids
527 evaluated by a new colorimetric microassay. Biochim. Biophys. Acta. 1672, 184–
528 191.

529 Salah, R.B., Ghamghui, H., Miled, N., Mejdoub, H., Gargouri, Y., 2007. Production of
530 butyl acetate ester by lipase from novel strain of *Rhizopus oryzae*. J. Biosci. Bioeng.
531 103, 368–372.

532 Salameh, M.A., Wiegel, J., 2007. Purification and characterization of two highly
533 thermophilic alkaline lipases from *Thermosyntropha lipolytica*. Appl. Environ.
534 Microbiol. 73, 7725–7731.

535 Schmidt-Dannert, C., Rua, M.L., Atomi, H., Schmid, R.D., 1996. Thermoalkalophilic
536 lipase of *Bacillus thermocatenuatus* I. Molecular cloning, nucleotide sequence,
537 purification and some properties. Biochim. Biophys. Acta. 1301, 105–114.

538 Schrag, J.D., Li, Y., Wu, S., Cygler, M., 1991. Ser-His-Glu triad forms the catalytic site
539 of the lipase from *Geotrichum candidum*. *Nature* 351, 761–764.

540 Sharma, R., Soni, S.K., Vohra, R.M., Gupta, L.K., Gupta, J.K., 2002. Purification and
541 characterization of a thermostable alkaline lipase from a new thermophilic *Bacillus*
542 sp. RSJ-1. *Process Biochem.* 37, 1075–1084.

543 Sharma, R., Chisti, Y., Banerjee, U.C., 2001. Production, purification, characterization,
544 and applications of lipases. *Biotechnol. Adv.* 19, 627-662.

545 Silva, W.O.B., Santi, L., Berger, M., Pinto, A.F.M., Guimaraes, J.A., Schrank, A.,
546 Vainstein, M.H., 2009. Characterization of a spore surface lipase from the
547 biocontrol agent *Metarhizium anisopliae*. *Process Biochem.* 44, 829–834.

548 Soliman, N.A., Knoll, M., Abdel-Fattah, Y.R., Schmid, R.D., Lange, S., 2007. Molecular
549 cloning and characterization of thermostable esterase and lipase from *Geobacillus*
550 *thermoleovorans* YN isolated from desert soil in Egypt. *Process Biochem.* 42,
551 1090–1100.

552 Sommer, P., Bormann, C., Gotz, F., 1997. Genetic and biochemical characterization of a
553 new extracellular lipase from *Streptomyces cinnamomeus*. *Appl. Environ.*
554 *Microbiol.* 63, 3553–3560.

555 Sugihara, A., Ueshima, M., Shimada, Y., Tsunasawa, S., Tominaga, Y., 1992.
556 Purification and characterization of a novel thermostable lipase from *Pseudomonas*
557 *cepacia*. *J. Biochem.* 112, 598–603.

558 Sztajer, H., Maliszewska, I., Wierczorek, J., 1988. Production of exogenous lipases by
559 bacteria, fungi, and actinomycetes. *Enzyme Microb. Technol.* 10, 492–497.

560 Tan, T., Lu, J., Nie, K., Deng, L., Wang, F., 2010. Biodiesel production with immobilized
561 lipase: A review. *Biotech. Adv.* 28, 628–634.

562 Wang, X., Yu, X., Xu, Y., 2009. Homologous expression, purification and
563 characterization of a novel high-alkaline and thermal stable lipase from
564 *Burkholderia cepacia* ATCC 25416. *Enzyme Microb. Technol.* 45, 94–102.

565 Wei, Y., Swenson, L., Castro, C., Derewenda, U., Minor, W., Arai, H., Aoki, J., Inoue,
566 K., Servin-Gonzalez, L., Derewenda, Z.S., 1998. Structure of a microbial
567 homologue of mammalian plateletactivating factor acetylhydrolases: *Streptomyces*
568 *exfoliatus* lipase at 1.9 Å resolution. *Structure*, 6, 511–519.

569 Winkler, U.K., Stuckman. M., 1979. Glycogen, hyaluronate and some other
570 polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*.
571 *J. Bacteriol.* 138, 663–679.

