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

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

p-NPP: *p*-Nitrophenyl Palmitate

Abstract

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

Keywords: Actinomycete lipase; *Amycolatopsis mediterranei*; Purification; 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

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

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

2.3.2 Titrimetric assay

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

2.4 Purification of AML

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

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

2.5 Gel electrophoresis and zymography

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

2.6 N-terminal sequence analysis

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

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

2.7 The effect of pH and temperature on activity and stability

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

2.8 Determination of kinetic constants

Initial rates measurements with 0.59 µg of purified AML were performed in 50 mM Tris–HCl buffer, pH 8.0 at 60°C with increasing concentration of *p*-NPP (0.02–0.93 mM). Kinetic constants were obtained by fitting experimental data to the Michaelis-Menten equation using EnzFitter (Biosoft, Cambridge, UK) to obtain estimates of Michaelis constant (K_m^{app}) and maximal velocity ($V_{\max}^{app}$). Turnover number k_{cat}^{app} was calculated using 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**

The effect of various detergents, oxidizing-reducing agents, chelating agents, free 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+}) on purified AML activity was analyzed by incubating the pure enzyme in 1 mM of these effectors for 1 h at 30°C in 50 mM Tris-HCl buffer (pH 8.0). The effect of urea was assessed at 6.0 M. The effect of group specific reagents (*N*-acetylimidazole (NAI), *N*-bromosuccinimide (NBS), phenylmethyl-sulfonylfluoride (PMSF), diethylpyrocarbonate (DEPC), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), iodoacetate (IA), citraconic anhydride (CA), phenylglyoxal (PG)) on AML was determined by incubating the purified enzyme in presence of 5 mM of these reagents for 1 h at 30°C. The effect of various organic solvents (40%, v/v) on AML activity was determined by incubating 1 ml of purified enzyme solution in 1.5 ml of the different organic solvents in airtight vials at 30°C, 200 rpm for 24 h. The control used was an enzyme sample without reagent/organic solvent under the same experimental conditions. Residual activity was measured using the spectrophotometric assay at 60°C, pH 8.0.

2.12 Potential of purified AML in ester synthesis

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

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

2.12.1 Quantification of ester synthesis

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

3. Results

3.1 Lipase purification

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

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

3.2 Gel electrophoresis and zymography

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

3.3 N-terminal sequence of AML

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

3.4 The effect of pH and temperature on activity and stability

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

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

3.5 Determination of kinetic constants

The kinetic analysis of purified AML performed on standard assay substrate, *p*-NPP at 60°C produced a Lineweaver Burk plot corroborating the Michaelis-Menten behavior of 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 1467.59 ± 34.86/s.

3.6 Substrate range

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

3.7 Position specificity

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

3.8 Effect of various reagents and organic solvents

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

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

3.9 Potential of purified AML in ester synthesis

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

5. Conclusions

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

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

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Table 1

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

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

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

Table 2

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

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

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

Table 3

Effect of effector molecules on AML activity

Effector molecule	Relative activity (% ± 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

[a] Purified AML was incubated in the presence of effector molecules (1 mM) at 30^{o}C for 1 h. The activity is expressed as a percentage of the activity of untreated control. Values represent the mean of three replicates ± standard deviation (SD).

[b] Urea concentration in incubation mixture.

Table 4

Effect of group specific reagents on AML activity

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

Purified AML (10 µg) was incubated with reagents (5 mM) specific to different amino acid functional groups. After 1 h at 30°C, residual AML activity was determined. The activity is expressed as a percentage of the activity of untreated control. Values represent the mean of three replicates ± standard deviation (SD).

Legends for figures

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

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

Fig. 2 Relative activities of AML towards various substrates

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

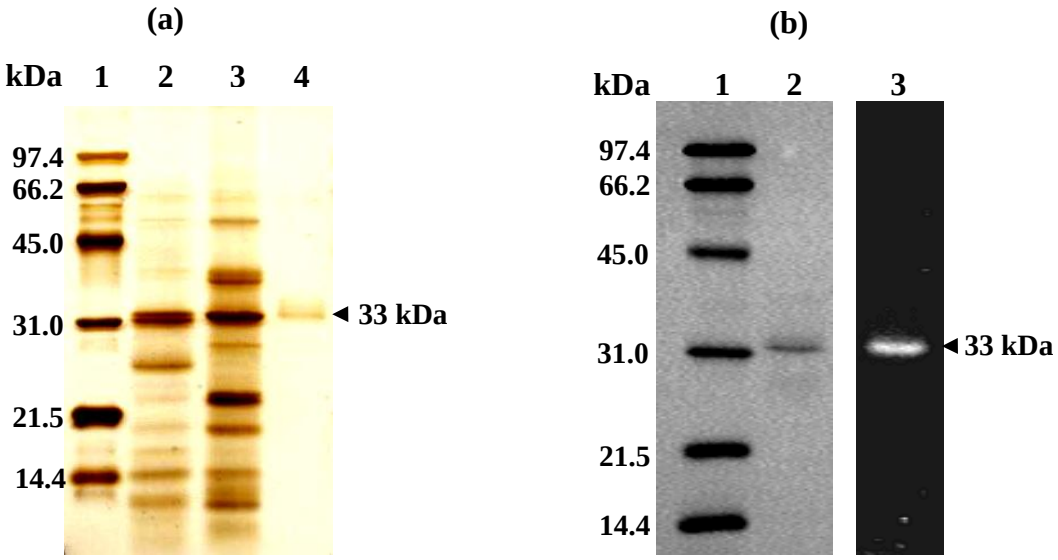
Fig. 3 Thin-layer chromatography of products of triolein hydrolysis by AML

