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Novel lipase isolated from a *Pseudomonas* strain and its application in the synthesis of *S*(+)-2-*O*-benzylglycerol-1-acetate

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Abstract: Isolation of a novel microbial lipase (EC 3.1.1.3) having specific catalytic activity for the synthesis of optically pure 2-*O*-benzylglycerol-1-acetate, the building block for the preparation of many β-blockers, phospholipase A2 inhibitors and other biologically active compounds was the aim of this investigation. A *Pseudomonas* (strain G6), recently isolated from soil, produced an extracellular lipase. SDS–PAGE analysis showed that the lipase protein was a hexamer. The molecular weight of the sub-units of the lipase protein were 10, 19, 29, 30, 47 and 53. The catalytic activity of the lipase was exploited for the synthesis of 2-*O*-benzylglycerol-1-acetate from 2-*O*-benzylglycerol through transesterification using vinyl acetate as acylating agent. High selectivity of the lipase towards the monoacetate product was demonstrated. A 97% enantiomeric excess (ee) of *S*(+)-2-*O*-benzylglycerol-1-acetate was obtained when the reaction was carried out at room temperature with shaking. The lipase was highly active in anhydrous organic microenvironments and in non-polar organic solvents with log *P* values above 2.5.

Keywords: *Pseudomonas*; lipase; 2-*O*-benzylglycerol-1-acetate; immobilization

INTRODUCTION

The demand for enzymes with high specific catalytic activity for the synthesis of optically pure chiral compounds stimulates the search for new enzyme sources. Lipase (EC 3.1.1.3, triacylglycerolhydrolase), an extensively used industrial enzyme, is isolated from plants, microbes and other organisms. However, from the commercial perspective, attention has recently been focused on the production of lipases from bacteria and yeasts.[1,2] The versatile bacterial strains, *Pseudomonas*, produce lipases of different characteristics.[3] Catalytic properties of the *Pseudomonas* lipases have been exploited for the synthesis of optically pure drugs and other commercially important compounds.[4–7] 2-*O*-benzylglycerol-1-acetate is a potential chiral intermediate used for the synthesis of cardiovascular drugs (β-blockers) and other biologically active compounds such as platelet-activating factors, phospholipase A2 inhibitors, sphingoglycolipids, etc.[8] Chemical synthesis of racemic 2-*O*-benzylglycerol-1-acetate has been reported.[9] However, synthesis of enantiomerically pure 2-*O*-benzylglycerol-1-acetate appeared to be a challenging task. Although, synthesis of chiral compounds by enzyme catalysis has been established as an attractive tool, very few reports are available on the enzyme-mediated chiral synthesis of 2-*O*-benzylglycerol-1-acetate.[8,10,11] In this paper we report the partial characterization of a newly isolated *Pseudomonas* lipase and its application in the synthesis of optically pure 2-*O*-benzylglycerol-1-acetate through a transesterification reaction.

MATERIALS AND METHODS

Organism and culture conditions

A soil sample collected from a petroleum hydrocarbon-contaminated area of the Borhola Oil Field, Assam, India, was screened for extracellular lipase-producing bacteria by dilution plating on nutrient agar plates containing emulsified glycerol tributyrate, as described previously.[12]

The inoculated agar plates were incubated at 30 °C for 24–48 h. The formation of a zone of clearance around an individual colony was considered as the indicator of extracellular lipase production. A pure strain picked up from a colony with a large zone of

