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Journal of Molecular Catalysis B: Enzymatic 36 (2005) 30–35
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enantioselective hydrolysis of methoxyphenyl glycidic acid methyl ester [(±)-MPGM] by a thermostable and alkalostable lipase from *Pseudomonas aeruginosa*

Sawraj Singh, U.C. Banerjee *

Biocatalysis Laboratory, Department of Pharmaceutical Technology (Biotechnology), National Institute for Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar 160062, Punjab, India

Received 13 May 2005; received in revised form 18 July 2005; accepted 18 July 2005
Available online 16 September 2005

Abstract

A potent bacterial strain, *Pseudomonas aeruginosa,* has been isolated from the soil which produces extracellular lipase that can carry out the excellent stereospecific hydrolysis of *trans*-3-(4-methoxyphenyl)glycidic acid methyl ester [(±)-MPGM)] to give [(−)-MPGM], an intermediate required in the synthesis of cardiovascular drug, diltiazem. As a preliminary experiment for enzymatic resolution, we characterized the fractionated enzyme. The enzyme had a pH and temperature optima of 8.0 and 60 °C, respectively. The enzyme showed high degree of thermostability. Also, the enzyme was found to be stable in alkaline condition and in organic solvents. The activity of the enzyme increased by the addition of magnesium ions. The small-scale hydrolysis of (±)-MPGM (250 mg) with partially purified enzyme (21,000 U) gave (−)-MPGM with good isolated yield (44%) and excellent enantiomeric excess (99.9%) in a very short time (12 h).

Keywords: Lipase; Thermostable; Alkalostable; (±)-MPGM; Diltiazem

1. Introduction

Lipases (triacyl glycerol acyl hydrolases EC3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and fatty acids at oil–water interface. They are widely distributed in animals, plants and microorganisms [1]. Microbial lipases have been receiving particular attention because of their potential applications in the detergent, oil and fat and drug and pharmaceutical industries. Interest in the microbial lipases has increased marketedly in the last two decades owing to the potential industrial applications [2,3]. One of the applications is the synthesis of chirally important drugs and drug intermediates [4,5]. The stringent FDA guidelines regarding the chirally pure drugs has made it essential for the pharmaceutical industry to produce these in a more environment-friendly way.

Diltiazem hydrochloride, (2*S*,3*S*)-3-acetoxy-5-[2-(dimethylamino) ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5H)-one hydrochloride, is a typical calcium channel blocker and has been clinically used as an effective anti-anginal and anti-hypertensive agents [6,7] in more than 100 countries for over than 20 years. Conventional production was carried out using a chemical synthetic process through nine steps from 4-anisaldehyde and chloroacetic acid methyl ester [8]. However, in the chemical process, a large amount of loss in the raw material occurs, as the optical resolution was carried out at the later stage of the process using a high molecular weight compound, i.e. *threo*-2-hydroxy-3-(4-methoxyphenyl)-3-(2-nitrophenylthio)-propanoic acid. A few lipase catalyzed resolution of (±)-MPGM have been reported using the lipases from the commercial sources and using the enzyme from *Serratia marcescens* [9,10]. The process has been developed on the industrial scale using the lipase from *S. marcescens* [8]. A maximum of 48.2% yield along with an

* Corresponding author. Tel.: +91 172 2214682–87; fax: +91 172 2214692.
E-mail address: ucbanerjee@niper.ac.in (U.C. Banerjee).

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doi:10.1016/j.molcatb.2005.07.005

Table 1
Reported organisms capable of resolution of (±)-MPGM

Organism	Conversion (%)	ee (%)	Reference
Serratia marcescens sr41 8000	48.2	89	[8]
Pseudomonas putida ATCC 17426	44.2	75.1	[8]
Cornybacterium promorioxydans ATCC 31015	44.6	75.6	[8]
Pseudomonas mutabilis ATCC 31014	44.7	74.7	[8]
Cornybacterium alkanolyticum ATCC 21511	45.8	77.4	[8]
Candida cylinderacea	82	50	[8]
Bacillus licheniformis	41	57	[8]
Rhizopus japonicus	58.4	42.8	[8]
Pseudomonas sp.	49.8	68.6	[8]
Serratia marcescens ECU1010	–	97	[9]
Candida cylindradea	45	99	[18]

enantiomeric excess of >89% was obtained (Table 1). In view of the potential uses of this enzyme, it is desirable to study this enzyme from different microbial sources. In order to further improve the hydrolytic process in terms of conversion and enantiomeric excess and the reaction time, we conducted an extensive screening for the microorganisms that can carry out the stereospecific resolution of (±)-MPGM. As a result of this screening a bacterial strain, *Pseudomonas aeruginosa* was obtained that is capable of carrying out the stereospecific resolution of (±)-MPGM giving good yield (44%) and excellent ee (99.9%). We herein report the screening of the microbial strain, characterization of the enzyme and its subsequent exploitation in the stereospecific resolution of (±)-MPGM.

