Characterization of a Novel Lipase from the Soil Isolate-Burkholderia cepacia SS-16

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Abstract: In the process of screening of lipase producing bacteria from the soil of vegetable oil industries, a particular gram negative, rod shaped lipase synthesising bacterial strain, Burkholderia cepacia SS-16 was obtained. The maximum lipase activity of the bacteria was obtained after 72 h of growth. The optimal temperature at pH 6.5-7.0 and the optimal pH at a temperature of 35°C were 60°C and 7.0, respectively. The lipase was stable to a temperature of 70°C and a pH of 8.0. The bacterial lipase was most stable in water miscible solvent isopropanol and cleaved triacylglycerol in a random manner.

Key words: Extracellular lipase, Burkholderia cepacia SS-16, organic solvent stability, positional specificity

1 Introduction

Lipases that mainly catalyze the hydrolysis of triglycerols to glycerol and free fatty acids and the synthesis of partially substituted glycerides have considerable applications in industry and medicine1-6. Lipases have been obtained from several bacterial strains, such as Flavobacterium odoratum7, Pseudomonas fluorescens8, Streptomyces sp9, Pseudomonas pseudoalcaligenes10, Pseudomonas aeruginosa11, Bacillus circulans12, Bacillus subtilis, Bacillus coagulans, Bacillus megaterium13, Aeromonas sobria LPOO 414, some thermophilic Bacillus sp.15. Other lipases from Pseudomonas sp.16,17 used for molecular cloning were also reported. Several mycelial fungi which include Rhizopus delemar18, Aspergillus oryzae19, Neurospora sp.20, etc., are also capable of synthesizing the enzyme. New lipases from several strains of yeast have also been reported sporadically21.

This paper presents the screening of lipase producing bacterial strains from soil and characterization of a novel lipase produced by the isolated lipase synthesizing bacteria Burkholderia cepacia SS-16.

2 Experimental Procedure

2.1 Microorganism and Culture Conditions

Several microbial strains were initially isolated from the soil of vegetable oil industries of and around Calcutta by serial dilution. For lipase production they were inoculated from slant cultures (for bacteria, nutrient agar slants contained 1.0 g beef extract, 2.0 g yeast extract, 5.0 g polypeptone, 0.5 g NaCl and 20.0 g agar per litre medium, pH-7.0; for fungus, potato-dextrose-agar slants contained extract of 200 g potato, 5.0 g yeast extract, 20.0 g dextrose and 20.0 g agar per litre medium) into a medium that consisted of (in g/L) K2HPO4-3.0, KNO3-5.0, MgSO4.7H2O-1.3, Na-borate.10 H2O-0.5, polypeptone-1.0 and olive oil-20.0 for bacteria and K2HPO4-2.0, MgSO4.7H2O-1.0, FeSO4.7H2O-0.1, sucrose-10.0, olive oil-10.0 for fungus. They were allowed to grow for 96 h at 35°C in a B.O.D. shaker (120 strokes/min). With the culture medium, the activity of the enzyme produced by the microbial strains was determined by the Cupric-Acetate method22.

2.2 Cupric-Acetate Method
The Cupric-pyridine reagent was prepared as follows: 5% (w/w) aqueous solution of cupric acetate was prepared and filtered, and pH adjusted to 6.0 with pyridine.

**Standard Curves of Free fatty Acids:** Samples containing 2.0-5.0 μmole free fatty acids-butyric, caproic, caprylic, capric, lauric, myristic, palmitic, stearic and oleic acids each were prepared by dissolving them in test tubes with 5 ml of isooctane. Slight warming was necessary to make solutions for stearic, palmitic and myristic acids. Then 1ml of cupric-acetate pyridine reagent was added and the two phases thus formed were mixed vigorously for 90sec in a vortex mixer. The mixture was allowed to stand for about 10-20 seconds until the aqueous phase was sedimented clearly from the solution of isooctane and fatty acid. The standard curve of free fatty acids vs. absorbance was determined by measuring the absorbance of isooctane solution at 715nm against the control which contained no free fatty acids.

