ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 12, December 2014

Isolation and Identification of a Novel Lipase Producing Bacteria from Oil Spilled Soil

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ABSTRACT: Lipases are glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Oil spilled soil samples collected from Dharmapuri and Salem districts were screened for a novel lipase producing bacteria by screening in Tributyrin agar plate and Rhodamine olive oil agar and lipase screening liquid medium. Among the two hundred isolates thirty two isolates exhibited high lipolytic activity on TBA plate assay and twenty isolate exhibited high lipolytic activity on ROA plate assay The isolate BLP 141 produces lipase more than 12 U/ml in the further lipase screening liquid medium was selected as a novel lipase producing bacteria and identified by morphological and biochemical characteristics as *Pseudomonas.* The 16S rRNA gene amplified by bacterial universal primer 27F and 1492R yielded 1478 bp was analysed by BLAST confirmed it as *Pseudomonas gessardii* and was submitted in GenBank with accession number KJ547711.

KEY WORDS: Oil spilled soil, Screening, lipase, *Pseudomonas*, 16S rRNA.

I.INTRODUCTION

Lipase (EC 3.1.1.3) is an ester hydrolase, which catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids [1]. Lipases are ubiquitous enzymes which are found in animals, plants, fungi and bacteria. Because of substrate specificity and a variety of different enzymatic properties, such as broad sources, short cycle, wide pH, wide range of temperature, microbial lipase had played a more important role than animal and plant lipases in enzymatic theoretical research as well as practical application, including hydrolysis, esterification, trans este-rification, and ester chiral synthesis [2,3] .

Bacterial enzymes are more preferred over fungal enzymes because of their higher activities and neutral or alkaline pH optima. In order to increase the cell yields and the enzymatic activities of the cells or to produce altered enzymes, genetic and environmental manipulations can be performed more readily on bacterial cells due to their short generation times, their simple nutritional needs and easy screening procedures for desired properties [4].Greater part of bacterial lipases comes from Gram-negative bacteria and the most important Gram-negative genus is *Pseudomonas* which contains at least seven lipase producing species, that are *P.aeruginosa, P. alcaligenes, P. fragi, P. glumae, P .cepacia, P. fluorescens and P. putida* [5,6]. Enzymes of *P. aeruginosa, P. cepacia* and *P. fluorescens* obtained in industrial conditions and are used in organic synthesis, including catalysis of reactions in aqueous solutions [7].

Several methods have been developed for the measurement of lipase activity in crude or purified lipase preparations. A simple and reliable method for detecting lipase activity in microorganisms uses tributyrin as a substrate and lipase production is indicated by the formation of clear halos around the colonies grown on tributyrin- containing agar plates [8,9]. Colorimetric methods are widely used as reference method for determination of the specificity and the kinetic parameters of lipases. Several authors reported the assay of microbial lipase by colorimetric copper soap method [10,11,12]. Industries are still seeking strains of bacteria that produce a high yield of potent lipase with

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 12, December 2014

excellent properties using cost-effective methods. Therefore, the present study was conducted to isolate a novel lipase producing bacteria from oil spilled soil.

II. MATERIALS AND METHODS

A. Isolation of bacteria from oil spilled soil

Oil spilled soil samples were collected from different oil mills located at Dharmapuri, Palacode, Pennagaram, Harur and Pappireddipatti taluks of Dharmapuri district and Attur, Gangavalli, Salem, Sankagiri and Valapady taluks of Salem districts, Tamilnadu by adapting aseptic technique from a depth of 5-10 cm [13]. The soil samples collected were aseptically subjected to serial dilutions and plated on Nutrient Agar (NA) by spread plate method and incubated at 37°C for upto 72 hrs. After incubation predominant bacterial colonies were isolated and were given the code BLP (Bacterial Lipase Producer). Then they maintained in a sterile LB agar slants for further use.

B. Tributyrin Plate Assay

The bacterial isolates were screened for lipolytic activity on tributyrin agar plates. A loopful of isolate was streaked into the tributyrin medium and incubated at 37°C for 24 hours. After incubation the isolates were observed for lipolysis i.e zone of hydrolysis around the colony. Lipase production is indicated by the formation of clear halos around the colonies grown on tributyrin- containing agar plates [5,8].

C. Rhodamine B-Olive oil plate assay (ROA)

A sensitive and specific plate assay for detection of lipase producing bacteria makes use of rhodamine B-olive oil-agar plate assay [14].To identify the specific bacterial lipase producer ROA medium plate was used. The bacterial isolates were inoculated into the medium by streak plate method. Lipase producing isolates were identified after incubation for 48 hr at 37°C. The hydrolysis of substrate causes the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation.

