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

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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, AANPYERGPDPTTASIEATR, 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

1. Introduction

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

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

3

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 Na_2CO_3. Liberated *p*-nitrophenol

83 (*p*-NP) was determined at 410 nm ($\varepsilon_{410\ nm}$: 0.0169/µmol 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

4

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 ×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 ×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 ×g for 10 min at 4°C) and the supernatant was applied to

106 a Q Sepharose HP column (2.5 × 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

5

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**

8

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

9

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

11

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 ± 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-dioleylglycerol (1,3-DO), 1,2(2,3)-

271   dioleylglycerol (1,2(2,3)-DO) and 1(2)-monooleylglycerol (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, cystein 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 (dimethyformamide, 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).

14

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

15

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

16

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 (Leščić 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 Klibanov, 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**

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583 **Table 1**

584 Purification of AML

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

591 [a] One International Unit (IU): 1 µmol of p-NP released per min using p-NPP as substrate.

592 [b] Protein concentration was estimated by Bradford method (Bradford 1979).

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598 **Table 2**

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

600 AML[*]	AANPYERGP**D**PT**T**ASIEA**T**R	This study
601 *S. exfoliatus* lipase	AANPYERGPAPTNASIEASR	(Wei et al. 1998)

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

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621 **Table 3**

622 Effect of effector molecules on AML activity

Effector molecule	Relative activity (% $\pm$ SD)[a]
Control	100.0 ± 1.8
EDTA	99.1 ± 2.5
Sodium citrate	99.5 ± 3.8
SDS	99.7 ± 1.5
Digitonin	142.5 ± 2.4
Sodium deoxycholate	241.3 ± 1.0
β-Mercaptoethanol	136.4 ± 2.2
1,4-Dithiothreitol	147.6 ± 0.9
Ascorbic acid	124.4 ± 3.4
Capric acid	89.7 ± 0.5
Myristic acid	97.1 ± 1.9
Palmitic acid	99.6 ± 1.4
Urea (6.0 M)[b]	98.9 ± 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.

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644 **Table 4**

645 Effect of group specific reagents on AML activity

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

655 Purified AML (10 µg) 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 ± standard deviation (SD).

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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
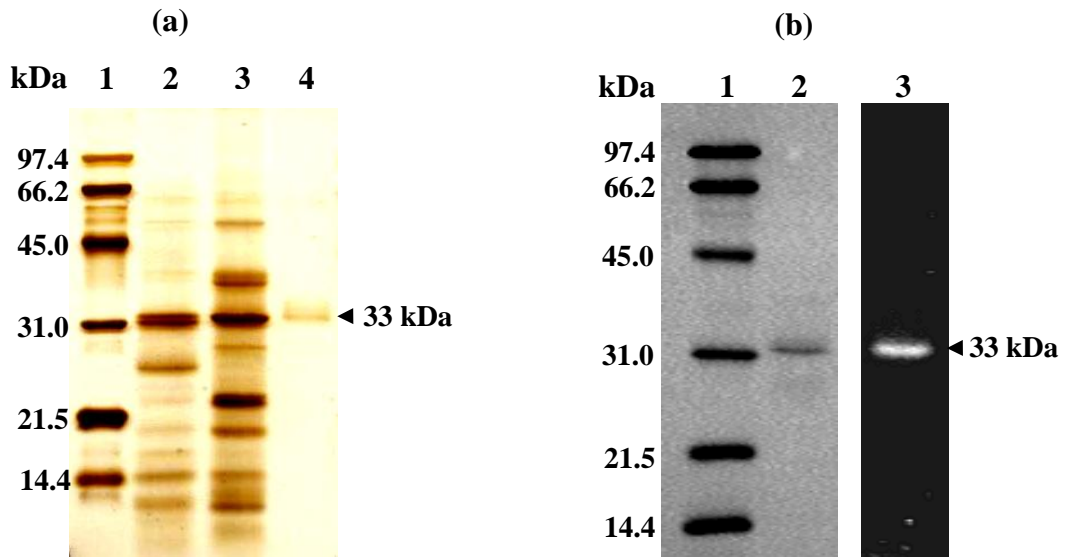
685 deviations.