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clearance was selected for further study. The strain was identified as *Pseudomonas*, based on cellular, cultural and biochemical tests following the procedures described in *Bergey's Manual of Determinative Bacteriology*,[13] and named as *Pseudomonas* species G6. The strain could grow on *n*-hexadecane as the sole carbon substrate, and was maintained in mineral agar slants containing *n*-hexadecane ($10\,cm^3\,dm^{-3}$). The mineral medium used in this study was a partial modification of that described earlier[14] and contained ($g\,dm^{-3}$): $NaNO_3$, 4; Na_2HPO_4, 3.61; KH_2PO_4, 1.75; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.001 and ($\mu g\,dm^{-3}$): $CuSO_4{\cdot}5H_2O$, 50; MoO_3, 10; $MnSO_4$, 10; pH 6.8. Lipase production was carried out by incubating the strain in $50\,cm^3$ sterile mineral medium containing $20\,cm^3\,dm^{-3}$ *n*-hexadecane (Sigma Chemical Co, St Louis, USA) in $250\,cm^3$ Erlenmeyer flasks at $34 \pm 1\,°C$ and a shaking rate of 200 rpm.

Isolation of lipase

The culture broth harvested at 36 h was centrifuged at $10000 \times g$ for 10 min at 4 °C, followed by filtration through a 0.45 μm Millipore membrane filter to remove the cells and residual substrates. The supernatant obtained was fractionated with $600\,g\,dm^{-3}$ ammonium sulfate and kept standing overnight at 4 °C. The precipitate was collected by centrifuging at $10000 \times g$ for 5 min at 4 °C, dialyzed and then lyophilized to obtained dry lipase powder. Protein in the lipase powder was measured by Hartree's method.[15]

Assay of lipase

Lipase activity was determined by the glycerol tributyrate – polyvinyl alcohol (PVA) emulsion method, which is a partial modification of the method described by Ibrahim *et al.* [16]

The reaction mixture consisted of $4\,cm^3$ of the emulsion ($10\,mg\,cm^{-3}$ aqueous polyvinyl alcohol containing $20\,mg\,cm^{-3}$ glycerol tributyrate), $5\,cm^3$ of 0.1 M phosphate buffer (pH 7.0) and $1\,cm^3$ of the enzyme solution. Incubation was performed at 30 °C for 1 h at a shaking rate of 200 rpm. The enzyme reaction was terminated by adding $20\,cm^3$ of a mixture of acetone and ethanol (1:1, v/v). Free fatty acid liberated was titrated against 0.02 N sodium hydroxide solution using phenolphthalein as indicator. One unit of lipase activity was defined as the amount of enzyme which liberates 1 μmol of fatty acid per min.

Electrophoresis

The cell-free supernatant and the lipase powder obtained by ammonium sulfate fractionation were analyzed by native and SDS–PAGE following the method of Laemmli.[17] Native PAGE was done using $35\,g\,dm^{-3}$ stacking gel and $80\,g\,dm^{-3}$ resolving gel. The gel was run at 35 mA for 120 min. The lipase activity in the gel was detected as follows: the washed gel was laid in a small glass tray containing tributyrin agar (Tris–HCl buffer, pH 7.5; tributyrin, $10\,g\,dm^{-3}$ and agar, $15\,g\,dm^{-3}$). Lipase activity appeared as a clear zone after 4 h of incubation at 37 °C. SDS–PAGE of each sample was determined by using $35\,g\,dm^{-3}$ stacking gel and $100\,g\,dm^{-3}$ resolving gel. The gel was initially equilibrated at a current of 20 mA and then run with a current of 35 mA for 120 min. Proteins in the gels were stained with Coomassie Brilliant Blue.

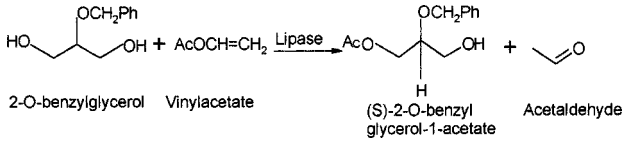
Amino acid analysis

A purified salt-free preparation of the lipase (400 μg) was reduced with $1\,cm^3$ of $5\,cm^3\,dm^{-3}$ aqueous β-mercaptoethanol, carboxymethylated with $0.5\,cm^3$ of 1 M iodoacetate, and hydrolyzed with $1\,cm^3$ of 6N HCl at 110 °C for 22 h. Cystine and cysteine were determined as cysteic acid after oxidation of the lipase with performic acid. The oxidation was allowed to proceed at 0 °C for 4 h. At the end of the reaction time most of the reagent was removed under reduced pressure at 30 °C in a rotatory vacuum evaporator (RVE) and hydrolyzed with $1\,cm^3$ of HCl under the conditions stated earlier. The hydrolyzates were analyzed with a Pharmacia LKB Alpha Plus analyzer (Pharmacia, Uppsala, Sweden).