572 Yu, L., Xu, Y., Yu, X., 2009. Purification and properties of a highly enantioselective L-
573 menthyl acetate hydrolase from *Burkholderia cepacia*. *J. Mol. Catal. B:Enzym.* 57,
574 27–33.

575 Zaks, A., Klibanov, A.M., 1998. Enzymatic catalysis in nonaqueous solvents. *J. Bio.*
576 *Chem.* 263, 3194–3201.

577 Zhang, J., Hou, Z., Yao, C., Yu, J., 2002. Purification and properties of lipase from a
578 *Bacillus* strain for catalytic resolution of (R)-naproxen. *J. Mol. Catal. B:Enzym.* 18,
579 205–210.

580 Zhang, Y., Meg, K., Wang, Y., Luo, H., Yang, P., Shi, P., Wu, N., Fan, Y., Li, J., Yao, B.,
581 2008. A novel proteolysis-resistant lipase from keratinolytic *Streptomyces fradiae*
582 var. k11. *Enzyme Microb. Technol.* 42, 346–352.

583 **Table 1**

584 Purification of AML

585	Purification step	Total activity ^a	Total protein ^b	Specific activity	Purification	Yield
586		(IU)	(mg)	(IU/mg)	(fold)	(%)
587	Culture filtrate	212.0	108.0	1.96	1.00	100
588	Ammonium sulphate precipitation	196.16	27.34	7.17	3.66	92.52
589	Q Sepharose HP	115.82	4.96	23.35	11.91	54.63
590	Toyopearl Phenyl-650M	76.6	0.098	781.63	398.79	36.13

591 ^a One International Unit (IU): 1 μ mol of *p*-NP released per min using *p*-NPP as substrate.592 ^b Protein concentration was estimated by Bradford method (Bradford 1979).

593

594

595

596

597

598 **Table 2**

599 N-terminal sequence comparison of AML with *Streptomyces exfoliatus* lipase

600	AML*	AANPYERGP <u>D</u> PT T ASIEA T R	This study
601	<i>S. exfoliatus</i> lipase	AANPYERGPAPTNASIEASR	(Wei et al. 1998)

602 *Non-matching amino acid residues are underlined and highlighted in bold.

603

604

605

606

607

608

609

610

611

612

613

614

615

616

617

618

619

620

621 **Table 3**

622 Effect of effector molecules on AML activity

623	Effector molecule	Relative activity (% \pm SD) ^a
624	Control	100.0 \pm 1.8
625	EDTA	99.1 \pm 2.5
626	Sodium citrate	99.5 \pm 3.8
627	SDS	99.7 \pm 1.5
628	Digitonin	142.5 \pm 2.4
629	Sodium deoxycholate	241.3 \pm 1.0
630	β -Mercaptoethanol	136.4 \pm 2.2
631	1,4-Dithiothreitol	147.6 \pm 0.9
632	Ascorbic acid	124.4 \pm 3.4
633	Capric acid	89.7 \pm 0.5
634	Myristic acid	97.1 \pm 1.9
635	Palmitic acid	99.6 \pm 1.4
636	Urea (6.0 M) ^b	98.9 \pm 3.5

637 ^a Purified AML was incubated in the presence of effector molecules (1 mM) at 30°C for 1 h. The
638 activity is expressed as a percentage of the activity of untreated control. Values represent the
639 mean of three replicates \pm standard deviation (SD).

640 ^b Urea concentration in incubation mixture.

641

642

643

644 **Table 4**

645 Effect of group specific reagents on AML activity

646	Reagent	Possible reaction site	Residual activity (% \pm SD)
647	CA	Lys	98.8 \pm 3.2
648	DEPC	His	00.0
649	EDAC	Asx/Glx	00.0
650	IA	Cys	92.3 \pm 3.1
651	NAI	Tyr	99.2 \pm 2.7
652	NBS	Trp	34.4 \pm 1.5
653	PG	Arg	99.5 \pm 1.2
654	PMSF	Ser	11.6 \pm 1.8

655 Purified AML (10 μ g) was incubated with reagents (5 mM) specific to different amino acid

656 functional groups. After 1 h at 30°C, residual AML activity was determined. The activity is

657 expressed as a percentage of the activity of untreated control. Values represent the mean of three

658 replicates \pm standard deviation (SD).