**Specific Activity of Microbial Lipase:** To monitor the enzyme reaction, 1ml of the culture supernatant was taken in a screw capped test tube, which was solubilised in 1ml of standard phosphate buffer, pH 7.0, followed by the addition of four drops of groundnut oil(GNO). The test-tube was then shaken vigorously at 162 strokes/min in a shaker at 37°C for 30min. At the end of the reaction, 6(N) HCl and 5ml of isooctane was added to stop the reaction and to extract the free fatty acids(FFA). Then 1 ml of clear isooctane in the upper layer was carefully drawn into a test-tube and its free fatty acid content was determined from the standard curve by the above method. (1U of enzyme activity = 1 μ-mole of FFA produced/min).

Of all the microbial isolates, a particular gram negative, rod shaped strain, SS-16 was found to synthesize the enzyme to a considerable extent. This bacterium was thus identified and its enzyme characterized. The total protein content of the enzyme in the strain SS-16 was assayed by the Folin Lowry method.

**2-3 Culture Age and Activity of SS-16 Lipase**

Aliquots of the culture medium were drawn off and enzyme activity measured after every 24 h till 144 h.

**2-4 Determination of Optimal Reaction Temperature and Thermal Stability of SS-16**

The optimal reaction temperature and thermal stability of the enzyme were determined by assaying the enzyme activity with GNO as substrate after incubating the culture medium containing enzyme in phosphate buffer (pH-7.0)(1:1 v/v) for 1 h at different temperatures viz-30°C, 40°C, 50°C, 60°C, 80°C and 90°C.

**2-5 Determination of Optimal Reaction pH.**

Enzyme activity and stability were assayed by the cupric-acetate method with GNO as substrate, in buffer at pH values ranging from 4.0 to 10.0. Very slight deviations in the results of the three experimental sets were obtained and the values given indicate the average of the three sets.

**2-6 Effect of Organic Solvents on Lipase Activity**

10 ml of lipase solution was mixed with 5 ml of organic solvents such as isopropanol, methanol and acetone separately, kept at 4°C for 1 h and then centrifuged at 1,000 g at 4°C. Lipase activity of the supernatants was assayed in phosphate buffer(pH-7.0) and protein contents of the supernatants were also determined.

**2-7 Preparation of the Acetone Powdered Form of the Lipase of SS-16**

60 ml of the culture medium (72 h) was mixed with 60 ml acetone and kept for 1 h at 0°C in a refrigerator. This was then centrifuged in a cold centrifuge at 4°C at 15,000 g for 20 min. The clear liquid at the top was decanted and the whole residual mass was dried in a vacuum at 40°C for 1 hour. This formed the acetone powdered form of the enzyme and it was stored at −30°C.

**2-8 Determination of Positional Specificity of the Lipase of SS-16**

Three sets of reactions with oil were carried out to examine the positional specificity of lipase. The first reaction mixture was composed of 20 mg of pure, refined, bleached and deodorised olive oil, 1 ml phosphate buffer (pH-7.0) and 10 U of lipase (acetone powdered). The second reaction mixture contained 20 mg of palm oil, the other components of the reaction being the same. The reaction mixtures were incubated
at 30°C for 30 min. with shaking, isomerisation of the reaction products being considered negligible because of the short reaction time\(^\text{20,24}\). After incubation, the products were extracted with 3 ml petroleum ether. Aliquots of petroleum ether were applied to thin layer chromatography (TLC) plates which were then developed with a 70:30 (vol/vol) hexane-diethylether mixture. Pure free fatty acid, monoacylglycerol and diacylglycerol were used as reference glycerides and the spots were visualised by introducing the plate into an iodine chamber.

The third reaction mixture contained 60 mg of palm oil, the other conditions being the same as before. The monoacylglycerols and free fatty acids were extracted separately by diethyl ether from the TLC plates and were converted into their corresponding methyl esters\(^\text{25}\). Gas liquid chromatographic separation\(^\text{26}\) of the methyl esters was done and the fatty acid composition of the methyl esters of the free fatty acids and monoacylglycerol obtained by the action of the bacterial lipase upon the substrate palm oil was determined.

### 3 Results and Discussion

During the process of screening of microorganisms from the soil of vegetable oil industries, a large number of fungi and bacteria were obtained. Of these microbes, a few (the SS strains numbered serially according to their isolation) were found to synthesize lipase, and their activities as determined by the cupric-acetate method are shown in Table 1.

#### 3.1 Specific Activity of SS-16 Lipase

Comparing the microbial lipase activities it is seen that the gm negative, rod shaped SS-16 bacterial strain, identified as *Burkholderia cepacia* had the highest lipase activity, being 0.74 U/ml at 35°C. The total protein content of its culture medium is 226.1 µg/ml.

