Enrichment, isolation and optimization of lipase-producing *Staphylococcus* sp. from oil mill waste (Oil cake)

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Abstract

The production of commercial enzymes, including lipase from bacteria has always been the industrial choice due to its economical and commercial feasibility. Twelve samples of oil cakes were enriched to isolate lipase producing bacteria and were screened for their lipolytic activity. The best producer (OC2) was characterized and identified as *Staphylococcus* sp. and was studied for lipase activity. The culture conditions were optimized for maximum enzyme production by isolate OC2 and were found to be 40°C at pH of 7.0 with agitation of 140 rpm and 48 hours of incubation time for achieving maximum enzyme activity of 13.92 U/ml in crude enzyme extract and 34.78 U/ml after partial purification of enzyme, with about 80% enzyme recovery. The purified enzyme showed maximum enzyme activity at 40°C and pH 7.0. The study provides a good mesophilic bacterial candidate for potential industrial production of lipase.

Keywords: Lipase, Enrichment, Isolation, *Staphylococcus*, Mesophilic

INTRODUCTION

Lipases (acyl glycerol hydrolases EC 3.1.1.3) are a class of serine hydrolases which belongs to the α/β - hydrolases super family. They catalyze the hydrolysis of triglycerides to mono- and diglycerides, glycerol and free fatty acids. In nature, lipases are ubiquitously produced in plants, animals and microorganisms. However, lipases of microbial origin are the most versatile and more thermostable and are known to bring about a range of bioconversions including alcoholysis, aminolysis, hydrolysis, esterification and inter-esterification (Pandey et al, 1999). Besides, microbes can be easily cultivated and microbial lipases are mostly extracellular and hence have received particular attention with respect to their industrial production and applications (Elboll and Ozer, 2000; Kamini et al., 2000; Kumar and Sharma, 2012). Lipases find their applications in various industrial sectors like processing of fats and oils, detergents and degreasing formulations, food processing, chemical and pharmaceuticals, paper mills, etc (Rubin and Dennis, 1997a,b; Kazlauskas and Bornscheuer, 1998; Masse et al., 2001; Takamoto et al., 2001).

Lipase production from microbial source, in general, and from bacteria, in particular, has always been a matter of study for finding lipases with novel and specific properties alongwith lower production and processing cost. So, the present study was carried out to enrich, isolate and characterize lipase producing bacteria from oil cake samples (oil mill waste). Also, the different bioprocess parameters were optimized for maximal enzyme activity.

MATERIALS AND METHODS

Sample Collection

For the present study 12 samples of oil mill wastes (oil cakes) were collected in sterile glass vials from different oil mills in and around Gwalior city of Madhya Pradesh, India and were stored at 4°C till further investigations. All the reagents and media were of analytical/microbiological grade, obtained from commercial vendors.

Enrichment of lipolytic Bacteria

20 gm of each sample (OC1 to OC12) was dissolved in 100 ml of double distilled water and filtered with the help of sieve. 5 ml of the filtrate was inoculated into 45 ml of sterile enrichment media broth [5g/l (NH4)2SO4, 0.5 g/l KH2PO4, 0.3 g/l MgSO4.7H2O, 5% Yeast extract, 5.0 g/l Olive oil; pH 7.2]. The inoculated broth was incubated for 48 hours at 37°C and 120 rpm in rotary shaker incubator (CIS 24BL Remi, Mumbai).

The enriched cultures thus obtained, were subjected to subsequent 7 rounds of sub-culturing using the same enrichment media and incubation conditions as for the 1st round but the concentration of yeast extract (carbon source) was serially decreased to 0.1% (w/v) and that of pectin was increased serially by 2.5g/l at every round of sub-culturing. The microbial growth was measured spectrophotometrically by measuring optical density (O.D.) at wavelength of 590nm using spectrophotometer (Systronic 106). All the experiments were carried in triplicates with control.