659

660

661

662

663

664

665

666

667 **Legends for figures**

668 **Fig. 1** SDS-PAGE of purified AML and zymogram analysis

669 (a) Lane 1: standard proteins; lane 2: culture supernatant; lane 3: culture supernatant precipitated
670 with 40% ammonium sulphate and dialyzed; lane 4: purified protein after Toyopearl Phenyl-
671 650M chromatography. (b) Zymogram from an SDS-PAGE of purified AML analyzed for
672 activity by MUF-butyrate (right) and subsequently stained with silver nitrate (left). The samples
673 loaded correspond to molecular weight standards (lane 1) and purified AML (lanes 2 and 3).

674 **Fig. 2** Relative activities of AML towards various substrates

675 Lipase activities are expressed as the percentage of that of *p*-NP caprylate (C:8) (a) or olive oil (b
676 and c). Values represent the mean of three independent experiments and error bars indicate
677 standard deviations.

678 **Fig. 3** Thin-layer chromatography of products of triolein hydrolysis by AML

679 Lane 1: 1(2)-monooleylglycerol (1(2)-MO); lane 2: oleic acid; lane 3: 1,2(2,3)-dioleylglycerol
680 (1,2(2,3)-DO) with traces of 1,3-dioleylglycerol; lane 4: 1,3-dioleylglycerol (1,3-DO); lane 5:
681 triacylglycerol (TO); lane 6: standard mixture; lane 7: control (without enzyme); lane 8: 20 IU of
682 purified AML.

683 **Fig. 4** Isoamyl acetate synthesis by free (●) and celite-immobilized (○) AML

