EXCELLENT PUBLISHERS

International Journal of Current Research and Academic Review

ISSN: 2347-3215 Volume 3 Number 8 (August-2015) pp. 56-66

www.ijcrar.com

Isolation and characterization of lipase producing bacteria for biodegradation of oil contamination

P. Pallavi[1], T. Ravikumar[2] and S. Ram Reddy[3]

[1]Department of Microbiology, Government Degree College for Women, Nalgonda – 508001, Telangana, India
[2]Department of Computer Science Kakatiya Degree & P.G College Nalgonda-508001, Telangana, India
[3]Department of Microbiology, Kakatiya University, Warangal - 506009 Telangana, India

Corresponding author

KEYWORDS

Lipase,
Tributyrin,
Bacillus subtilis
Y-IVI

A B S T R A C T

In view of several biotechnological applications of microbial lipases, an attempt was made to isolate better strain to produce lipase for degradation of oil contaminations. Of different isolation an efficient strain *Bacillus subtilis* Y-IVI, is isolated from oil contaminated soil based on 16Sr-DNA analysis. Maximum lipase production was observed at 48h of growth (8.5 Eu/ml).

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1. 1.3) are part of the family of hydrolases that act on carboxylic ester bonds. Their physiological role is to hydrolyze triglycerides to diglycerides monoglycerides, fatty acids and glycerol (Pallavi Pogaku *et al.*, 2010). Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential. They constitute the most important group of biocatalyst for biotechnological applications (Pallavi *et al.*, 2012). Lipase-catalyzed processes are reported to offer cost-effectiveness in comparison with traditional downstream processing in which energy consumption and toxic by-products might often pose problem (Jansen *et al.*, 1996). The alkaline thermophilic lipases find application in detergent industry many fatty food stains and human serum contain triglycerides which are hydrolyzed by lipases to produce fatty acids, monoglycerides and diglyceries, which are easier to remove than unhydrolyzed triglycerides (Ram Reddy and Pallavi, 2012).

Lipases were generally added to the detergents primarily in combination with proteases and cellulases. However, other enzymes such as amylases, peroxidases and oxidases are also reported to be added in detergent preparations. Removal of oil/fatty deposits by lipase is attractive owing to its

suitability under milder washing conditions. To be a suitable additive in detergents, lipases should be both thermostable as well as alkalophilic and capable of functioning in the presence of the various components of washing powder formulations (Ram Reddy and Pallavi, 2012). Lipases have been used for the degradation of wastewater contaminants such as olive oil from oil mills (Vitolo *et al.,* 1998). The treatment process involved the cultivation of lipase-producing microbial strains in the effluents. The microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases. They cultivated pure and mixed microbial flora known to produce lipases and other enzymes. Acinetobacter sp. was the most effective of the pure cultures, typically degrading 60–65% of the fatty material (Wakelin and Forster, 1997).

During our investigations, a number of bacterial strains were isolated from oil contaminated soils which were found to be efficient lipase producers. In this regard, an effort was made to optimize the cultural parameters for maximum production of lipase for degradation of oil stains. Results pertaining to the isolations, lipolytic potentials and production of lipase by selected strains are presented in this paper.

Materials and Methods

Samples collection

Isolation of lipolytic bacteria was made from three types of soil samples: i. Normal soils, ii. Oil contaminated soils and iii. Stored oil samples. For the first two types of soils, samples from a depth of 5cm were collected aseptically from different localities representing normal soils and oil soaked soils and enriched soils of Warangal district. The third type, oil samples were collected from oil mills, merchants, stores and households.

Isolation

Isolations of lipase producing bacteria were made by spread plate method using serial dilutions on nutrient agar medium supplemented with olive oil as substrate (Nutrient agar with 1% olive oil).

Screening

The primary screening of isolated bacterial strains was made by the methods suggested by Limpon (Bora Limpon *et al.,* 2006). The isolated strains were screened for lipolytic activity and lipolytic potential (R/r), using tributyrin agar medium and spirit blue agar medium. The strains were spread on tributyrin agar and spirit blue agar medium.

Lipolytic potential = hydrolytic zone diameter / colony diameter.