Synthesis of optically pure 2-*O*-benzylglycerol-1-acetate

The prochiral selective catalytic activity of the lipase for the synthesis of (*S*)-2-*O*-benzylglycerol-1-acetate (Scheme 1) from the corresponding achiral diol through the transesterification reaction was studied by carrying out the reaction in a $50\,cm^3$ round bottom flask fitted with a glass stopper at 30 °C under constant stirring by a magnetic stirrer for 4 h.

The reaction mixture consisted of 2-*O*-benzylglycerol (Fluka, Switzerland) and vinyl acetate (Fluka, Switzerland) at concentrations of 50 mmol and 100 mmol, respectively. The amount of lipase powder used for the reaction was 1500 U. At the end of the reaction the lipase powder was separated by centrifugation at $10000 \times g$ for 5 min. The lipase-free supernatant was concentrated using an RVE (Eyela, Tokyo, Japan) at a pressure of 3.73 kDa and temperature of 30 °C. The concentrated aliquot consisted of the desired product, namely, 2-O-benzylglycerol-1-acetate along with the undesired 2-O-benzylglycerol-1,3-diacetate and unconverted substrate. The monoacetate was purified by silica gel chromatography (60–120 mesh; Qualigens, Mumbai, India) in a column (1 m × 2 mm) using the following solvent system: dichloromethane, ethyl acetate and *n*-hexane in the ratio of 42.5:15:42.5 (v/v). The purified 2-O-benzylglycerol-1-acetate was concentrated by the RVE and the purity and quantity were determined by GLC. The operating



Scheme 1

conditions of the GLC (Varian 3700); (Varian Co, Polo-Alto, CA, USA) were as follows: oven temperature 150 °C; injection temperature 220 °C; column temperature (programmed), 150 °C to 220 °C at 2 °C min^{-1}; FID temperature 230 °C, chart speed 0.5 cm min^{-1}. The flow rates were: carrier gas (N_2) 30 cm^3 min^{-1}, H_2 30 cm^3 min^{-1}, and O_2 60 cm^3 min^{-1}; injection volume 1 µl. The structure of the molecule was verified by GC–MS (Finnigan, Austin, TX, USA) and 300 MHz NMR spectroscopy (Bruker Spectrospin, Zug, Switzerland). Mass spectra of the compounds were taken using an SLD PROBE (50–280 °C).

The optical purity of the purified monoacetate was determined by HPLC (Waters 510, 486 tunable absorbance UV detector, recorded on a 746 data module) (Waters Ges MbH, Vienna, Austria) using a chiral column (0.46 cm × 25 cm, Chiralcel OD) (Daicel Chemical Industries Ltd, Tokyo, Japan) with 2-propanol in *n*-hexane (1:9) as the solvent at a flow rate of 1 cm^3 min^{-1} with detection at a wavelength of 254 nm. The pressure was maintained at 158 psi at ambient temperature (25 °C). The retention times for (*S*)-monoacetate and (*R*)-monoacetate were 17.20 and 15.80 min, respectively.

Pre-equilibration of the water activity

The reaction medium was equilibrated using saturated salt solutions at 25 °C in separate containers. The salts used were: LiBr (water activity, $a_w = 0.064$), LiCl ($a_w = 0.113$), $MgCl_2$ ($a_w = 0.328$), $Mg(NO_3)_2$ ($a_w = 0.529$), NaCl ($a_w = 0.753$) and K_2SO_4($a_w = 0.973$). Equilibration was performed for 24 h.