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690   **Fig. 1**

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(a) **(b)**

(a) SDS-PAGE lanes labeled: kDa, 1, 2, 3, 4 with markers 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 and arrow marking ◄ 33 kDa

(b) lanes labeled: kDa, 1, 2, 3 with markers 97.4, 66.2, 45.0, 31.0, 21.5, 14.4 and arrow marking ◄ 33 kDa

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707   **Fig. 2**
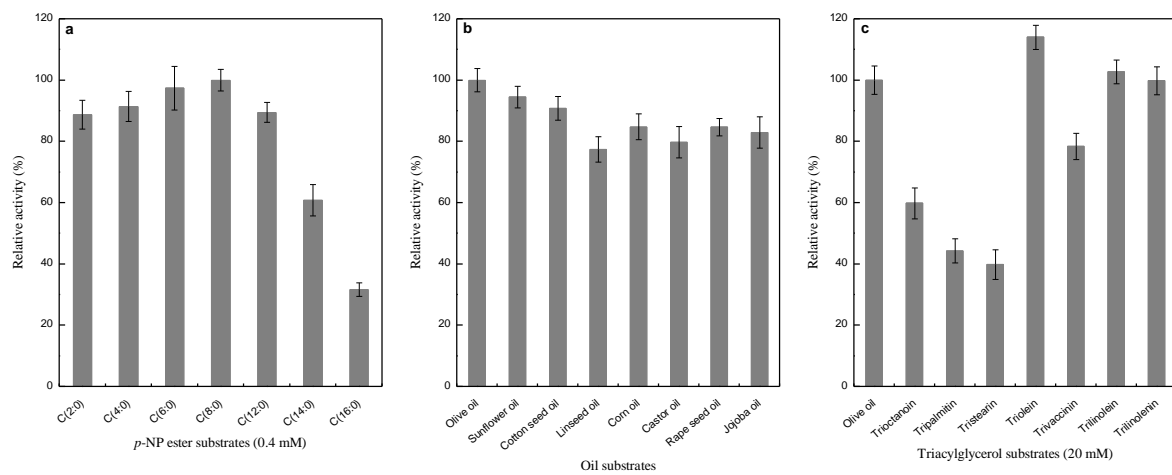
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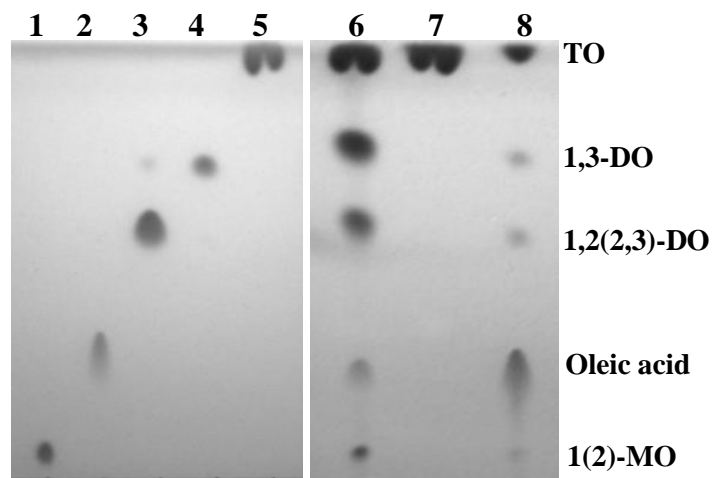
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Fig. 3

739 **Fig. 4**

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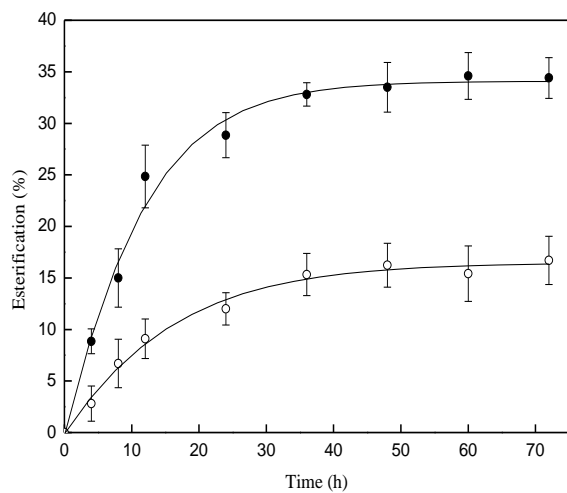
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