2. Materials and methods

2.1. Chemicals and medium

(±)-Methoxyphenyl glycidic acid methyl ester was prepared from 4-anisaldehyde and methyl chloroacetate in presence of sodium methoxide according to the reported procedure [11]. *p*-Methoxyphenylacetaldehyde was also synthesized in laboratory (M^+) (*m*/*e*) 151. ^{1}H NMR δ ($CDCl_3$, 300 MHz), 3.57 (2H, M CH_2CHO), 3.75 (3H, S, OCH_3), 6.85 (2H, d, J = 8.5 Hz, Ar–H), 7.09 (2H, d, J = 8.5 Hz, Ar–H), 9.67 (1H, S, CHO). All other chemicals were purchased from various commercial sources and were of analytical grade. Soil samples were collected from different areas of Punjab region of India. Minimal salt medium (MSM) consisting of disodium hydrogen phosphate (0.2%), potassium dihydrogen orthophosphate (0.1%), ammonium chloride (0.04%) and magnesium chloride (0.04%) was used with (±)-MPGM as the sole source of carbon and energy. Agar plates were prepared by supplementing MSM with 2 mM (±)-MPGM. The production medium consisted of peptone (0.5%), beef extract (0.15%), yeast extract (0.15%), NaCl (0.5%) and glucose (1%).

2.2. Isolation of microorganism

A soil suspension was prepared by adding 10 ml tap water to 1 g of the soil sample. It was vortexed and 1 ml supernatant was used as an inoculum in 100 ml MSM containing 2 mM (±)-MPGM medium and was incubated at 30 °C in an orbital shaker (200 rpm) for 5–7 days. Enriched samples were streaked on selective plates (the plates containing [(±)-MPGM])] in MSM agar. The organisms so obtained were purified and maintained on nutrient agar plates (0.5% peptone, 0.15% yeast extract, 0.5% beef extract, 0.5% sodium chloride, agar 1.5%, pH 8). These organisms were further grown in nutrient broth (0.5% peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sodium chloride, pH 8). The enzyme activity, the hydrolytic potential and the enantioselectivity for (±)-MPGM hydrolysis were determined. Out of these, the organisms showing the maximum hydrolytic activity and enantioselectivity were chosen for further studies. The microbial strain showing the best hydrolytic potential was identified according to the general procedures of Bergey's Manual of Systematic Bacteriology [12] at Microbial Type Culture Collection (MTCC), Chandigarh, India.

2.3. Analytical methods and enzyme assay

Enzyme assay was carried out by the method of Winkler and Stuckmann [13] with a little modification using *p*-nitrophenyl palmitate as a substrate. The substrate was dissolved in iso-propanol (3 mg/ml). It was then emulsified with aqueous solution (9 ml) of gum arabic (0.11%) and triton X-100 (0.44%). This emulsion (0.9 ml) was mixed with 1.5 ml Tris–HCl buffer (50 mM, pH 8) and 0.5 ml $CaCl_2$ (75 mM). The mixture was preincubated at 60 °C for 5 min and 100 μl of appropriately diluted enzyme was added and incubation was continued for further 10 min. The optically density was taken at 410 nm spectrophotometrically with proper enzyme and substrate blanks. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol/min of *p*-nitrophenol under the standard assay condition.

Conversion and the enantiomeric excess of the hydrolytic reaction were monitored by HPLC performed on a Shimadzu 10AVP Instrument equipped with UV detector using a Chiracel ODH column (0.46 mm × 250 mm, 5 μm, Diacel). The mobile phase used was hexane:isopropyl alcohol in the ratio of 85:15 (v/v) at a flow rate of 0.5 ml/min and detected at 254 nm. ^{1}H NMR spectra was recorded on a Bruker Advance DPX 300 NMR spectrometer. Chemical shifts were reported in parts per million (ppm, δ) using TMS as an internal standard. LCMS was performed on mass spectrometer (LCQ Finnigan MAT, England) using Chiracel ODH column (0.46 mm × 250 mm, 5 μm, Diacel) with mobile phase consisting of hexane: isopropyl alcohol

in the ratio of 85:15 (v/v). Optical rotations were measured on a Rudolph polarimeter (Rudolph Research Autopol IV).

