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ORIGINAL ARTICLE

Purification and characterization of lipase by *Bacillus methylotrophicus* PS3 under submerged fermentation and its application in detergent industry

Pushpinder Sharma, Nivedita Sharma *, Shruti Pathania, Shweta Handa

University of Horticulture and Forestry, Nauni, Solan, HP 173230, India

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KEYWORDS

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Abstract Lipase production bacterial isolate was isolated from soil of service station and identified as *Bacillus methylotrophicus* PS3 by 16SrRNA with accession number |LN999829.1|. Lipase enzyme was purified by sequential methods of ammonium sulfate precipitation and Sephadex G-100 gel column chromatography. The molecular weight of purified enzyme was 31.40 kDa on SDS-PAGE. This purification procedure resulted in 2.90-fold purification of lipase with a 24.10% final yield. The purified lipase presented maximal hydrolytic activity at a temperature of 55 °C, and pH of 7.0. Lipase activity was stimulated by Triton X-100 and SDS with Mg^{2+} and Ca^{2+} metals employ a positive effect and outlast its stable in organic solvent i.e. methanol and ethanol.

1. Introduction

The biosphere is occupied by a wide variety of microorganisms that carry out important functions such as global primary energy, element cycling and they form the largest part of living organisms in the sense of total biomass and species diversity. This diversity of microorganisms is the most common source of genes which can be used in several industrial and research applications [1]. Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes the hydrolysis of the carboxyl ester bonds in triacylglycerols to produce diacylglycerols, monoacylglycerols, fatty acids and glycerol under aqueous conditions and the synthesis of esters in organic solvents [2]. Under the controlled conditions, lipases are able to catalyze a large number of reactions [3]. Lipases of microbial origin are of considerable commercial importance, because of the high versatility and high stability, moreover, the advantage of being readily produced in high yields [4]. Many microbial lipases have been commercially available in free or immobilized form. Numerous species of bacteria (*Bacillus, Pseudomonas, and Burkholderia*), yeasts (*Candida rugosa, Yarrowia lipolytica, and Candida antarctica*) and molds (*Aspergillus, Trichoderma viride*) produce lipases with different enzymological properties and specificities but microbes are known to be more potent lipase

* Corresponding author.
E-mail addresses: apushpindersharma@gmail.com (P. Sharma), niveditashaarma@yahoo.co.in (N. Sharma), shrutipathania89@gmail.com (S. Pathania), shwetahanda137@gmail.com (S. Handa).

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producer [5]. Lipases have broad variety of industrial applications such as food industry (improvement of flavor) [6], detergent (hydrolysis of oil and fats) [7], pharmaceutical (synthesis of chiral drugs), paper (control of pitch), medicine (triglyceride measurement), cosmetics (exclusion of lipids), wastewater (decomposition and removal oil), leather (elimination of fat from animal skin)[8]. However, the major contributions of microbial lipases are in the detergent formulations. The main reason for the steadily growing interest in lipases is because of their enantio-selective, regio-selective and chemo-selective nature [9]. Therefore in the present study an attempt has been made for the isolation of potential lipase producing bacteria, its purification and characterization and its application as a detergent.

2. Material and methods

2.1. Organism and inoculum preparation

Soil of service station for automobiles was collected from the nearby areas of Solan, Himachal Pradesh, India and stored at 4 °C. The soil samples were enriched by adding 1 % of tributyrin into the sample, kept at 37 °C and incubated for 3 days. The samples were serially diluted with enriched sample on nutrient agar medium containing 1 % tributyrin, kept at 37 °C for 24 h of incubation. The pure cultures obtained were maintained at 4 °C on PDA medium. Different bacterial strains were screened for lipase production using Tributyrin hydrolysis test to screen hyper-lipolytic bacteria [10].The zone forming bacterial strains were further screened for quantitative analysis of lipase using titrimetric method [11]. The genomic DNA was isolated using DNA Kit DNA prep kit (Bangalore Genei, India Pvt. Ltd. make). The 16S rRNA gene was selectively amplified from genomic DNA using PCR with oligonucleotide universal primers. The strain was identified on the basis of 16S rRNA gene sequences using appropriate software (nucleotide BLAST) in "National Centre for Biotechnology Information" resource. The lipase producing bacteria was grown in nutrient agar medium containing 1% tributyrin medium as carbon source and the pH was maintained at 7.0. The culture was incubated at 40 °C for 72 h with 10% inoculum size with divalent ion Ca^{2+}, tween 80 as surfactant with 1% substrate concentration. The culture medium was removed after 12, 24, 36, 48, 60 and 72 h for determining the growth pattern and lipase activity. The growth patterns of bacterial strains were taken at O.D. at 540 nm with a spectrophotometer (Cary, 100 Bio, Varian Co, Australia).

