Isolation, production and application of lipase/esterase from *Bacillus* sp. strain DVL43

Davender Kumar¹a, Lalit Kumar¹, Sushil Nagar¹, Chand Raina², Rajinder Parshad², Vijay K Gupta¹*

¹Department of Biochemistry, Kurukshetra University, Kurukshetra-136119, India
²Indian Institute of Integrative Medicine (IIIM), Canal Road, Jammu-Tawi (CSIR), India

ABSTRACT

A lipolytic isolate *Bacillus* sp. DVL43 was screened from oil contaminated area of common city garbage site using tributyrin as substrate. The qualitative analysis using tributyrin, rhodamine-agar, and Tween20/ Tween 80 indicated that this isolate produced both lipase as well as esterase. The effect of three different production media (PM1, PM2 and PM3) on quantitative production of lipase and esterase by *Bacillus* sp. DVL43 revealed maximum intracellular and extracellular lipase activity in PM2 after 24 and 36 h respectively. The highest esterase activity in culture filtrate (extracellular) and cell free extract (intracellular) was observed in the medium PM2 whereas the highest activity in cell pellet was in PM3. The maximum esterase activity in the culture filtrate, cell free extract and cell pellet was recorded after 24h, 48h and 36h of fermentation respectively. The partially purified lipase from *Bacillus* sp. DVL43 catalyzed esterification between oleic acid and ethanol in hexane resulting in the formation of ethyl oleate as confirmed by thin layer chromatography. The esters of oleic acid are useful for biodiesel production. The production of both lipase and esterase activities by the isolated bacterial strain would likely to be useful in its industrial application.

Keywords: Lipase/Esterase, Isolation, Esterification, Tributyrin, Ethyl-oleate

INTRODUCTION

Lipases (EC 3.1.1.3, triacyl glycerol acylhydrolases) and esterases (EC 3.1.1.1, carboxyl ester hydrolases) are a diverse group of enzymes that catalyze the hydrolysis of ester bonds in triacylglycerides to glycerol and fatty acids [1, 2]. However, lipases act mainly on triacylglycerols composed by long chain fatty acids whereas esterases preferentially hydrolyze triacylglycerols composed by fatty acids shorter than C10. These enzymes contain a catalytic triad that usually consists of a serine, a histidine and an aspartic acid, with the serine embedded in the consensus sequence G-X-S-X-G at the active site [3]. In non-aqueous systems, lipases catalyze the reverse reaction, namely ester synthesis and transesterification [4, 5]. Lipase catalyzed reactions are stereoselective and regioselective in nature [6,1]. Therefore, there is an increasing interest for lipases and lipase-producing strains, as lipase-catalyzed reactions show high selectivity under mild conditions, with no requirement for added cofactors [7]. Lipolytic enzymes have numerous industrial applications [8]. The number of lipases has been increasing as a result of the developments in cloning and expression of enzymes from microorganisms, but still the demand for the biocatalysts with novel and specific properties such as specificity, stability, pH, and temperature is increasing. This has led to enhanced interest in isolation of new lipolytic micro-organisms from diverse habitat [9, 10]. In particular, microbial
lipases have a wide range of enzymatic properties and substrate specificities, making them very useful for industrial applications, such as the processing of fats and oils, additives, detergents, cosmetics, paper manufacturing, and pharmaceuticals [6, 11]. Digestive aids, treatment of malignant tumors, gastrointestinal disturbances therapy, and dyspepsia therapy are some of the medical applications of lipases [6]. Lipase-producing microorganisms have been found in diverse habitat like oil-contaminated soils, oil processing factories, industrial wastes etc. Lipases are ubiquitous in nature and produced by animals, plants and microorganisms. Currently, microbial lipases are receiving a great deal of attention due to the availability of a wide range of hydrolytic and synthetic activities, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and easy cultivation of microbes on inexpensive media [12, 13]. Microbial lipases may be intracellular, extracellular or membrane bound. The extensive and persistent screening for new microorganisms and their lipolytic enzyme will open new, simple routes for synthetic processes. In view of the above facts, there is a great urge to explore novel lipases/esterases of industrial uses. Biodiesel is one of the most popular biofuels because it is completely compatible with fossil diesel and the synthesis process is commercially available. As a traditional chemical method of alternative technologies and green energy-saving, enzyme-catalyzed synthesis of synthetic chemicals and fuels becomes an important development direction [14]. The present study reports isolation and production of a lipase/esterase-producing bacterial strain DVL43 from oil contaminated soil samples and evaluation of its enzymatic potential in esterification reaction of oleic acid and ethanol. Ethyl oleate formed as a result of lipase- catalyzed esterification would be useful in biodiesel production.