2.4. Hydrolysis of [(±)-MPGM] by P. aeruginosa

The microbial strain was grown on nutrient agar plate (pH 8) for 24 h. Single colony was then inoculated in 50 ml nutrient broth and incubated in an orbital shaker (200 rpm) at 30 °C for 16 h. This seed culture (1%, v/v) was used as an inoculum to carry out the enzyme production in 100 ml nutrient broth (pH 8). The flasks were then incubated for 24 h (200 rpm, 30 °C). Since the enzyme produced is extracellular in nature, the cells were centrifuged out (10,000 × *g* for 10 min) and supernatant was concentrated. Supernatant (100 ml) with 301 U/ml lipase activity was precipitated with acetone in ratio of 1:2. For precipitation the mixture was kept at 4 °C for 12 h at 40 rpm and centrifuged at 12,000 × *g* for 15 min. The precipitates were resuspended in 10 ml Tris–HCl buffer (50 mM, pH 8) having 21,000 U of total lipase activity. The enzyme so obtained was used for the hydrolysis of [(±)-MPGM]. Twenty one thousand lipase units were used to hydrolyze 250 mg of substrate. It was carried out in toluene: lipase (1:1) two-phase system at pH 8 and 30 °C on a magnetic stirrer (1000 rpm) in a tightly plugged flask. Samples (toluene layer) were taken out after every 2 h and evaporated using rotavapour. Light yellow oily residue obtained was analyzed by HPLC. The identity of the product was further confirmed by LCMS and was assigned configuration on the basis of optical rotation.

2.5. Characterization of the lipase

The concentrated enzyme was used to find out the substrate specificity, optimum temperature, thermostability, optimum pH, pH stability, stability in the presence of organic solvents and effect of different metal ions. All the experiments were carried out thrice in duplicate and standard deviation from mean is shown in graphs.

2.5.1. Substrate specificity

To determine the substrate specificity of lipase, the enzyme activity was determined using a series of esters like *p*-nitrophenyl acetate, *p*-nitrophenyl propionate, *p*-nitrophenyl butyrate, *p*-nitrophenyl palmitate and *p*-nitrophenyl stearate with appropriate substrate and enzyme blank. In each case, the enzyme activity was determined using the individual substrate dissolved in iso-propanol (3 mg/ml). As short chain fatty esters (*p*-nitrophenyl acetate, *p*-nitrophenyl propionate, *p*-nitrophenyl butyrate) are not stable at 60 °C, the substrate specificity experiments were carried out at 30 °C. Except the assay temperature, all other conditions in these experiments followed the standard enzyme assay procedure.

2.5.2. Effect of temperature and pH

The temperature optimum of the enzyme was evaluated by measuring the lipase activity at different temperatures (25–75 °C) in 50 mM Tris–HCl buffer (pH 8.0) using *p*-nitrophenyl palmitate as substrate. The thermostability of lipase was tested by preincubating it at temperatures ranging from 50–70 °C and determining the residual lipase activity at different time intervals (0–48 h) according to the standard assay protocol. To ascertain the pH optimum of the enzyme, its activity was measured at different pH using 50 mM buffers, citrate (pH 6) and Tris–HCl for a pH range from 7–9. Similarly, the pH stability was investigated over the pH range of 7–9 using Tris–HCl buffer (pH 7–9).

2.5.3. Effect of solvents

The effect of various solvents (toluene, hexane and petroleum ether) on lipase activity was checked by incubating the enzyme with the solvent in the ratio of 1:1 at 30 °C on a magnetic stirrer (1000 rpm). The samples were withdrawn at regular interval (2.5 h) and lipolytic activity was determined according to the standard assay protocol.

2.5.4. Effect of metal ions

Lipase activity was determined in the presence of metal ions like calcium, copper, iron, potassium, magnesium, manganese, nickel and zinc (2 mM each). The enzyme solution was pre-incubated with metal ions (60 °C, 5 min), and lipase activity was determined according to the standard assay protocol using *p*-nitrophenyl palmitate as a substrate. Separate blanks with individual metal ions were prepared.