RESULTS

The homogeneity of the ammonium sulfate-precipitated lipase protein, produced extracellularly by *Pseudomonas* species G6, was checked by native PAGE which showed a single protein band in the gel. SDS–PAGE analysis showed that the lipase protein was a hexamer with corresponding sub-unit molecular weights of 10, 19, 29, 30, 47 and 53 (Fig 1). Amino acid analysis showed that phenyl alanine (Phe), alanine (Ala), aspartic acid (Asp), serine (Ser), glycine (Gly), leucine (Leu) and threonine (Thr) were the predominant amino acids (Table 1). The polar: apolar amino acid ratio of the lipase protein was 1.17:1.

The isolated lipase effectively catalyzed the synthesis of 2-O-benzylglycerol-1-acetate from the corresponding diol by transesterification. Data on the spectral analysis of the purified product were as follows: *NMR*: δ 3.10–3.60 (m, 5H, —$CH(CH_2O—)_2$, 4.40 (s, 2, OCH_2), 6.80–7.20 (m, 5H, —C_6H_6), 2.00 (s, 3, $COCH_3$). *Mass*: *m/z* 224 (M^+), 183/181 (M − 41/M − 43), 165 (M − $OCOCH_3$), 43(CH_3CO).

The ratio of monoacetate: diacetate: remaining diol obtained at the end of the reaction time was 31.8:0.1:68.0. A 97% ee of the *S*(+)-2-*O*-benzylglycerol-1-acetate was obtained by the lipase-catalyzed reaction.

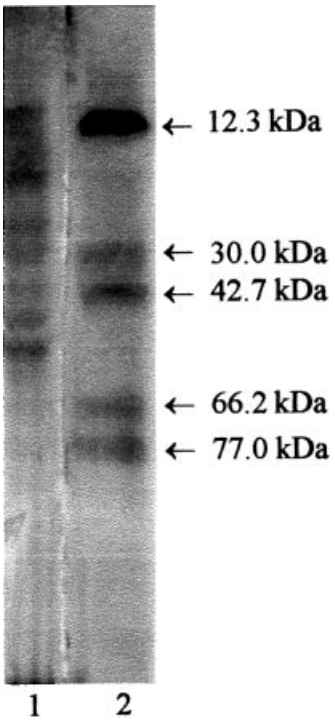


Figure 1. SDS–PAGE of ammonium sulfate-precipitated lipase protein (lane 1) of *Pseudomonas* G6 and standard protein marker (lane 2).

The effect of solvents on the catalytic activity of the lipase was studied (Fig 2). The lipase was highly active in non-polar solvents. Significant lowering of activity was observed for solvents with log *P* values less than 2.5. The optical purity of the product was also not much altered upon addition of organic solvents with log *P* values above 2.5, but was profoundly affected by polar solvents. However, the effect was not directly proportional to solvent polarity.

The activity of the lipase in the transesterification

Table 1. Amino acid composition of the *Pseudomonas* species G6 lipase protein

Amino acid	*Mol %*	*Residues mole*$^{-1}$
Asp	10.71	12
Thr	8.37	9
Ser	10.64	11
Glu	5.80	6
Pro	3.67	4
Gly	9.97	11
Ala	11.07	12
Val	5.14	6
Met	2.65	3
Ile	2.65	3
Leu	6.98	10
Tyr	2.47	3
Phe	11.80	13
His	1.55	2
Lys	4.10	4
Arg	3.06	3