2.2. Assay for lipolytic activity [11]

Lipase activity was determined titrimetrically on the basis of olive oil hydrolysis. One ml of the culture supernatant was added to the reaction mixture containing 1 ml of 0.1 M Tris–HCl buffer (pH 8.0), 2.5 ml of deionized water and 3 ml of olive oil and incubated at 37 °C for 30 min. Both test (in which all the reaction mixture were added with enzyme) and blank (in which all the reaction mixture were added without enzyme) were performed. After 30 min the test solution was transferred to a 50 ml Erlenmeyer flask. 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acids were titrated against 0.1 M NaOH using phenolphthalein as an indicator. End point was an appearance of pink color. A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per min under specified assay conditions.

2.3. Lipase purification

Bacterial culture grown in nutrient medium and 1% tributyrin was centrifuged at 10,000 rpm for 20 min at 4 °C in a refrigerated centrifuge. Cell free supernatant was saturated with (0–70%) ammonium sulfate with continuous stirring at 4 °C followed by centrifugation at 14,000 rpm for 20 min. Ammonium sulfate fraction was dialyzed against phosphate buffer (pH 7.0) for 6 h at 4 °C in a dialysis bag. The concentrated enzyme after dialysis was loaded onto Sephadex G-100 column. The lipase was eluted from the column at a flow rate of 3 ml/min. Enzyme fractions (5 ml each) were collected and the protein content was measured spectrophotometrically at 280 nm. Lipase assay was performed using fractions containing highest protein content.

2.4. Characterization of lipase enzyme

2.4.1. Effect of pH on the activity and stability

The effect of pH on enzyme activity was determined by incubating the reaction mixture at various pHs ranging from 4.0 to 11.0 at 50 ± 2 °C for 30 min. The buffers used were citrate phosphate buffer (pH 4.0 to 7.0), Tris HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0 to 11.0).

2.4.2. Temperature optimum and thermal stability

To evaluate the optimal temperature for the enzyme activity, the assay was conducted at varying temperatures ranging from 35 to 121 °C. The lipase was pre-incubated at different temperatures ranging from 30, 40 ……121 °C for 0–180 min.

2.4.3. Effect of metal ions

0.5 ml of purified lipase in 2.5 ml 20 mM Tris HCl buffer (pH 8.0) was incubated for 30 min with various metal ions (1 mM) Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+}.

2.4.4. Shelf stability of lipase

Shelf stability of lipase was determined by pre-incubating the enzyme at 4 °C in 20 mM Tris HCl buffer (pH 8.0). Enzyme activity was determined every 3 days till 9 days.

2.4.5. Effect of media additives

To determine the influence of different additives viz. SDS, EDTA, CTAB, Tween 20, Tween 80, Triton X 100 and Glycerol etc., purified lipase in 1 M phosphate buffer (pH 7.0) was pre-incubated for 30 min at 50 ± 2 °C.

2.5. Application of purified lipase as a laundry additive lipase from B. Methylotrophicus PS3

Application of lipase as a detergent additive had been evaluated in terms of its washing performance on white cotton cloth pieces (5 × 5 cm) that were stained separately with different oils i.e. olive oil, black grease, butter, vegetable oil, and white grease. The stained cloth pieces were taken in separate petriplates and the following washing performances sets were