Isolation and Screening of Lipolytic Bacteria

The Round 7th enriched cultures were serially diluted and were plated onto Tributyrin agar base containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 1% (w/v) Tributyrin and 2% agar with pH 7.0 by spread plate method (Sarda et al., 1998). The inoculated plates were incubated for 48 hours at 37°C and zone of clearance were observed due to hydrolysis of tributyrin by lipase (Sztajer et al.,...
The isolate (OC2) showing the maximum zone of clearance was selected for further analysis. The isolate OC2 was analysed for morphological and biochemical characteristics for its identification, according to the Bergey’s Manual of determinative bacteriology (Holt et al., 1996).

Lipase Assay

The selected bacterium (OC2) was assayed for extracellular lipase production using titrimetric method using olive oil as a substrate. Olive oil (10% v/v) was emulsified with gum Arabic (5% w/v) in 100 mM potassium phosphate buffer with pH 7.0. 10 µl of cellular extract/partially purified lipase was added to the emulsion and incubated for 15 min. at 37°C. The reaction was stopped and fatty acids were extracted by addition of 1.0 ml of acetone: ethanol solution (1:1). The amount of fatty acid liberated was estimated by titrating with 0.05M NaOH until pH 10.5 using phenolphthalein as indicator (Jensen, 1993). One unit of lipase activity is defined as the amount of enzyme required to hydrolyse µmol of fatty acids from triglycerides.

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\text{Lipase Activity (Unit/mL)} = \frac{\text{Vol of alkali consumed} \times \text{Normality of NaOH}}{\text{Time of incubation} \times \text{Vol of enzyme solution}}
\]

Optimization of Bioprocess parameters for Lipase production by Isolate OC2

a) Incubation period: Isolate OC2 was cultured in Tributyrin broth and 1% (w/v) olive oil at 37°C in orbital shaker at a rotary speed of 130 rpm for 4 days (96 hours). The crude broth was harvested, aseptically, at every 12 hours interval by high speed cooling centrifugation at 10,000 g for 30 min at 4°C. The supernatant collected was used as crude enzyme solution and was assayed for enzyme activity.

b) Temperature: For selecting optimum temperature for lipase production by isolate OC2, the incubation temperatures varying from 25°C-45°C were selected, keeping the remaining parameters same, except the incubation period as standardized above.

c) pH: A range of medium pH from 5.0 to 9.0 was scanned for determining the optimal pH for lipase production by the isolate OC2, keeping other parameters unchanged except for the incubation time and temperature, as optimized.

d) Agitation speed: To optimize the agitation speed for the maximum production of lipase by OC2, the isolate was cultured in orbital shaker incubator at varying rotary speed from 110-160 rpm alongwith a stationary culture. The other parameters were as per optimized conditions or kept unaltered.

e) Carbon source: To evaluate different C-source for maximum lipase production by the isolate OC2, olive oil (1% w/v) present in the culture media was replaced with different oils like sunflower oil, mustard oil, soyabean oil, coconut oil, groundnut oil, palm oil and ghee, with the respective final concentration of 1% (w/v). The other parameters were as per their respective optimized value.

Lipase purification

The partial purification of lipase, produced by the isolate OC2 under optimized condition, was carried out by salting out precipitation method. The supernatant of the fermented broth culture of OC2, containing extracellular lipase, was treated with 0.4M CaCl2 for precipitating the fatty acids. It was followed by centrifugation at 15,000 rpm and 4°C for 30 min. The supernatant was collected in a beaker and chilled acetone was added slowly to it, with continuous stirring, to obtain 70% (v/v) final concentration. It was then kept at 20°C for 6 hours to allow protein precipitation. The precipitate was harvested by centrifugation at 15,000 rpm and 4°C for 30 min. The pellets thus obtained were re-suspended in 35 ml of 20nM Tris-HCl buffer (pH 8.0) to allow the solubilization of proteins. The unsolubilized proteins were removed by centrifugation at 15,000 rpm and 4°C for 30 min. The supernatant was then subjected to overnight dialysis against the same buffer. The protein content [estimated by Folin’s Lowry Method (Lowry et al, 1951; Hartree, 1972)] and lipase activity (as described earlier) were determined after each step.