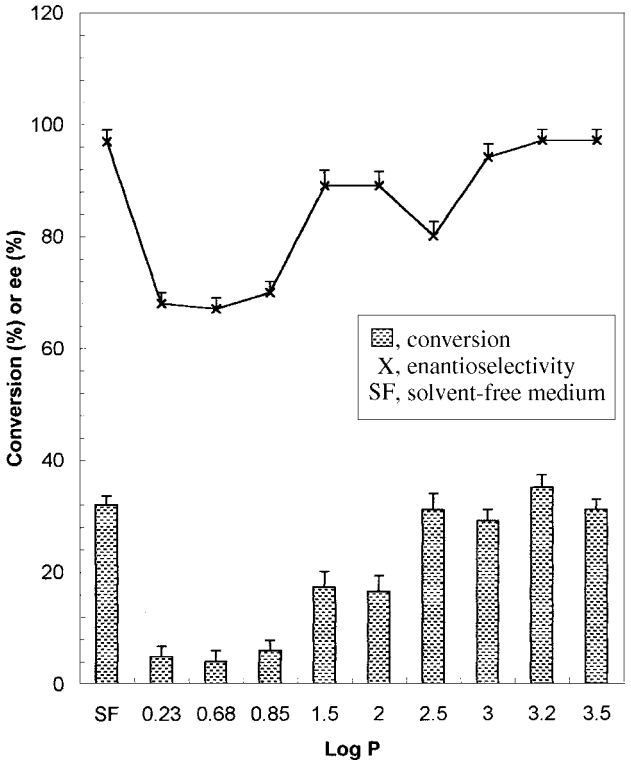


Figure 2. Catalytic activity of lipase G6 on the conversion and enantioselectivity in the presence of different solvents in the reaction mixture. The solvents used, along with their log *P* values, are: hexane, 3.5; cyclohexane, 3.2; CCl_4, 3.0; toluene, 2.5; $CHCl_3$, 2.0; CH_2Cl_2, 1.5; diethylether, 0.85; ethyl acetate, 0.68, and acetone, 0.23. The log *P* values were taken from Laane *et al.*[18] Data points represent mean values (n=4) and the error bars the standard deviation ($p < 0.05$).

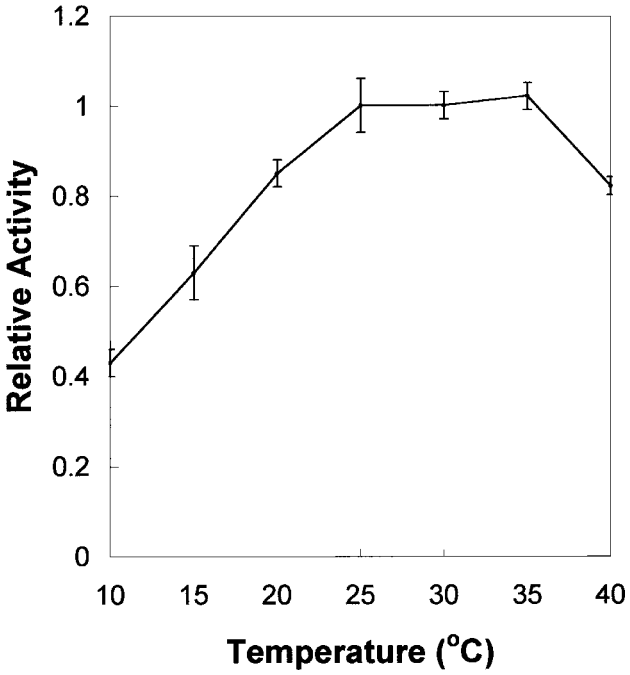


Figure 3. Effect of temperature on the activity, relative to that at 30 °C, of lipase G6. Data points represent mean values (n=4) and the error bars the standard deviation ($p < 0.05$).

reaction was highest over the temperature range of 25 to 35 °C (Fig 3). No significant differences of the conversions within the range were detected at $p < 0.05$. The enantioselectivity of the lipase, which was 97% ee, however, did not change with changes in the reaction temperature. The conversion decreased with increases in the water concentration in the reaction mixture (Fig 4).