684 Values represent the means of three independent experiments and error bars indicate standard
685 deviations.

686

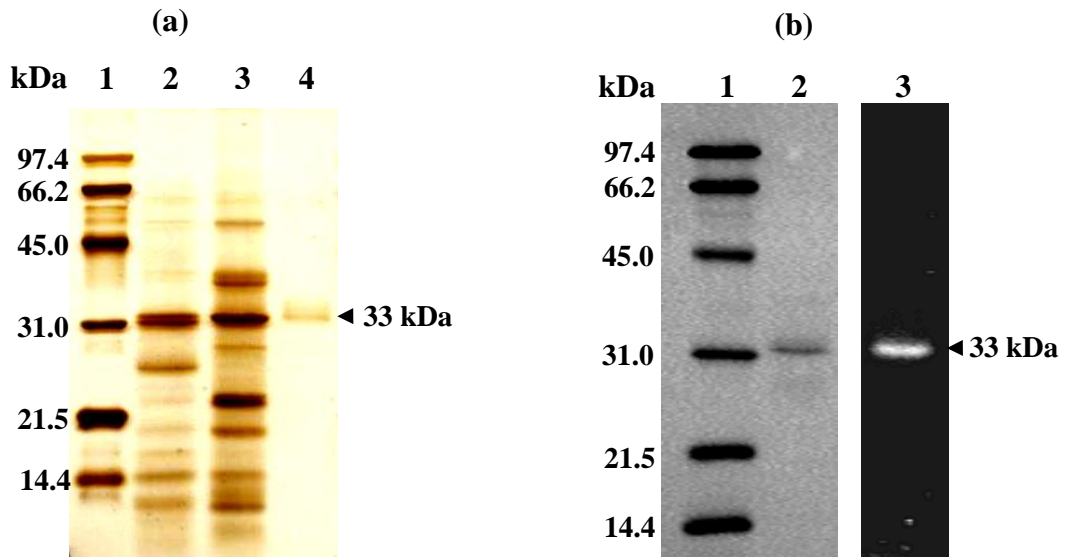
687

688

689

690 **Fig. 1**

691



692

693

694

695

696

697

698

699

700

701

702

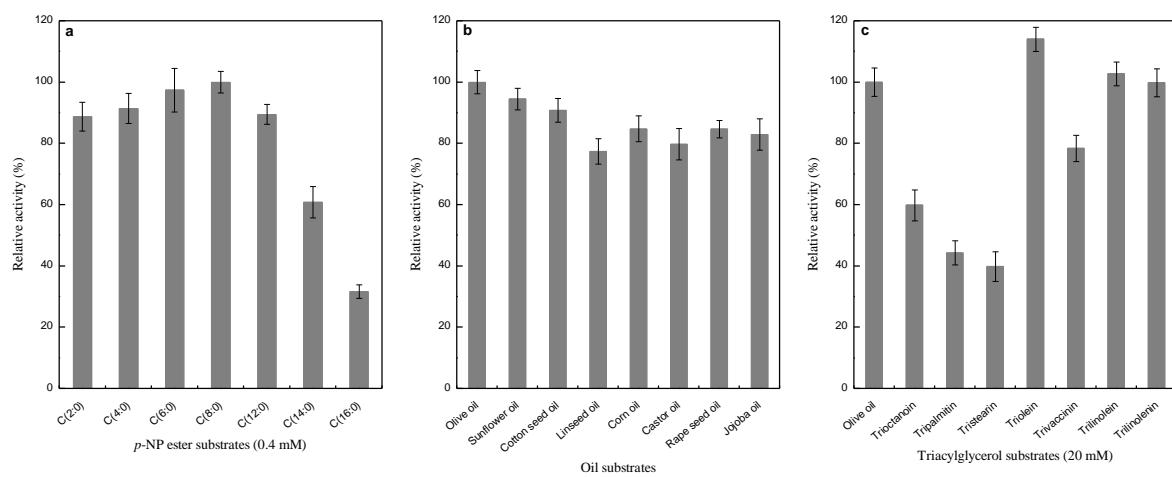
703

704

705

706

707 **Fig. 2**



708
709

710

711

712

713

714

715

716

717

718

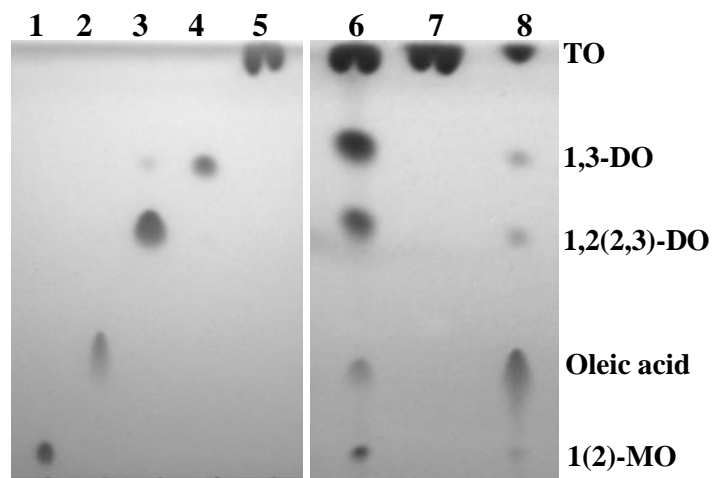
719

720

721

722 **Fig. 3**

723



724

725

726

727

728

729

730

731

732

733

734

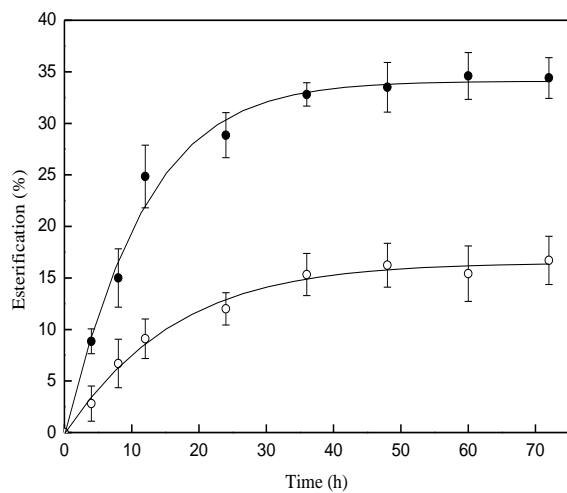
735

736

737

738

739 **Fig. 4**



740

741

742