**MATERIALS AND METHODS**

**Sample collection**

Samples were collected from oil contaminated area of common city garbage site of Karnal district of Haryana (India).

**Isolation of lipase/esterase producing microorganisms**

Samples were serially diluted with sterile distilled water and spread on the nutrient agar plates followed by incubation for 24-48 h at 37°C for the growth of microorganisms. Microbial colonies, which appeared on nutrient agar plates, were purified and subjected to qualitative screening for identification of lipase/esterase producing micro-organisms on tributyrin agar (TBA), rhodamine olive oil (ROA), tween 20 and tween 80 agar plates.

**Tributyrin agar plate assay**

Lipase/esterase producing micro-organisms produced a zone of clearance (hydrolysis) when their appropriate dilutions were spread on the TBA medium containing per liter of peptone, 5g; beef extract, 3g; tributyrin, 10ml and agar-agar, 20g. The zone size was measured after 12, 24, 36 and 48 h of incubation at 37°C.

**Rhodamine-olive oil agar plate assay**

A sensitive and specific plate assay for detection of lipase producing bacteria makes use of rhodamine-olive oil-agar medium. The growth medium contained (g/L): nutrient broth, 8.0; NaCl, 4.0 and agar-agar 20. The medium was adjusted to pH 7.0, autoclaved and cooled to about 60 °C. Then 31.25 ml of olive oil and 10 ml of rhodamine B solution (1.0 mg/ml distilled water and sterilized by filtration) was added with vigorous stirring. It was then poured into petri plates under aseptic conditions and allowed to solidify. The bacterial culture was inoculated on to the medium in these plates. Lipase producing strains were identified on spread plates after incubation for 48 h at 37 °C. The hydrolysis of substrate causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation.

**Hydrolytic activity on tween 20/ tween 80**

The hydrolytic activity of *Bacillus* sp. DVL43 lipase was done on tween 20/ tween 80 medium composed of (g/L): peptone, 10; NaCl, 5; CaCl$_2$.2H$_2$O, 0.1; agar-agar, 20; tween 20 or tween 80, 10 mL (v/v).

**Submerged fermentation**

The effect of production media and incubation period on the production of lipase/esterase by the isolate *Bacillus* sp. DVL43 was studied in submerged fermentation using three different production media (PM1, PM2, PM3) for 24, 36 and 48 h. The composition of these media is shown in Table 1. Inoculum size, pH of the production medium and incubation period selected for fermentation were optimum for the bacterial growth.
Table 1: The composition of different production media used for lipase/esterase production

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Composition of different production media (g/L)</th>
<th>PM1</th>
<th>PM2</th>
<th>PM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Peptone</td>
<td>3</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Beef extract</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Yeast extract</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Olive oil</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>Tributyrin</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Tryptone</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Glucose</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Tween80</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Sucrose</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Enzyme extraction**

Lipase/esterase was determined in three different fractions viz. extracellular, cell pellet and intracellular. Extracellular lipase/esterase was extracted from the production medium after desired incubation time (24, 36 and 48 h) by its centrifugation at 10000 x g for 30 min in a refrigerated centrifuge followed by collection of the supernatant, which contained the enzyme. The pellet was also collected and total cell biomass was calculated. The cell pellet was used the assay of cell bound lipase/esterase. It was stored at −20°C for recovery of maximum enzyme. The sonicated cell suspension was centrifuged (15000 x g for 30 min) and cell free extract (intracellular lipase/esterase) was collected for activity assay.

**Enzyme assay**

**Assay of Esterase**

The esterase activity was measured using modified titrimetric method as described by Beisson et al. [15]. The titrable solution containing 13.5 mL of 1% (v/v) tributyrin (substrate) in 1% gum acacia solution, 0.5 mL of 2% calcium chloride and 1.0 mL of 1M NaCl. The reaction mixture was titrated against 10mM NaOH. The enzyme activity was calculated using following formula:

\[
\text{Esterase activity} = \frac{\text{Volume of NaOH consumed (mL) \times Molarity of NaOH}}{\text{Volume of enzyme (mL) \times Reaction time (min)}}
\]

One IU of esterase activity was defined as the amount of enzyme that liberated 1 µmol fatty acid min⁻¹ at 30°C and pH 7 under the assay conditions.

**Assay of Lipase**

The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as substrate according to the method of Nawani et al. [16] with some modifications. The reaction mixture containing 0.3 mL of 0.05M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control was run simultaneously, which contained the same contents but the reaction was terminated prior to the addition of enzyme. Absorbance of the resulting yellow colored product was measured at 410 nm in a spectrophotometer. One International Unit (IU) of lipase activity was defined as the amount of enzyme catalyzing the release of 1 µmole of p-nitrophenol min⁻¹ from p-NPP under the standard assay conditions.