DISCUSSION

The molecular weight of the lipase protein appears to be considerably different from that of other *Pseudomonas* lipases reported in the literature.[3] The lipase was highly selective towards the 2-*O*-benzylglycerol-1-acetate during the transesterification of 2-*O*-benzylglycerol. Formation of the undesired by-product, namely 2-*O*-benzylglycerol-1,3-diacetate, was significantly lower than that of other *Pseudomonas* lipase-catalyzed conversions.[8,10,19] Although, the enantiomeric excess (97%) of the (*S*)-monoacetate obtained by us was higher than[8,10] or comparable[11,19] to reported values, formation of a very low concentration of diacetate might be indicative of higher stereoselectivity of the lipase towards (*S*)-monoacetate than (*R*)-monoacetate. Wang *et al*[8] reported that the stereochemical preference for the acetylation of the (*R*)-monoacetate was higher than that for the corresponding (*S*)-monoacetate. Consequently, the (*R*)-monoacetate formed as a result of less specific lipase catalysis is further acetylated, resulting in the enhanced accumulation of diacetate with concomitant increase of optical purity of the (*S*)-monoacetate in the product. The Effects of solvents and temperature on the lipase-catalyzed transesterification reactions for the synthesis of optically pure 2-*O*-benzylglycerol-1-acetate have not been examined, although they are

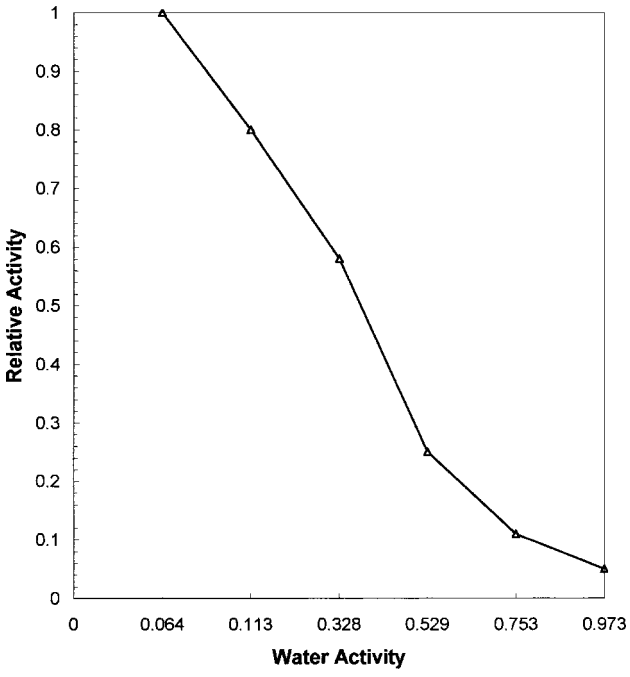


Figure 4. Effect of water activity (relative to that at a_w 0.064) on the activity of lipase G6.

considered as important parameters for optimization of a process. Low-polarity solvents with log *P* values above 2.5 did not have a significant effect on the optical purity of the monoacetate product. Significantly lower enantioselectivity of the lipase was observed when toluene was used as the solvent. Loss of catalytic efficiency of enzymes in the presence of toluene during reaction with other branched substrates has been reported by Parida and Dordick.[20] It is suggested that the decreasing activity of the lipase upon addition of polar solvent to the reaction mixture is caused by the stripping of the enzyme-bound water which is essential for the activity of the lipase. Lowering of lipase activity at a_w above the critical level (0.064) might be caused by the partitioning of the non-polar acylating agent from the active site of the lipase. The use of vinyl acetate as acylating agent is advantageous since the vinyl alcohol formed as a result of the reaction is tautomerized to acetaldehyde which is volatile, thus preventing the reverse reaction.[8] The maximum activity of the isolated lipase was sustained up to 35 °C starting from room temperature (25 °C), thus offering flexibility in the use of the lipase at different reaction temperatures within this range. Further investigations on the kinetics of the lipase-catalyzed transesterification reaction for the synthesis of (*S*)-2-*O*-benzylglycerol-1-acetate are in progress.

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