D. Lipase Screening liquid medium

In order to select the best lipase producer for enzyme purification and characterization, Isolates were cultured in lipase Screening medium [15] with slight modification. Inoculum was prepared in the screening medium, devoid of oil. Triplicate flasks were inoculated for each isolate with 1%v/v of inoculum to a 100 ml of medium in 500 ml Erlenmeyer flasks and incubated on a rotary shaker at 150 rpm for 48hr. Samples were withdrawn and the cells were removed by centrifugation at 20,000 rpm for 10 min. The lipase production in the supernatant was estimated by colorimetric method.

E. Assay of lipase enzyme by Colorimetric method

Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range (λmax 715 nm), yielding a blue color. The reaction mixture consist of 1.0ml of crude enzyme, 2.5ml olive oil emulsion, 0.02 ml of 20 mmol $CaCl_2 \cdot 2H_2O$, was incubated for 30 min with shaking at 200 rpm at 37°C. Then the reaction was stopped by adding 1.0ml of 6N HCl and 5.0ml isooctane. The upper layer (4ml) was pipetted out into a test tube, and 1.0ml cupric acetate pyridine was added. The FFA dissolved in isooctane was determined by measuring the absorbency of isooctane solution at 715nm blanks in a UV-Visible spectrophotometer (Shimadzu UV 1800, Japan). Lipase activity was determined by measuring the amount of FFA from the standard curves of oleic acid.

F. Identification of lipase producing Bacteria

The isolate which shows maximal lipase activityTBA plate assay and on ROA plate assay are considered as positive colonies for lipase enzyme production. The isolate BLP141 which produces maximum lipase production in the lipase screening liquid medium was selected as a novel lipase producing bacteria was identified by phenotypic characterization based on morphological, biochemical and physiological characters [16]. Genomic DNA of BLP141 was isolated by CTAB method [17]. A PCR was performed in the Thermal cycler (Eppendorf, Germany) using the genomic DNA from BLP141 and 16S rDNA specific primers 27F: AGA GTT TGA TCM TGG CTC AG and 1492R: CGG TTA CCT TGT TAC GAC TT(Sigma genosys, Bangalore, India). The sequences of the PCR products were determined using the Big Dye Terminator Cycle Sequencing v2.0 kit on an ABI310 automatic DNA sequencer (Applied Biosystems, CA and USA). The identity of the sequence obtained was established by comparing with the

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 12, December 2014

gene sequences in the database using BLAST software [18]. The 16S rDNA sequence was multiple aligned using CLUSTAL W, version 1.7 [19] with a selection of *Pseudomonas* reference sequences obtained from GenBank. The alignment was corrected manually and converted to a distance matrix. The distance matrix was converted to a phylogenetic tree by NJ method using MEGA 5.0 [20] with multiple substitutions corrected and positions with gaps excluded.

III. RESULT AND DISCUSSION

Soil is a reservoir of a large and diverse microbial population. Lipase producers have been isolated mainly from soil that contains vegetable oil [21]. Lipases are currently used in different industrial products and processes and new areas of applications are constantly being added, which include the production of single cell protein, cosmetics, pulping, lubricants etc [22]. Bioremediation, the use of microorganisms or microbial process to detoxify and degrade the oil effluents is among the innovative technologies. Different microbes producing lipase are used for the oil effluent remediation process [23]. Hence in the present study an attempt has been made to isolate lipase degrading microorganism from the oil spilled soil of Dharmapuri and Salem districts and to investigate the ability of these microorganisms to produce lipases. Oil spilled soil samples were collected from different taluks of Dharmapuri and Salem districts of Tamilnadu. All the samples were aseptically subjected to serial dilutions and plated on Nutrient Agar (NA) by spread plate method. After incubation two hundred morphologically distinct predominant bacterial colonies were isolated.

A.TBA plate assay

The production of lipase by the bacterial isolate was evident by the formation of clear halos around the colonies grown on tributyrin agar. Among the two hundred isolates screened for lipolytic activity thirty two isolates exhibited high lipolytic activity (> 50 mm), ten isolate showed moderate activity (25 to below 50 mm), fifty three isolates showed low lipolytic activity (< 25 mm) and one hundred and five isolates have no lipolytic activity (Table 1). Although, different screening strategies have been proposed for the determination of lipase activity, assays using agar plates are highly recommended, because it is an easier method with lower cost [24]. Tributyrin agar plate assay is the most common methods reported for measuring the activity of lipases by the appearance of degradation haloes on culture media supplemented with desired substrates tributyrin [25,26].