**Partial purification by ammonium sulphate fractionation**

The intracellular lipase was partially purified by ammonium sulphate fractionation for use in esterification. The cell free extract was subjected to protein fractionation by addition of small increments of solid ammonium sulphate at 4°C with constant stirring to obtain three fractions i.e. 0-30%, 30-70% and 70-90%. When all the ammonium sulphate was dissolved at the end of each fractionation range, the mixture was allowed to stand for 30 minutes to 1 h followed by its centrifugation at 10,000 x g for 30 minutes at 4°C. The pellet was collected and the supernatant was used as starting material for next fractionation. The collected precipitate of each fractionation range was re-suspended in small volume of 0.05M phosphate buffer (pH 7.0) and checked for the enzyme activity and protein content.
Lipase-catalyzed esterification

Partially purified intracellular lipase from the isolate DVL 43 was used as biocatalyst for esterification of oleic acid with ethanol in 1:1 (v/v) ratio in hexane. The reaction was carried out at 30°C with shaking at 100 rpm for 24 h with heat inactivated free enzyme (incubated at 100 °C for 5 min) as control. The ester formation was identified by analytical thin layer chromatography (TLC) performed using pre-coated silica gel 60 F254 (20x20 cm) plates. After chromatography, TLC plates were visualized by immersion in 10% (v/v) H2SO4 in ethanol solution followed by heating on a hot plate.

RESULTS AND DISCUSSION

Screening and isolation of lipolytic Bacillus sp. DVL43

In the present investigation, the lipolytic bacterial strain Bacillus sp. DVL43 was isolated from oil contaminated area. Screening was carried out by spreading different dilutions of samples on nutrient agar plates. The colonies which appeared on these plates were purified and streaked on Tributyrin-agar (TBA). The isolate Bacillus sp. DVL 43 produced a good zone of hydrolysis on TBA (Fig. 1a). The hydrolysis of tributyrin (C4:0) indicated the production of esterase by the isolate since esterase is able to hydrolyze shorter chain acylglycerols (≤ 10 carbon atom) while lipases hydrolyze long-chain (≥10 carbon atoms) acylglycerols [1]. To test the production of lipase, Bacillus sp. DVL43 strain was streaked on rhodamine-olive oil agar plate. The hydrolysis of olive oil (substrate) caused by DVL43 lipase, resulted in the formation of orange fluorescent halos around bacterial colonies (Fig. 1b) visible upon UV irradiation indicating the production of lipase by this bacterial strain [17, 18].

Lipolytic microorganisms can also be detected in agar media using tweens (fatty acid esters of polyoxyethylene sorbitan) as substrate [19]. Lipase/esterase producing micro-organisms show precipitation on tween agar plates, which is due to the formation of calcium salt of the fatty acids released by hydrolysis of tween 20/tween 80. Fatty acids liberated as a result of lipase/esterase action on tweens, bind with calcium incorporated into the medium. The calcium complex is visible as insoluble crystals around the inoculation site. Tween 80 is mostly hydrolyzed by lipase as it contain esters of oleic acid and rarely by esterases while tween 20 is easily hydrolyzed by esterase as it contains esters of lower chain fatty acids viz. lauric acid. The production of zone of precipitation on tween 80 and tween 20 agar plates by Bacillus sp. DVL43 strain (Fig. 1c&d) implies that the enzyme produced by this isolate has both esterase and lipase activity. A number of Bacillus sp. strains producing lipase have been isolated by many researchers from different sources. Takaç and Marul [20] isolated Bacillus sp. strain from soil by serial dilution technique on tributyrin agar (TBA) plates on the basis of zone of hydrolysis. Ertugrul et al. [21] isolated 17 bacterial strains using olive oil mill waste as substrate. Carvalho et al. [22] isolated a bacterial strain from petroleum-contaminated soil. Abada [23] produced lipase from a strain of B. stearothermophilus AB-1 isolated from air. Thermostable extracellular lipase producing Bacillus strains have been isolated from hot springs in Malaysia [24]. In the present study, qualitative screening on different media has been employed for isolation of lipase and esterase producing strain Bacillus sp. DVL43.

Production media and incubation time

The isolate Bacillus sp. DVL43 was subjected to submerged fermentation in three different production media (PM1, PM2 and PM3) for 24, 36 and 48 h. The activity of esterase/lipase was then determined in the culture filtrate (CF; extracellular), the cell free extract (CFE; intracellular) and cell pellet. The isolate DVL43 was found to produce both lipase as well as esterase. The lipase activity (estimated using p-NPP) was found in CF as well as CFE whereas esterase activity (estimated by titrimetric method) was found in CF, CFE and cell pellet in all the three media used (Fig. 2). A comparison of the esterase activity in different media showed that the highest activity in CF and CFE was observed in the medium PM2 whereas the highest activity in cell pellet was in the medium PM3. Further, the maximum activity in CF (34 IU/L), CFE (2700 IU/L), and cell pellet (62 IU/g) was recorded after 24h, 48h and 36h of fermentation respectively.

