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A novel alkaline lipase from *Ralstonia* with potential application in biodiesel production

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ABSTRACT

With the aim of isolating a biocatalyst able to catalyze biodiesel production from microbial source, *Ralstonia* sp. CS274 was isolated and a lipase from the strain (RL74) was purified. Molecular weight of RL74 was estimated to be 28,000 Da by SDS–PAGE. The activity was highest at 50–55 °C and pH 8.0–9.5 and was stable at pH 7.0–12.0 and up to 45 °C. It was resistant to oxidizing and reducing agents and the activity was enhanced by detergents. RL74 was 1,3 specific and K_m and V_{max} for *p*-nitrophenyl palmitate were 2.73 ± 0.6 mM and 101.4 ± 1.9 mM/min mg, respectively. N-terminal amino acid sequence showed partial homology with that of *Penicillium* lipases. RL74 produced biodiesel more efficiently in palm oil than in soybean oil; and the production was highest at pH 8.0, at 5% methanol and at 20% water content.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), one of the most important industrial enzymes, hydrolyze fatty acid ester bonds in aqueous medium and synthesize them in non-aqueous medium. They are immensely used in various facets of biotechnology such as in fats and oil hydrolysis, food and detergent industries, peptide synthesis, pharmaceutical industries and so on (Hasan et al., 2009). In recent years, lipases draw significant attentions because they display exquisite chemoselectivity, stereoselectivity and regioselectivity; they can be produced in large scale using various microbes; and crystal structures of many lipases, which is highly desirable to execute engineering strategies, have been solved (Jaeger and Eggert, 2002). Applications of lipases in non-aqueous media have scores of advantages, such as in organic synthesis, but in general, they are unstable in such media. Various physical and chemical methods have been employed to make them suitable for organic media; therefore, naturally organic solvent stable lipase will be an invaluable alternative of the modification processes. One of the strikingly important applications of lipase is in the production of biodiesel, which is generally carried out in non-aqueous media through the process of transesterification. Biodiesel is a fatty acid ester, which is synthesized from alcohols and variety of oils, animal fats or waste oil *via* a reaction catalyzed by acid, alkali or biocatalyst. The use of enzyme catalysts (lipases) in biodiesel production is being increasingly studied because of some advantages it has over chemical or thermal hydrolysis, because enzyme reactions require lower temperatures, thereby preventing the degradation of the products and reducing energy costs. Furthermore, enzymes are biodegradable and are therefore less polluting than chemical catalysts. The disadvantages of enzyme catalysis are mainly its longer reaction time and the higher cost of the biocatalysts (Du et al., 2008; Cavalcanti-Oliveira et al., 2011). Biodiesel is a non-toxic, biodegradable, environmental friendly and non fossil fuel (renewable) that produces significantly lower emissions than petroleum-based diesel when it is burned, whether used in its pure form or blended with petroleum diesel. Importantly, biodiesel does not contribute to a net rise in the level of carbon dioxide in the atmosphere and leads to minimize the intensity of greenhouse effect and they are better than diesel fuel in terms of sulfur content, aromatic content, flash point and biodegradability (Vicente et al., 2004; Antolin et al., 2002). An ideal lipase to be used in biodiesel production should have the following characteristics: able to utilize all mono, di, and triglycerides as well as high yield of free fatty acids, low product inhibition, high activity and yield in non-aqueous media, low reaction time, temperature and alcohol stable, reusability of immobilized enzyme, etc. (Bajaj et al., 2010). For the search of lipases with potential

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biotechnological applications, various bacteria, yeasts, and fungi have been focused. Among bacteria, *Pseudomonas*, *Staphylococcus*, *Chromobacterium*, *Bacillus*, *Acinetobacter*, *Corynebacterium*, and *Streptomyces* have been extensively studied (Saxena et al., 2003; Abramic et al., 1999; Hasan et al., 2009). We have screened *Ralstonia* sp. CS274 as a potent lipase producing microbe from 700 soil isolates preserved in our laboratory. A lipase secreted by the strain, termed as RL74, produced biodiesel from methanol and soybean or palm oils. To the best of our knowledge, this is the first report of lipase from *Ralstonia* able to produce biodiesel. Here, we describe purification, characterization and application of RL74 in biodiesel production.

2. Methods

2.1. Materials

p-Nitrophenol (*p*NP), *p*-nitrophenyl acetate (*p*NPA), *p*-ntrophenyl butyrate (*p*NPB), *p*-nitrophenyl decanoate (*p*NPD), *p*-nitrophenyl palmitate (*p*NPP), triolein were acquired from Sigma–Aldrich (St. Louis, MO, USA). Phenyl Sepharose CL-4B was obtained from Pharmacia (Uppsala, Sweden). Thin layer chromatography (TLC) silica gel 60 F_{254} plates were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

2.2. Screening, isolation and identification of the strain

With the aim of isolating applicable lipase for the production of biodiesel from microbial sources, strains CS133, CS274, CS326 and CS628 were screened from seven hundreds soil isolates preserved in our laboratory. The strain CS274, producing the most pronounced lipolytic activity as well as biodiesel production among the four strains, was selected for the further study. It was identified based on morphological and 16S rRNA sequence, according to our previous report (Simkhada et al., 2010a).