3. Results and discussion

3.1. Screening of [(±)-MPGM] hydrolyzing microorganisms

The lipase catalyzed stereospecific resolution of [(±)-MPGM] is shown in Fig. 1. [(±)-MPGM] is known to degrade at pH lower than 8.0 [8], hence to hydrolyze this substrate biochemically, an enzyme stable in alkaline condition is required. In order to find out such an enzyme, extensive screening of eighty different bacterial isolates was done for their stereospecific hydrolytic potential. Out of these, only four microorganisms showed good hydrolytic potential for (±)-MPGM. Among them only one bacterial strain, was selected for further study due to its good conversion (49.6%) and excellent enantioselectivity (ee > 99.9%).

The cells of the strain are small rods, non-sporulating, motile and fluorescent. They showed gram-negative behaviour. Additionally, the microorganism utilized glucose, arabinose and mannitol. All the taxonomic studies of the bacterial strain indicated that it belongs to the genus *Pseudomonas* and were identified as *P. aeruginosa*. The results have been confirmed by 16S rRNA analysis. The strain is deposited at MTCC in the Institute of Microbial Technology, Chandigarh, India with an accession number of 5113.

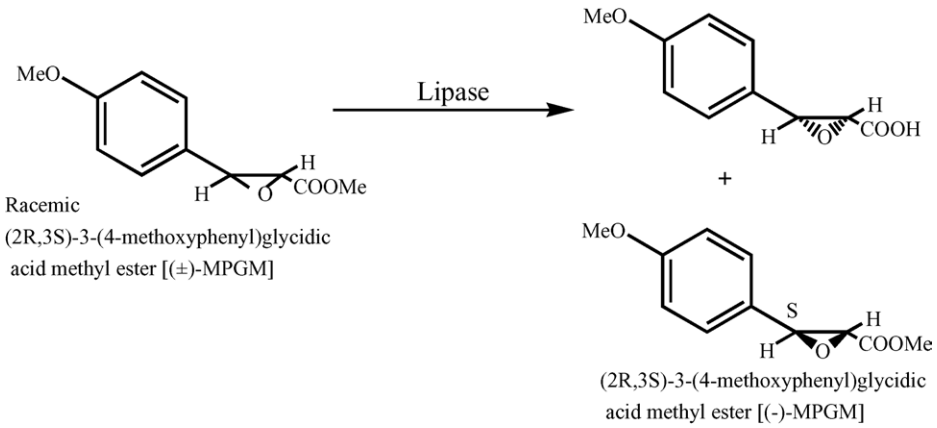


Fig. 1. Schematic diagram for the enzymatic resolution of [(±)-MPGM]. (Reaction has been carried out using 21,000 units of lipase to hydrolyze 250 mg of (±)-MPGM in toluene: buffer (Tris–HCl, 50 mM, pH 8) at 30 °C in an emulsion system at 1000 rpm.)

3.2. Resolution of (±)-MPGM

The resolution of (±)-MPGM was carried out by lipase from *P. aeruginosa* and it was observed after 12 h the isolated yield of (−)-MPGM was 44% with an excellent enantioselectivity (ee = 99.9%, Chiracel ODH). Mass (*m*/*e*) M^+ 208. ^{1}H NMR δ ($CDCl_3$, 300 MHz) = (1H, d, J = 160 Hz) 3.81 (3H, S, $COOCH_3$) 3.82 (3H, S OCH_3) 4.05 (1H, d J = 1.4 Hz >$CHCOOCH_3$) 6.89 (2H, d, J = 8.6H Ar–H) 7.20 (2H, d, J = 8.6 H, ArH). In mass spectrometer a molecular ion (M^+) (*m*/*e*) 151 has been observed in the −ve modes of APCI and ESI in the reaction constituents that correspond to *p*-methoxyphenylacetaldehyde having a molecular weight of 150. Matsumae et al. [8] reported that the hydrolysis of [(±)-MPGM] yields (−)-MPGM and (+)-MPGM is hydrolysed to produce *p*-methoxyphenylacetaldehyde, which has been used for the calculation of yield in the present study. The same has been compared with the chemically synthesized *p*-methoxyphenylacetaldehyde mass (M^+) (*m*/*e*) 151. ^{1}H NMR δ ($CDCl_3$, 300 MHz), 3.57 (2H, M CH_2CHO), 3.75 (3H, S, OCH_3), 6.85 (2H, d, J = 8.5 Hz, Ar–H), 7.09 (2H, d, J = 8.5 Hz, Ar–H), 9.67 (1H, S, CHO). The optical purity of the (−)-MPGM formed was further confirmed by measuring the optical rotation. $[\alpha]_D^{25}$-25.1 (c = 1, MeOH).