RESULTS AND DISCUSSION

Enrichment, Screening & Isolation of Lipolytic Bacteria

5 out of 12 collected oil cake samples showed high microbial density after 7th round of enrichment (Fig.1) and were selected for screening. These samples (OC2, OC3, OC7, OC9 and OC10) when plated onto the tributyrin agar base for screening of their lipolytic activity showed OC2 with maximum zone of clearance, indicating maximum lipolytic activity. Therefore OC2 was selected for further study and its pure culture was obtained with its glycerol stock maintained at -20°C.

Identification of OC2

The isolate OC2 was identified as Staphylococcus sps., based on staining, morphological and biochemical characterization (Table 1). Lipolytic bacterial strains (Bacillus sps., Staphylococcus and Lactobacillus) have been reportedly isolated earlier from oil mill wastes (Mohan et al., 2008), oil contaminated soils (Sirisha et al., 2010), coconut milk (Padmapriya et al., 2011) with comparative enzyme yield and activity.

Optimization of Bioprocess parameters for Lipase production by Isolate OC2

Isolate OC2 showed maximum lipase activity after 48 hours of incubation (Fig. 2) at 40°C (Fig. 3) and pH 7.0 (Fig. 4) with 140 rpm agitation speed (Fig. 5). The enzyme activity was found to be maximum for olive oil followed by palm oil and groundnut oil (Fig. 6) while optimizing the process for C-source. The results show that lipase production by the isolate OC2 vary widely under different growth conditions and indicates that medium supplemented with triglycerides (olive oil) enhances the enzyme production. The results are in good accordance with earlier reports where triglycerides supplementation in the medium has been shown to be indispensable for lipase production (Del Rio et al., 1990; Marek and Dednarski, 1996; Changir and Sarikaya, 2004; Mohan et al., 2008; Sirisha et al., 2010; Padmapriya et al., 2011). The high agitation speed of 140 rpm, required for maximum lipase production by OC2 indicates its elevated aeration requirement. Also, shaking may also be required to create a condition for better bioavailability of nutrients, thus confirming the earlier reports (Nahas, 1998; Rao et al., 1993; Changir and Sarikaya, 2004; Sirisha et al., 2010).
Lipase purification

The partial purification of lipase enzyme from the crude culture filtrate of isolate OC2 resulted in almost 2.5 fold purification with about 80% recovery. The enzyme yield is good but requires further investigation for proper downstream processing, so that its commercial value could be increased.

Lipase characterization

The partially purified lipase enzyme produced by the isolate OC2 was found to show maximum activity at pH 7.0 (Fig. 7a) and temperature of 40°C (Fig.7b), after 1 hour of incubation with substrate. There have been earlier reports of microbial lipases with similar characteristics of being stable at temperature up to 50°C and in pH range of 2.0-10.5 (Rajeswari et al., 2010; Padmapriya et al., 2011).

Table 1. Morphological and Biochemical characteristics of isolate OC2

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test/Substrate/Parameter</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gram Staining</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Morphology (Negative staining)</td>
<td>Cocci</td>
</tr>
<tr>
<td>3</td>
<td>Citrate</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Nitrate reduction</td>
<td>Positive (Weak)</td>
</tr>
<tr>
<td>5</td>
<td>Catalase</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>Gelatin liquefaction</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Oxidase</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Glucose fermentation</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Sucrose fermentation</td>
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</tr>
<tr>
<td>10</td>
<td>Lactose fermentation</td>
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<tr>
<td>11</td>
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<td>12</td>
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<tr>
<td>13</td>
<td>Urease</td>
<td>Negative</td>
</tr>
<tr>
<td>14</td>
<td>Voges-Proskauer</td>
<td>Negative</td>
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</table>
CONCLUSION

It may be concluded from the present study that the isolate OC2, characterized as Staphylococcus sps., can be used as a potential bacterial source of lipase with high thermal stability and pose to be a promising bacterial candidate for exploration at higher scales of pilot and industrial level.

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REFERENCES