2.3. Enzyme production

In order to produce lipase, *Ralstonia* sp. CS274 was cultured at 28 °C for 120 h on a rotary shaker maintained at 180 rev min^{-1}. The culture medium (1 L) was supplemented with 10 g mannitol, 10 g trypton, 0.1 g $CaCO_3$ and distilled water.

2.4. Protein estimation and lipase assay

Protein concentration was determined at 595 nm according to Bradford method (Bradford, 1976) using bovine serum albumin as the standard. Lipase activity was determined by previously described methods (Gaur et al., 2008; Kilcawley et al., 2002) with slight modifications. Briefly, 350 μl solution (solution A) containing 1 mM $CaCl_2$ and 0.1% Triton X-100 in 0.1 M Tris/HCl buffer (pH 8.0) was preincubated at 55 °C for 5 min. Twenty-five microlitre suitably diluted enzyme sample and 25 μl substrate (10 mM *p*NPP in acetonitrile) were mixed to the solution A and incubated at 55 °C for 30 min. The reaction was terminated by adding 0.1 M $ZnSO_4$ solution (10 μl) and immersing in ice water for 5 min. After centrifugation at 10,000*g* for 10 min, the amount of liberated *p*NP was measured at 405 nm using a microplate reader (Thermo, Multiskan EX). Appropriate blanks were used to subtract the absorbance corresponding to the reaction mixture other than that produced by the specific hydrolysis of *p*NPP. A *p*NP standard curve was generated to assess the enzyme activity and 1 unit (U) lipase activity was defined as the amount of enzyme liberating 1 μM *p*NP per minute from *p*NPP under the assay conditions.

2.5. Enzyme purification

Ralstonia sp. CS274 was grown for 72 h and crude supernatant was obtained by centrifugation at 6000*g* for 1 h. Solid $(NH_4)_2SO_4$ at 0–30%, 30–75% and 75–95% saturation were added to the supernatant and the precipitated proteins were recovered by centrifugation at 6000*g* for 1 h. The enzyme specific activity was found highest at 30–75%; therefore, the fraction was dialyzed using 10 mM Tris/HCl (pH 8.0) with an ultra filtration membrane (YM 30, Millipore Corp, Amicon Danvers, MA, USA). A Phenyl Sepharose CL-4B column (1.6 × 18 cm) was equilibrated with 1 M $(NH_4)_2SO_4$ in Tris–HCl buffer (pH 8.0). Ammonium sulfate fraction after equilibrating with 1 M $(NH_4)_2SO_4$ was loaded onto the column and eluted with a decreasing linear gradient of 1 to zero mole ammonium sulfate at 30 ml/h (1.5 ml/fraction). Lipase active fractions were pooled, concentrated and analyzed for purity and then proceeded for further characterization.

2.6. Polyacrylamide gel electrophoresis

To determine purity and molecular weight of the enzyme, the purified enzyme was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% polyacrylamide slab gel, according to Laemmli (1970). Protein size maker (MBI, Fermentas) was used as reference proteins. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 and destaining with a solution containing methanol:glacial acetic acid:distilled water = 1:1:8 (by volume). Molecular weight was estimated by comparing the relative mobility of the reference proteins.

2.7. Biochemical characterization

2.7.1. Effects of pH and temperature

The relative enzyme activity was determined at various pH values using 100 mM buffers pH ranged from 3.0 to 14.0. Buffers used as standard were citric acid/sodium phosphate (pH 3.0–7.0), Tris/HCl (pH 7.0–9.5), $NaHCO_3$ (pH 9.5–11.0) and KCl/NaOH (pH 11.0–14.0). To evaluate the pH stability, aliquots of enzyme samples were incubated at 0–4 °C refrigerator for 24 h with respective pH buffers. Remaining enzyme activity was measured under standard assay protocol and calculated considering the initial activity as 100%. Temperature optimum of the enzyme was evaluated by assessing the enzyme assay at various temperatures at pH 8.5. In order to access thermal stability, enzyme solutions were incubated at various temperatures (45–80 °C) for various time intervals (up to 90 min). Aliquots of sample were withdrawn at various time intervals and the residual enzyme activity was measured under the standard assay protocol. Moreover, combined effect of pH and temperature on the enzyme activity was also determined within the range of pH and temperature with relatively higher activity (i.e. at 45, 50 and 55 °C, and pH 8.0, 8.5 and 9.0).

2.7.2. Effect of metal ions and detergents on lipase activity

Enzyme samples were incubated with various concentrations (0.1, 1.0 and 10 mM) of Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, K^{+} and Na^{+}. Relative enzyme activity was measured under the standard assay protocol. Similarly, effect of detergents Triton X-100, Tween-20, Tween-80, deoxycholic acid, SDS and polyxyethylen-4-laurylether was determined at 0.0625–0.5%.

2.7.3. Organic solvent stability of lipase

Organic solvent stability of the lipase was determined according to our previous report (Simkhada et al., 2010b). Besides this, enzyme stability in various concentrations of methanol and ethanol