3.3. Characterization of lipase

The enzyme produced by *P. aeruginosa* is confirmed for its lipase activity by carrying out the reactions with long chain fatty esters in emulsion system (at interface of biphasic system) that is essential for lipase activation [1]. The lipase produced was characterized using the acetone precipitate of the same. Most of the industrially important enzymes are not purified fully as it increases the cost. Among the various esters tested (Table 2) the enzyme gave maximum activity with *p*-nitrophenyl butyrate followed by *p*-nitrophenyl palmitate, *p*-nitrophenyl acetate, *p*-nitrophenyl propionate and *p*-nitrophenyl stearate. Since, *p*-nitrophenyl butyrate is not stable at higher temperature (60 °C), *p*-nitrophenyl palmitate was chosen for characterization studies. Substrate specificity experiments were carried out at 30 °C as short chain fatty esters were not stable at high temperatures. Activity with *p*-nitrophenyl butyrate was taken as 100% in this experiment.

3.4. Effect of temperature and pH

The optimum temperature of the lipase activity was 60 °C (Fig. 2). The enzyme retained 94 and 65% of its maximum activity at 55 and 65 °C, respectively. Other workers have reported optimum temperature for lipase to be 55 °C [14] and 60–70 °C [15]. The present lipase from *P. aeruginosa* showed a very high thermostability and it retained 100% activity after 20 h and more than 75% of its original activity after 47 h at 50 °C. The half-life of the enzyme was more than 7 and 17 h at 70 and 60 °C (Fig. 3). This lipase shows comparable thermostability to the *Bacillus* strain A-30-1 (ATCC 53841) as reported by Wang et al. [14] that retained 100% of its original activity at 75 °C for 30 min, with a half-life of 8 h. The enzyme showed activity in a pH range of 6–9 (Fig. 4). The optimum pH of this enzyme was found to be 8. Regarding the

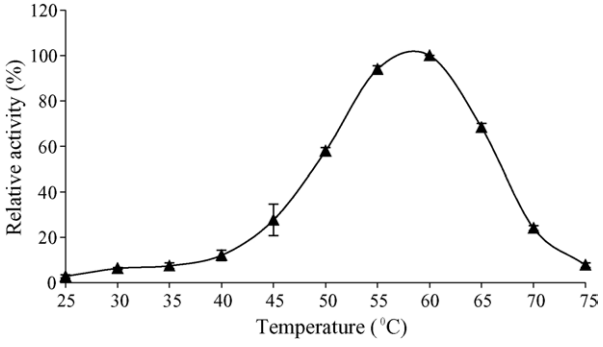
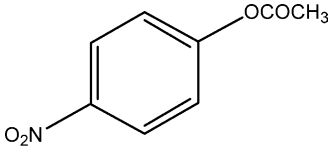
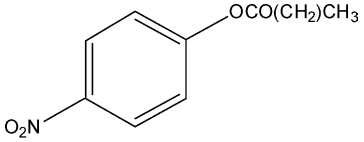
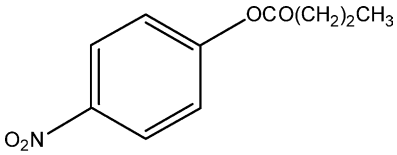
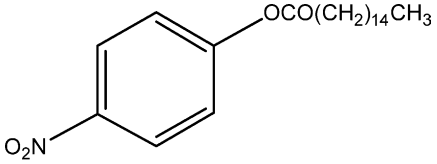
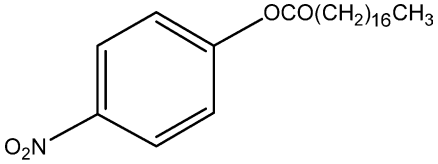


Fig. 2. Temperature optima of lipase produced by *P. aeruginosa*. (The activity was determined at different temperatures using *p*-nitrophenyl palmitate as a substrate in Tris–HCl buffer (50 mM, pH 8). The enzyme activity at 60 °C (2100 U/ml) was taken as 100%.)

