Molecular characterization of extracellular lipase producing soil bacteria, *Bacillus sp.* RV 12 and *Bacillus sp.* RV 18 and optimization for process parameters

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**Abstract**

Bacteria are prominent producers of lipases and they have a wide range of applications in the field of dairy, pharmaceuticals, food industries, detergent formulations, etc. In the present study, lipase producing bacteria were isolated from different oil contaminated soil samples from different sites of Davangere, Karnataka, India and isolates were preliminarily screened for lipase production on tributyrate agar media and confirmed on Rhodamine B olive oil agar media. Potential lipase producing isolates were subjected to optimization for different parameters such as temperature, pH, substrate concentration, incubation time and different oils as a carbon source. The potential bacterial isolates *Bacillus sp.* RV 12 and *Bacillus sp.* RV 18 showed maximum lipolytic activity when incubated at 45°C temperature, pH 7.2, 2.5% substrate concentration, 60 hr of incubation time and when butter is provided as a carbon source. Maximum activity showed isolates were subjected to biochemical tests and 16S rRNA molecular characterization. On molecular characterization of 16s rRNA confirmed that isolates belonged to *Bacillus sp.*

**Keywords**: Lipases, *Bacillus sp.*, 16S rRNA, phylogenetic tree and BLAST.

**Introduction**

Lipases (E.C. 3.1.1.3); lipid ferments are a group of hydrolytic enzymes which catalyses the hydrolysis of triacylglycerols to yield glycerol and free fatty acids in aqueous media. They act at lipid water interface (Sharma *et al.*, 2001). Microbial lipases are obtained as a result of the investigation of spoiled dairy products (Pahoja and Sethar, 2002). Many bacterial species are known to produce lipases, maybe they are thermophilic, psychrophilic, basophilic, halophilic or mesophilic in nature. The molecular weight of many bacterial lipases falls between the range 11.6 to 69 k D having wide range of applications in the field of dairy, pharmaceuticals (Vellard, 2003), food industries (Aravindan 2007 and 2009), detergent formulations (Jaeger *et al.*, 1999, Amara *et al.*, 2009 and Hasan *et al.*, 2010). Lipase biosensors are used in environmental pollution monitoring system to detect pesticides in water bodies (Kartal *et al.*, 2007).

In the present study, lipase producing bacteria were isolated and among them selected fast growers for their enzyme production and their properties. Oil contaminated soils are good source for isolation of lipolytic microorganisms, 12 different oil contaminated soil samples were collected from different sites of Davangere, Karnataka, India (altitude of 602.5 m from sea level in between 14.4644° N, 75.9218° E). The bacterial isolates for lipase production were identified using biochemical characteristics and 16S rRNA sequencing. The bacterial isolates optimized for process
parameters and identified which belongs to *Bacillus sp.* The bacterial lipases were subjected to downstream processing after production by submerged fermentation and their activity was measured.

**Materials and Methods**

2.1. **Sample Collection and Preparation**

Oil contaminated soil samples were collected from different sites in Davangere, Karnataka, India at a depth of 4 - 5 cm with the help of a sterile spatula in sterile containers and transported to the laboratory. They are kept in refrigerator at 4°C until further use. 1g of soil is added to 100ml sterile distilled water in sterile flasks and incubated in a rotary shaker at 120 rpm at 37 °C for one hr and serial dilution performed up to 10^-7 dilutions (Vinay Kumar and Ramalingappa, 2017).

2.2. **Isolation and screening of lipolytic bacteria:**

- **Primary Screening on Tributyrate Agar (TBA) media and Conformation on Rhodamine B Olive oil agar media and Biochemical characterization of the isolates :**

  100µl aliquots of each sample from dilutions 10^-6 were plated on TBA media of pH 7.2 and incubated at 37°C for 24 hr and observed for zone of lipolysis around the colonies. The lipase producing bacterial isolates on TBA media were selected and confirmed for their lipolytic activity on Rhodamine B olive oil agar media by observing orange fluorescence halo around the colonies under UV irradiation. The biochemical characterization of the potential lipase producing isolates (on Rhodamine B olive oil agar media based on zone of lipolysis) was carried out according to Bergey’s manual of systematic bacteriology (Vinay Kumar and Ramalingappa, 2017).

2.3. **Molecular characterization:**

For the molecular characterization of 16S rRNA genes, bacterial genomic DNA was isolated using the Insta Gene™ Matrix Genomic DNA isolation kit; as per the kit instruction mentioned on it. PCR amplification was done in MJ Research Peltier Thermal Cycler by using universal 16 S rRNA primers 27F (5’ AGAGTTTGATCMTGGCTCAG 3’) and 1492 R (5’ GGTACCTTGGTACGACTT 3’). Initial Denaturation 94°C for 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. Final Extension at 72°C for 10 min. DNA fragments were amplified about 1,400bp in the case of bacteria. Included a positive control (*E. coli* genomic DNA) and a negative control in the PCR. Further, positive amplification was confirmed through gel electrophoresis. Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using a ABI PRISM®Big Dye™ Terminato Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The 16S r RNA sequence was subjected to BLAST using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of BLAST results was performed followed by multiple sequence alignment and phylogenetic tree constructed in MEGA 6.06 software (Swamy et al., 2016).