B. ROA plate assay

Screening for true bacterial lipase producer using rhodamine B olive oil plate assay was evident by the formation of orange fluorescent halos around bacterial colonies visible upon UV irradiation. Twenty isolate exhibited high lipolytic activity (> 50 mm), six isolate showed moderate (25 to below 50 mm), forty eight isolates showed low lipolytic activity (< 25 mm) and one hundred and twenty six isolates have no lipolytic activity on rhodamine B olive oil plate assay (Table 1). Since both esterases and lipases can hydrolyze tributyrin, rhodamine B-olive oil-agar plate assay was performed in further screening in order to select efficient lipase producing bacteria [27]. Similar results was reported that 38 solvent-tolerant strains were isolated from different environment and 20 of them were lipase positive in tributyrin agar plates [28] and among which only 12 strains showed lipase activity in rhodamine B plate method.

C. Lipase screening liquid medium

The bacterial isolates which showed high lipolytic activity in the ROA plate assay was selected and screened further in tertiary liquid medium in order to select best lipase lipase producing bacteria. The results of the Phase III tertiary screening are presented in Fig -1. From the results it is inferred that the bacterial isolate BLP141 produces maximal lipase production 12 U/ml when compared to other isolate. Followed by BLP11,179 producing 4U/ml. The isolate BLP23, BLP86, BLP118, BLP156 and BLP194 produces 3U/ml lipase and BLP37, BLP60,BLP69, BLP92,BLP175 produces 2U/ml lipase while the remaining seven bacterial isolates BLP41, BLP45, BLP104, BLP123,BLP127,BLP152 and BLP 200 produces only 1U/ml lipase. Hence the isolate BLP141 was selected as potential strain for lipase production. In order to select best lipase producer for enzyme purification and characterization the isolates screened as lipase producer by plate assay was further selected in liquid screening medium and lipolytic activity was determined by colorimetric method.

The method is specific for fatty acids and more sensitive in comparison to titrimetric method [29,30]. Several authors

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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investigated production and optimization of lipase and measured lipase activity by colorimetric method [31,32].

Isolate	TBA	ROA	Isolate	TBA	ROA	Isolate	TBA	ROA	Isolate	TBA	ROA	Isolate	TBA	ROA
BLP1	+	-	BLP41	+++	+++	BLP81	-	-	BLP121	-	-	BLP161	-	-
BLP2	-	-	BLP42	-	-	BLP82	-	-	BLP122	-	-	BLP162	-	-
BLP3	++	++	BLP43	-	-	BLP83	+	+	BLP123	+++	+++	BLP163	+	+
BLP4	-	-	BLP44	+	-	BLP84	-	-	BLP124	-	-	BLP164	-	-
BLP5	-	-	BLP45	+++	+++	BLP85	-	-	BLP125	-	-	BLP165	-	-
BLP6	+	-	BLP46	-	-	BLP86	+++	+++	BLP126	-	-	BLP166	+	+
BLP7	-	-	BLP47	-	-	BLP87	-	-	BLP127	+++	+++	BLP167	+	-
BLP8	++	++	BLP48	+	+	BLP88	-	-	BLP128	-	-	BLP168	-	-
BLP9	-	-	BLP49	+	+	BLP89	+	+	BLP129	-	-	BLP169	+++	-
BLP10	-	-	BLP50	-	-	BLP90	+++	++	BLP130	-	-	BLP170	+	+
BLP11	+++	+++	BLP51	-	-	BLP91	-	-	BLP131	-	-	BLP171	+	+
BLP12	-	-	BLP52	+	-	BLP92	+++	+++	BLP132	+	+	BLP172	-	-
BLP13	-	-	BLP53	-	-	BLP93	-	-	BLP133	+++	+	BLP173	-	-
BLP14	-	-	BLP54	-	-	BLP94	+	+	BLP134	-	-	BLP174	+	+
BLP15	+	+	BLP55	+	+	BLP95	+++	-	BLP135	++	-	BLP175	+++	+++
BLP16	++	-	BLP56	++	++	BLP96	+	+	BLP136	-	-	BLP176	+	+
BLP17	-	-	BLP57	+	+	BLP97	+	-	BLP137	-	-	BLP177	-	-
BLP18	+	-	BLP58	-	-	BLP98	-	-	BLP138	+	+	BLP178	+	+
BLP19	-	-	BLP59	-	-	BLP99	+++	-	BLP139	-	-	BLP179	+++	+++
BLP20	+	+	BLP60	+++	+++	BLP100	+	+	BLP140	+	+	BLP180	-	-
BLP21	+	+	BLP61	-	-	BLP101	+	+	BLP141	+++	+++	BLP181	++	++
BLP22	-	-	BLP62	-	-	BLP102	+	+	BLP142	-	-	BLP182	+	+
BLP23	+++	+++	BLP63	-	-	BLP103	-	-	BLP143	+	+	BLP183	+	+
BLP24	-	-	BLP64	-	-	BLP104	+++	+++	BLP144	-	-	BLP184	-	-
BLP25	-	-	BLP65	++	-	BLP105	-	-	BLP145	-	-	BLP185	-	-
BLP26	-	-	BLP66	-	-	BLP106	-	-	BLP146	+	+	BLP186	+++	-
BLP27	-	-	BLP67	-	-	BLP107	-	-	BLP147	-	-	BLP187	+	+
BLP28	-	-	BLP68	+	+	BLP108	+++	+	BLP148	-	-	BLP188	-	-
BLP29	-	-	BLP69	+++	+++	BLP109	+	+	BLP149	+++	-	BLP189	+	+
BLP30	+	+	BLP70	-	-	BLP110	+	+	BLP150	-	-	BLP190	+	+
BLP31	-	-	BLP71	-	-	BLP111	-	-	BLP151	-	-	BLP191	-	-
BLP32	-	-	BLP72	+	+	BLP112	+++	-	BLP152	+++	+++	BLP192	-	-
BLP33	-	-	BLP73	+	-	BLP113	+	+	BLP153	-	-	BLP193	+	+
BLP34	+	+	BLP74	+++	+	BLP114	-	-	BLP154	++	++	BLP194	+++	+++
BLP35	+	+	BLP75	-	-	BLP115	-	-	BLP155	-	-	BLP195	-	-
BLP36	-	-	BLP76	-	-	BLP116	+	+	BLP156	+++	+++	BLP196	++	-
BLP37	+++	+++	BLP77	-	-	BLP117	-	-	BLP157	-	-	BLP197	-	-
BLP38	-	-	BLP78	+	+	BLP118	+++	+++	BLP158	+	+	BLP198	+++	-
BLP39	-	-	BLP79	-	-	BLP119	-	-	BLP159	+	+	BLP199	+	-
BLP40	-	-	BLP80	++	-	BLP120	+++	+	BLP160	-	-	BLP200	+++	+++