Table 2
Substrate specificity of *P. aeruginosa* lipase

Substrate	Relative enzyme activity (%)
 *p*-nitrophenyl acetate	6.24
 *p*-nitrophenyl propionate	88.9
 *p*-nitrophenyl butyrate	195
 *p*-nitrophenyl palmitate	100
 *p*-nitrophenyl almitate	65

The experiments for substrate specificity were carried out taking different substrates in emulsion system at 30 °C in Tris–HCl buffer (50 mM, pH 8). These experiments were carried out at 30 °C as short chain fatty esters (*p*-nitrophenyl acetate, *p*-nitrophenyl propionate, *p*-nitrophenyl butyrate) were not stable at higher temperatures. The absolute value of enzyme activity (130 U/ml) with *p*-nitrophenyl palmitate at 30 °C was taken as 100%.

pH stability of this enzyme, it was found that the enzyme was highly stable in the pH range (7–9) retaining 100% activity up to 29 h (Fig. 5). Relative activities were calculated taking the activity at 60 °C as 100% with *p*-nitrophenyl palmitate as substrate in Tris–HCl buffer (50 mM, pH 8). High stability in the alkaline condition is a desirable character for the lipase capable of hydrolyzing [(±)-MPGM] because this substrate is easily decomposed in aqueous solution under neutral to acidic condition. This may be due to the electron donating effect of a phenyl group on [(±-MPGM)] that makes an oxirane carbon bearing this group vulnerable to nucleophilic attack.

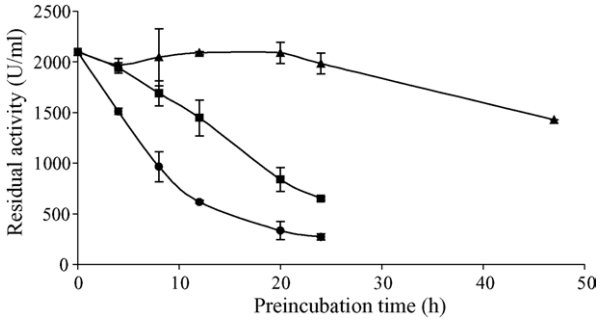


Fig. 3. Thermostability of lipase produced by *P. aeruginosa*: (▲) 50 °C (■) 60 °C (●) 70 °C. (The initial enzyme activity was 2100 U/ml. Enzyme was preincubated at various temperatures, samples were taken at different time interval and enzyme activity was measured according to the standard assay protocol using *p*-nitrophenyl palmitate as a substrate.)

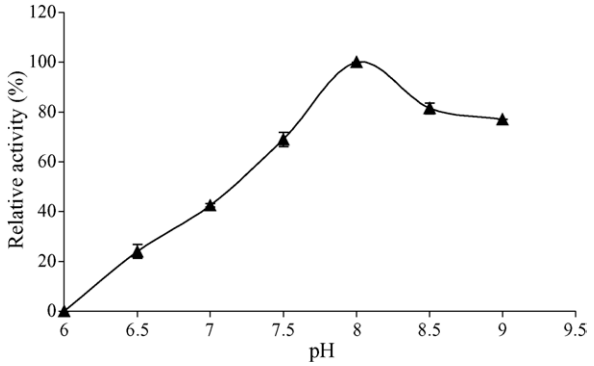


Fig. 4. pH optima of lipase produced by *P. aeruginosa*. (Activity was measured at different pH using 50 mM buffers, citrate (pH 6) and Tris–HCl for a pH range from 7–9. The enzyme activity at pH 8 (2100 U/ml) was taken as 100%.)

3.5. Effect of organic solvents and metal ions

Due to the insolubility of many compounds in aqueous media, reactions in organic solvent are desirable [16]. Keeping this in view, the effect of organic solvents on lipase

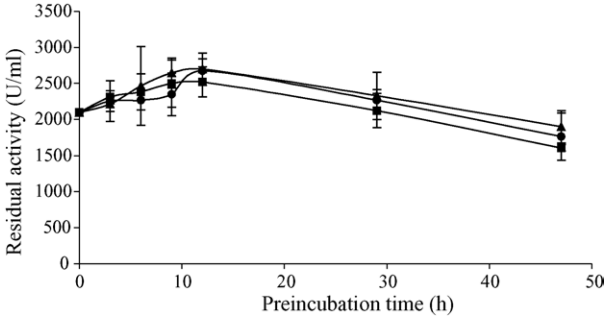


Fig. 5. pH stability of lipase produced by *P. aeruginosa* at (▲) pH 7; (●) pH 8; (■) pH 9. (Enzyme was preincubated in Tris–HCl buffer (50 mM) at different pHs 7–9 and samples were taken at different time interval. Enzyme activity was measured according to the standard assay protocol using *p*-nitrophenyl palmitate as a substrate. The initial enzyme activity was 2100 U/ml.)