2.4. **Submerged Fermentation**

A loop full of bacterial isolates *Bacillus sp.* RV 12 and *Bacillus sp.* RV18 were inoculated aseptically (separately) into 250ml Erlen-Meyer flasks containing 50 ml Luria-Bertani (LB) broth of pH 6.8 with 2% olive oil and incubated at 37 °C for 48 hrs in a rotary incubator at 220 rpm (Rabbani et al., 2013). A different process parameters were used for the optimization of production conditions.
2.5. Optimization of Process Parameters

The production, media with bacterial isolates were incubated at different temperatures ranging from 25 to 60ºC. The pH of the media was adjusted in the range 5.8 to 7.8. The different substrate concentrations ranging from 1% to 4.5% were provided by the difference of 0.5% variation. The different animal fats such as butter and ghee, and vegetable oils such as, sunflower oil, coconut oil, castor oil and olive oil at 2% concentration were provided as a carbon source. The media were incubated for different time periods of 12 to 60 hrs for the production of the enzyme.

The temperature was 37°C for all variables except for optimization of temperature. pH was 6.8 for all variables except for optimization of pH. Substrate concentration was 2% for all variables except for the optimization substrate concentration. Olive oil was used as a substrate for variables of all parameters except for optimization of parameter; different oils as carbon source. Incubation time was 48 hr for all parameters except for optimization of incubation time.

2.6. Extraction and Partial Purification

After incubation the production broth was centrifuged at 6000 rpm for 20 min and the bacterial pellet were removed. This process was repeated and the supernatant was used for further purification. The supernatant was subjected to Ammonium sulphate precipitation (Bollag and Edelstein, 1991). After precipitation, the obtained solution was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet of proteins was collected and dissolved in 10 ml of 50 mMTrisHCl solution. This concentrated protein solution was subjected to dialysis in Phosphate buffer of pH 7.2 and stirred with the help of magnetic stirrer for 24 hr by replacing the buffer after every 12 hr.

2.7. Enzyme Activity Assay

Lipase activity assay was performed according to Lopes et al., (2011) by using p- Nitrophenyl palmitate (p-NPP) (Sigma Aldrich) as substrate. The absorbance was measured at 410 nm spectrophotometrically (Systronics PC based double beam Spectrophotometer 2202). One unit of lipase activity was determined as amount of lipase that releases 1mmol p-Nitro Phenol at conditions provided.

Results and Discussion

The serial dilution followed by pour plate method was employed to isolate lipolytic bacteria from oil contaminated soil samples on tributyrate agar medium. On which 150 bacteria were isolated among them 30 isolates showed clear zone around the colonies which indicates the production of extracellular lipase or esterases. These 30 bacterial isolates also produced orange fluorescence halo around the colonies on Rhodamine B olive oil agar media under UV radiation which confirmed the production of true extracellular lipases. From these 30 isolates only 2 potential isolates (RV 12 and RV 18) were selected for the further tests because of their high lipolytic activity compared with other isolates (zone of lipolysis on TBA 3.2 and 3.6mm, respectively). The specific and sensitive plate assay method was employed to determine the bacterial lipase production; in this method they used olive oil instead of trioleoylglycerol as a carbon source. Then they confirmed the lipolytic activity on Rhodamine B olive oil agar medium (Kouker and Jaeger, 1987).

In the present study, for the isolation of lipolytic bacteria employed glycerol tributyrate and olive oil were used. Further, the maximum lipolytic activity was recorded in butter, an animal fat and lipase production was confirmed on Rhodamine B agar medium.

Further optimization of process for lipase production recorded that the bacterial isolate RV12 and RV 18 both showed maximum lipase activity at
45°C temperature, pH 7.2, 2.5% substrate concentration, 60 hr of incubation time and butter as a carbon source. The detailed optimization of different parameters such as pH, temperature, incubation time, substrate concentration and carbon source of bacterial isolates for lipase production were represented in graphical forms (Fig.1-5). From the biochemical tests, both of the isolates (RV12 and RV 18) were identified as they belong to the genus Bacillus (Vinay kumar and Ramlingappa, 2017). Earlier, lipase producing Bacillus megaterium AKG-1 have been reported from soil and hot springs (Sekhon et al., 2006; Akanbi et al., 2010; Festus and Phebe, 2017). In the present study, both Bacillus sp. RV12 and Bacillus sp. RV 18 showed...
maximum lipase production at 45°C. Further, *Bacillus* sp. RSJ-1 had been reported showing stable activity between pH range 8.0 - 9.0 (Sharma et al., 2003). In this study both the isolates *Bacillus* sp. RV12 and RV18 showed maximum lipase production at pH 7.2 indicating lipase production and enzyme activity high at alkaline pH. Moreover, *Bacillus* sp. RV12 and *Bacillus* sp. RV18 showed maximum lipase production at 2.5% substrate concentration and 60 hr of incubation time for both the isolates. Further, both *Bacillus* strains (RV12 and RV18) showed maximum lipase production when butter is supplied as a carbon source as compared with ghee and olive oil. The minimum lipase activity recorded in coconut oil, sunflower oil and castor oil respectively.

Molecular characterization by 16S rRNA gene sequencing and BLAST analysis for similarity sequences confirmed that both the bacterial isolates RV12 (Accession No: SUB2864248 Seq1 MH628234) and RV18 (Accession No: SUB4304454 Seq2 MH628230) showed 100% similarity with *Bacillus aryabhattai* strain B8W22 (Accession No: NR_115953.1) and *Bacillus megaterium* (Accession No: NR_117473.1). The phylogenetic tree clearly showed that bacterial isolates were more related to each other and also showed maximum similarity with *Bacillus aryabhattai* strain B8W22.

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**References**


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