Table .1. Lipolytic activity of bacterial Isolates in Tributyrin agar plate and Rodamine B olive oil plate
High activity (> 50 mm); ++, moderate activity (25 to below 50 mm);+. Low activity (< 25 mm); -, no activity

DOI: 10.15680/IJIRSET.2014.0312050

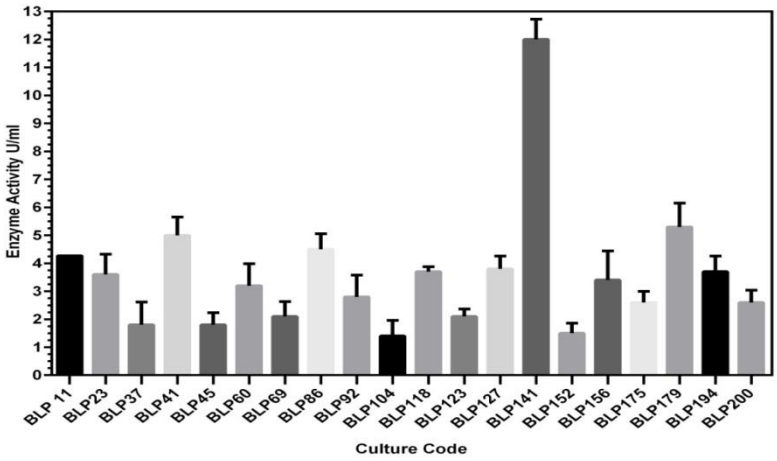
ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 12, December 2014

Fig.1. Enzyme activity of lipase positive bacteria



D. Identification of the bacterial isolate

Among the two hundred bacterial isolates screened for lipase production, twenty isolates were selected and were preliminarily examined for morphological characteristics by gram staining, shape, motility and for the presence of endospore. The selected isolate BLP141 which produced maximum lipase12U/ml than the other isolate was further characterized and identified by biochemical characteristics (Table 2). The isolate BLP141 was gram negative, rod shaped