The higher activity of esterase in CF and CFE in PM 2 as compared to the other two media could be due to the presence of Tween 80 and tributyrin. Tween-80 seems to have a dual effect on lipase/esterase activity i.e. it can act as both an inducer and surfactant. The presence of Tween-80 stimulates the release of the enzyme by affecting the cell permeability, increasing esterase/lipase activity recovered in the growth medium. Tween-80 can also be used as a sole carbon source in the culture medium, as it is miscible with water and does not inhibit bacterial growth [9]. Tributyrin may also act as inducer of lipase production [25].

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The activity of extracellular lipase after 24h of fermentation was highest in PM3 followed by PM1 but after 36h, the activity was highest in PM2. On the other hand, the intracellular lipase exhibited maximum activity (220 IU/L) in PM3 after 36h of fermentation. The maximum extracellular lipase activity in PM2 might be due to surfactant effect of tween 80 as it increases the permeability of the cell, thus increasing the secretion of intracellular and membrane bound lipase to the extracellular medium. The enhanced lipase activity in CF in the production medium PM3 might be due to the presence of olive oil, which acts as a carbon source for lipase production [26]. It was thus apparent from these results that the titre of lipase/esterase in different production media was found to vary with the time period of fermentation.

Partial purification using ammonium sulphate fractionation
Most of the lipase activity was recovered at 30-70% saturation. This partially purified lipase had specific activity of 21.8 IU mg⁻¹ protein with the total activity of 210 IU. The recovery of the enzyme was approximately 70 % with the purification fold of 11.3 as shown in Table 2.
Figure 2: Quantitative measurement of lipase/esterase production on three different production media (PM1, PM2, and PM3). CF and CFC represent culture filtrate (extracellular) and cell free extract (intracellular lipase) respectively. The activity of esterase is expressed as: CF (IU/100mL); Cell pellet (IU/100mg) and CFC (IU/mL) whereas activity of lipase is expressed as: CF (IU/100mL); CFC (IU/10mL).

Table 2: Partial purification table of the intracellular lipase from Bacillus sp. DVL43

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (IU/mL)</th>
<th>Total activity (IU)</th>
<th>Protein (mg/mL)</th>
<th>Total protein (mg)</th>
<th>Specific activity (IU/mg)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free extract</td>
<td>6</td>
<td>300</td>
<td>3.1</td>
<td>155.0</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>A.S. fractionation (30-70%)</td>
<td>70</td>
<td>210</td>
<td>3.2</td>
<td>9.6</td>
<td>21.8</td>
<td>11.5</td>
</tr>
</tbody>
</table>

*Ammonium sulphate precipitation

**Esterification**

Enzymatic synthesis of esters is generally preferred over chemical synthesis as it free from the contamination of other chemicals which make the purification of ester multistep and expensive. The partially purified lipase from Bacillus sp. DVL43 was used to catalyze esterification between oleic acid and ethanol in hexane resulting in the formation of ethyl oleate. The formation of ester was confirmed by thin layer chromatography (Fig. 3). The maximum amount of ester was formed after 24h of incubation. The practical applications of lipase-catalyzed esterification/transesterification have been discussed by many researchers [27]. The esters of oleic acid with ethanol are useful for production of biodiesel [28]. The maximum amount of ester was formed after 24h of incubation. The practical applications of lipase-catalyzed esterification/transesterification have been discussed by many researchers [27]. The esters of oleic acid with ethanol are useful for production of biodiesel [28].
CONCLUSION

A lipolytic bacterial strain Bacillus sp. DVL43 has been isolated from oil contaminated area. Its qualitative behavior on tributyrin-agar, rhodamine-agar and tween 20/ tween 80-agar media indicated that it produced both lipase as well as esterase. The quantitative analysis revealed the presence of lipase/esterase in the culture filtrate, cell free extract and cell pellet. The partially purified lipase showed its potential in catalyzing esterification between oleic acid and ethanol resulting in the formation of ethyl oleate, which is used in biodiesel production. The presence of both lipase/esterase activities in the same strain may be more helpful in its industrial application. Moreover, membrane bound lipase/esterase in cell pellet, regarded as naturally immobilized enzyme, might be more valuable than extracellular and intracellular enzyme for many applications like enantioselective resolution of drugs and fine chemical synthesis.

REFERENCES