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

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

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

Fig. 1


(a)
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707 **Fig. 2**

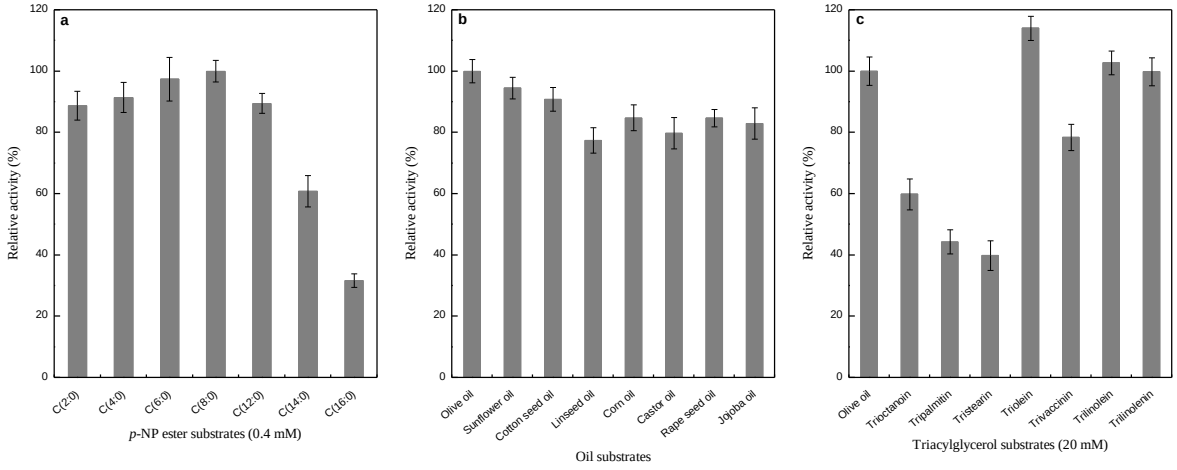

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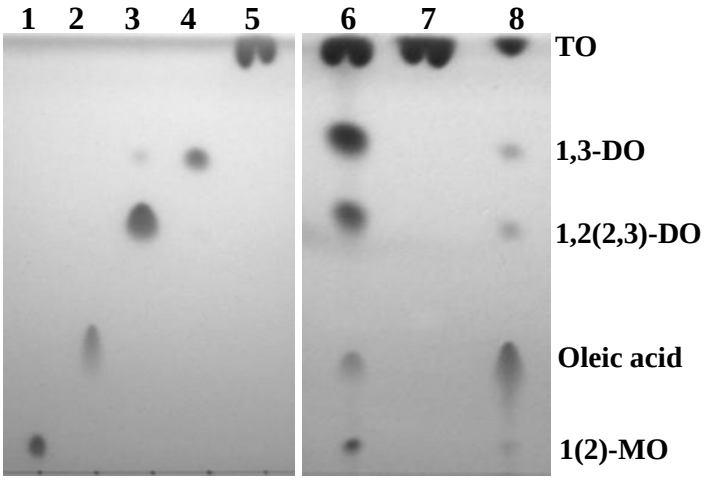
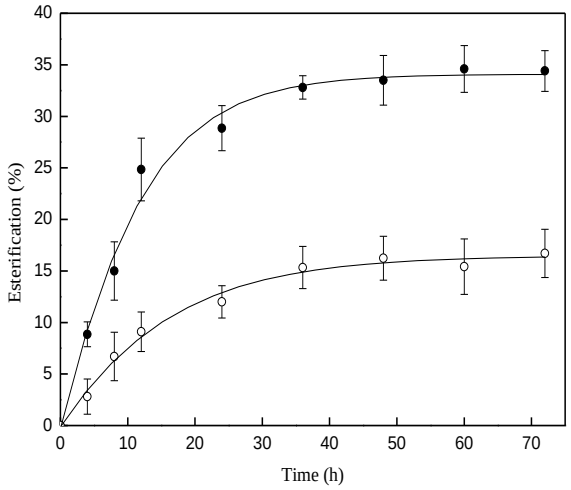
Fig. 3



739 **Fig. 4**



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