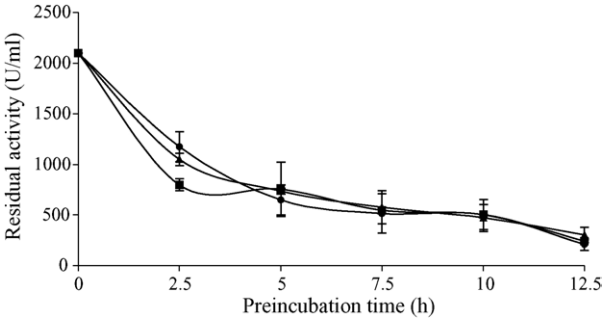


Fig. 6. Stability of lipase produced by *P. aeruginosa* in different organic solvents. (●) Petroleum ether; (■) toluene; (▲) hexane. (The effect of organic solvents on lipase stability was studied by mixing the enzyme in different organic solvents (hexane, toluene and petroleum ether) in the ratio of 1:1. This mixture was stirred at high speed (1000 rpm) for 12.5 h at 30 °C. Samples were taken at a regular time interval to carry out enzyme assay taking *p*-nitrophenyl palmitate as a substrate at 60 °C in Tris–HCl buffer (50 mM, pH 8). The initial enzyme activity was 2100 U/ml.)

Table 3
Effect of metal ions on the lipase activity of *P. aeruginosa*

Metal ion	Relative activity (%)
Ca^{2+}	100
Cu^{2+}	7
Fe^{2+}	30
K^{+}	100
Mg^{2+}	111
Mn^{2+}	55
Ni^{+}	37
Zn^{2+}	10

Enzyme activity (2100 U/ml) with calcium ion was taken as 100%. The enzyme was pre-incubated (60 °C, 5 min) with metal ions (2 mM) and lipase activity was determined according to the standard assay protocol using *p*-nitrophenyl palmitate as a substrate. Separate blanks with individual metal ions were prepared.

stability was studied by mixing the enzyme in different organic solvents like hexane, toluene and petroleum ether in the ratio of 1:1. This mixture was stirred at high speed (1000 rpm) for 12.5 h at room temperature. As shown in Fig. 6, the enzyme retained more than 80% of its maximum activity in all the three solvents up to 90 min.

It has been generally known that the activity of the ester hydrolase family is partly or significantly affected by the presence of cofactor divalent metal ion. The effect of different metal ions on the enzyme activity was tested. It has been found that (Table 3) the enzyme gave maximum activity in the presence of magnesium ions and was highly active in the presence of potassium and calcium ion. Lipase was least active in the presence of zinc and copper ions.

4. Conclusion

It may be concluded that the bacterial strain *P. aeruginosa* MTCC 5113 is a novel and attractive biocatalyst capable of carrying out the resolution of [(±)-MPGM] while exhibiting remarkable stereoselectivity. (−)-MPGM is a key intermediate in the synthesis of the important anti-anginal and anti-hypertensive agent diltiazem. Though a number of lipases have been reported to carry out the stereoselective hydrolysis of (±)-MPGM, the hydrolytic efficiency exhibited by lipase from the present strain is very competitive. It has resolved (±)-MPGM to give (−)-MPGM with a good isolated yield (44%) and excellent enantiomeric excess (ee = 99.9%) and conversion 49.6%. Moreover, the time required for the conversion was quite less (12 h). Apart from this, the lipase used in the present study has shown very good characteristics with respect to the temperature (stable for 20 h at 50 °C) and pH stability (stable for 30 h at pH 8.0). This makes this very bacterial strain a highly attractive candidate for the biocatalytic preparation of diltiazem. A patent has been filed for the same [17]. Studies on the further purification of this enzyme and exploiting the potential of this lipase for the other hydrolytic reactions are in progress.

Acknowledgement

One of the authors Sawraj Singh is thankful to the Council of Scientific and Industrial Research, Government of India for providing the financial assistance.

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