#### 3.2 Culture Age and Lipase Activity of *Burkholderia cepacia* SS-16 Lipase

Figure 1 indicates that the specific activity of *Burkholderia cepacia* SS-16 lipase at 35°C increased with the increase in the age of the bacterial culture from 24 h onwards and reached a maximum of 0.75 U/ml at 72 h. A 72 h culture of *Burkholderia cepacia* SS-16 is therefore needed for utilizing its lipase enzyme in various reactions.

#### 3.3 Optimal Reaction Temperature and Thermal Stability of *Burkholderia cepacia* SS-16

As shown in Fig. 2 the enzyme activity reached its maximum at about 60°C, being 1.34 U/ml as determined by the cupric-acetate method, and the enzyme retained its stability to 70°C, so that this bacterium is thermally more stable than *Aeromonas sobria* LPOO \(^\text{41}\) which retained its maximum enzyme activity at 55°C and is as thermally as stable as the thermophilic *Bacillus* sp. \(^\text{15}\) that retained its activity at 70°C.

**Table 1** Specific Activity of Microbial Isolates.

| Types of Microbial Isolates | Specific Activity of Lipase (U/ml) |
|-----------------------------|----------------------------------|
| SS-1                        | 0.39                             |
| SS-2                        | 0.12                             |
| SS-4                        | 0.23                             |
| SS-8                        | 0.43                             |
| SS-10                       | 0.11                             |
| SS-12                       | 0.03                             |
| SS-16                       | 0.74                             |
| SS-18                       | 0.37                             |
| SS-23                       | 0.51                             |
| SS-25                       | 0.36                             |
| SS-31                       | 0.41                             |
| SS-33                       | 0.03                             |
| SS-34                       | 0.14                             |

(1U of enzyme activity = 1 µ-mole of FFA produced/min)

**Fig. 1** Culture Age and Lipase Activity of *Burkholderia cepacia* SS-16.
3.4 Optimal Reaction pH.

The bacteria *Burkholderia cepacia* SS-16 resembled the thermostable lipase from thermophilic *Bacillus* Sp. that possessed the highest activity at a pH of 7.0 and retained its stability till pH 8.0, after which it slowly declined (Fig. 3).

3.5 Effect of Organic Solvents on Stability and Enzyme Content of *Burkholderia cepacia* SS-16 Lipase

In order to utilise lipase in various hydrolytic and transesterification reactions, the effect of organic solvents on its stability needed to be determined (Table 2). Here the lipase was most stable in the water miscible solvent isopropanol, where the lipase activity recovered was 2.90 U/ml with 50% acetone the activity obtained was 1.01 U/ml; and in methanol it was 0.79 U/ml.

In aqueous medium at 35°C and after 72 h of growth the total protein content of the bacterial strain SS-16 was 226.1 μg/ml. In 50% isopropanol, the protein content was 125.61 μg/ml, in 50% methanol it was 140.1 μg/ml and in acetone it was 139.9 μg/ml.

During the treatment of culture medium of SS-16 with organic solvents the protein content had decreased due to precipitation of some proteins except lipase, as supported by the previous observations of Kosugi et al.27). The lipase activity of the protein deposited during centrifugation was also found to be negligible, so this served as partial purification of the enzyme by the organic solvents.

The lipase activity was highest when treated with 50% isopropanol, being 23.2 U/mg of protein compared to 5.6 U/mg and 7.2 U/mg with 50% methanol and 50% acetone respectively. Thus isopropanol could serve best in lipase enzyme purification. The recently reported *Neurospora* sp. TT-24130) lipase was also stable in most of the water miscible solvents such as *Burkholderia cepacia* SS-16 lipase, but its stability in the three solvents was in the order methanol > isopropanol > acetone.