Table .2. Morphological and biochemical characteristic of the isolate BLP141

Test	Result
Gram's Staining	Gram Negative
Shape	Rod
Motility	Positive
Spore Staining	Negative
Oxidase Test	Positive
O/FGlucose test	Oxidative
King's B medium Fluorescent diffusible pigment	Positive
King's A medium Non- Fluorescent diffusible pigment	Negative
Nitrate Reduction test	Positive
Catalase test	Positive
Levan Production Test	Positive
Indole Test	Negative
Methyl Red Test	Negative
Voges-Proskauer test	Negative
Citrate Test	Positive
Lecithinase Test	Positive

motile bacteria. It is positive for oxidase test, O/F glucose test,fluorescent diffusible pigment, nitrate reduction, catalase, levan production, citrate utilization and lecithinase test. It gives negative result for spore staining, non fluorescent diffusible pigment, indole, methyl red and VP test. From these results it is inferred that the selected isolate BLP141 belongs to *Pseudomonas* sp.

DOI: 10.15680/IJIRSET.2014.0312050

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

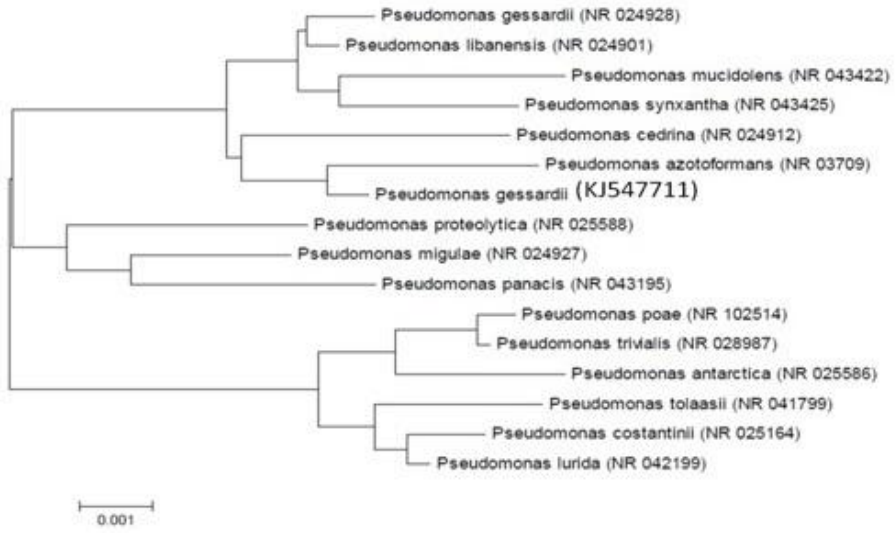
(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 12, December 2014

E. Molecular Identification

Molecular techniques utilizing amplification of target DNA provide alternate methods for diagnosis and identification [33]. To identify the experimental strain exactly according to 16S rRNA sequence analysis as well as taxonomical studies, genomic DNA of the strain was used as template to amplify partial 16S rRNA using universal bacterial primer pairs 27F and 1492R. Finally, the obtained partial 16S rRNA sequence1478bp of this strain was analyzed with BLAST. It was found to have 95- 99% identity with different strains of *Pseudomonas.* Among them, it showed high similarity (99%) with *Pseudomonas gessardii* strain CIP 105469 and the nucleotide sequence was deposited in Genbank with Accession Number KJ547711. Therefore, it could be concluded that the BLP141 strain was *Pseudomonas gessardi.* A phylogenetic tree was also constructed based on the homology of known 16S rRNA sequences (Fig 2). The evolutionary history was inferred using the Neighbor-Joining method.. The optimal tree with the sum of branch length = 0.04449125 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1462 positions in the final dataset. Evolutionary analyses were conducted inMEGA5.

Fig 2. Phylogenetic tree showing relationship of a novel lipase producing *Pseudomoas gessardii* (KJ547711) with other *Pseudomoas* sp.



The *Pseudomonas* genus demonstrates a great deal of metabolic diversity, and consequently *Pseudomonas* sp. are able to colonise a wide range of niches [34]. Their ease of culture and the availability of an increasing number of *Pseudomonas* strain genome sequences have made the genus a focus for scientific research [35].

IV.CONCLUSION

Pseudomonas exhibit the ability to bio-transform a wide range of organic compounds and are able to degrade various chemical pollutants such as simple hydrocarbons, aromatic hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics etc. Therefore the present study was focused to isolate a novel lipase degrading bacteria *P. gessardi* from the oil spilled soil which can be useful for the remediation of oil contaminated soil.

DOI: 10.15680/IJIRSET.2014.0312050

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 3, Issue 12, December 2014

V. ACKNOWLEDGEMENT

The authors are thankful to the Management, K.S.R.College of Arts & Science, Tiruchengode for their support.

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ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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