3.6 Positional Specificity of *Burkholderia cepacia* SS-16 Lipase

In the first two reactions, where 20 mg of substrate oil was present in the reaction mixtures, the product mixture contained free fatty acids exclu-
Table 3  Fatty Acid Composition of Palm Oil Before and After Reaction with *Burkholderia cepacia* SS-16.

| Fatty Acid | Fatty acid (%) w/w in Original Oil | Fatty acid (%) w/w in Monoglyceride | Fatty acid (%) w/w in Free Fatty Acid |
|------------|-------------------------------|-----------------------------------|-------------------------------------|
| C<sub>16:0</sub> | 46.4                          | 43.9                                | 45.4                                |
| C<sub>18:0</sub> | 3.6                           | 3.8                                  | 3.5                                  |
| C<sub>18:1</sub> | 40.1                          | 41.6                                  | 40.8                                  |
| C<sub>18:2</sub> | 9.9                            | 10.7                                  | 10.3                                  |

sively and negligible amounts of monoacylglycerol, diacylglycerol and triacylglycerol, as was revealed from the spots on the TLC plates. The two oils were, in fact, completely hydrolysed within 30 min. reaction time, (isomerisation of the reaction products being considered negligible because of the short reaction time<sup>20,24</sup>) indicating thereby the random or non-specific nature of the lipase unlike *Neurospora* sp.TT-241<sup>20</sup>) with low 1,3 specificity or *F. heterosporum*<sup>28</sup>) and *Py. ultimum*<sup>29</sup>) with low 1,3-positional specificity.

In the third reaction, when the substrate (oil) amount was greater (60 mg) relative to the amount of the active enzyme, the formation of free fatty acids was obviously less and amounts of mono and diacylglycerols were observed on the TLC plates. The fatty acid compositions of the monoacylglycerol fraction and of the free fatty acid portion were almost identical to that of the total fatty acid composition of the oil(Table 3). This undoubtedly confirms the random nature of the lipase from *Burkholderia cepacia* SS-16, as one can see that the positional specificity of the lipase would not have resulted in a similar fatty acid composition of the reactants (original oils) and the hydrolysed products.

### 4 Conclusion

From our preliminary observations it can therefore be concluded that the gram negative, rod shaped bacterial strain SS-16 is capable of secreting lipase with a considerable enzyme activity, quite close to that of *Candida rugosa*<sup>30,31</sup>) or *Bacillus stearothermophilus*<sup>25</sup>) that is thermally stable, can withstand solvents and cleaves triacylglycerols in a random manner.

For commercial exploitation of this SS-16 lipase after its purification with isopropanol, medium conditions are to be standardized for its enhanced specific activity, its physical properties are to be determined and further work to increase its productivity is to be carried out.

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【報文】 土壌から分離した *Burkholderia cepacia* SS-16 の新規リバーゼの特性

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土壌からリバーゼを産生するバクテリアを分離しスクリーニングする過程で、グラム陰性、桿状リバーゼ合成菌，*Burkholderia cepacia* SS-16 を見いだした。このバクテリアのリバーゼの最大活性は培養 72 時間で得られ，
pH6.5 ～ 7.0 における至適温度，および 35℃における至適 pH はそれぞれ 60℃ と 7.0 であった。リバーゼは 70℃，
および pH8.0 の条件下でも安定であった。このバクテリア由来のリバーゼが最も安定なのは水と混和しやすいイソプロパノール中であり，トリオシルグリセリドに対し無作為に触媒活性を示した。
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【報文】 *Trans*- アネートール誘導体と数種のモノテルペン化合物との総合反応ならびにチロシナーゼ活性阻害，活性酸素抑制効果およびヒアルロンダーゼ活性阻害について

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ウイキョウの精油成分の一つである *trans*- アネートールを出発物質として用い，エポキシ化，オキシア化などの
反応により 2 種類のアミン体へ誘導した。ついで，これら 2 種類のアミン体と塩化クロロアセチルとの付加反応
を行ったのち，4 種類のモノテルペンアミン (2- (2,3- トリメチルシクロペンタン -3- エニル) エチルアミン，
2- (2,3,3- トリメチルシクロペンタン -1- エニル) エチルアミン，4- イソプロペンアル -1- シクロヘキセン -1- カルボニルアミンおよび 6,6- ジメチルビシクロ [3.1.1] ペプト -2- エニル -2- カルボニルアミン）との総合反応に
より，8 種類の新規化合物を合成することができた。これら化合物について，*in vitro* での各酵素によるチロシナーゼ
活性阻害試験，活性酸素抑制効果試験およびヒアルロンダーゼ活性阻害試験を実施し，その活性阻害の有無を
検討した。その結果，チロシナーゼ活性阻害試験において，モノテルペン基を導入した化合物の方が市販品で
あるアルブチンの活性阻害値よりも高い値を示すことがわかった。
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