The strains which had exhibited high lipolytic potential were selected and screened further for efficient lipase production.

Identification

Bacterial isolates were identified by morphological and biochemical tests and by referring Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

Secondary screening

The strain was tested for lipase production were assessed first in 25ml of enrichment medium (peptone-10g/l, beef extract-3g/l, NaCl-5g/l, 1% olive oil and pH-7). Lipase production in different reported media under submerged fermentation conditions was carried out and lipase content was assayed. After

incubation for 24 hours the preculture formed was inoculated into production media. The culture was then incubated for 72 hours in an orbital shaker at 100 rpm at 30^{o}C.The cells were then harvested by centrifugation at 5000 rpm for 15 min and the supernatant was used for further assay at regular interval of 24 hours, 48 hours, and 72 hours. Bacterial growth was determined by measuring the absorbance at 550 nm (Sangiliyandi and Gunasekaran, 1996).

Lipase assay

The lipase activity in the culture filterate was assayed by titrimetry (Venkateshwarlu and Reddy, 1993). The reaction mixture that included 2ml of enzyme, 5ml of citrate phosphate buffer (pH 8.0), 2ml of triacetin was incubated at 37^{o}C for 3hours. At the end of incubation period the reaction was terminated by adding 10ml of ethanol and the mixture was titrated against 0.05M NaOH using phenolphthalein indicator. The activity of enzyme was expressed in terms of enzyme units. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µmol of equivalent fatty acid (ml /min) under the standard assay conditions.

Gel diffusion assay

The tributyrin agar containing 0.25% tributyrin that had been emulsified by sonication for 6min in 1.5% agar. Each plate contained 20ml of agar with 4mm diameter wells to which 30ml of sterile supernatant from the inoculated broth after centrifugation at 6000 rpm for 15 mins was added. Lipase activities were determined during aerobic incubation at 30^{0}C for 24hours by measuring the diameter (mm) of the zone of clearing after substracting the well diameter (Blake *et al.*, 1996).

16S rDNA sequence identification and phylogenetic tree analysis

The best producer of lipase was identified to species level where genomic DNA extraction was utilized as a template for the performance of PCR amplification for 16S rDNA identification with a set of universal primers that are highly conserved among prokaryotes and could amplify 1,500bp. The universal primers used were as follows:

8F: 5' AGA GTT TGA TCC TGG CTC AG 3'
1492R: 5' ACG GCT ACC TTG TTA CGA CTT 3'

The sequencing of PCR was carried out by using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer DNA homology search was conducted using the Genbank database (http://WWW. ncbi.nih.gov) and a phylogenetic tree was constructed using Tree Top phylogenetic Tree prediction software (http://WWW.genebee.msu.su).

Statistical analysis

The data obtained was subjected to relevant statistical analysis using Statistical Package for the Social Sciences (SPSS) 12.0 Software version

Results and Discussions

Distribution of lipase producing bacteria

Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, oil

contaminated soils, oil seed cakes, decaying food, compost heaps, coal tips and hot springs.

In the present investigations, the distribution of lipase producers in different soils of Warangal district was determined and the results are precised in table 1. A critical perusal of table 1 reveals that lipase producing bacteria are distributed in all the samples tested. However the percentage of occurrence varied with the nature of the soil. Different soils under investigation supported lipase producers ranging from 5-48 x 10^6 CFU g^{-1} of soil. The table reveals the total number of colonies, lipolytic colonies and the percentage of lipolytic colonies. The soils from Autonagar, Mandibazar, Warangal Chowrastha, Under bridge, Kothawada, harboured maximum number of lipase producers. Autonagar soil harboured maximum percentage of lipolytic colonies i.e. 76.19%. Next to Autonagar, the soils which supported maximum percentage of lipase producers were from Mandibazar. Hanamkonda Chowrastha soil harboured a minimum percentage i.e. 13.76% of lipase producers.

It is evident from the table 2 that the lipase producers were found to be high in oil soaked soils of ranging from 24 to 105 x10^6 CFU g^{-1} and their percentage ranged from 100% to 83.63%. Oil mill underbridge, Sai traders, Patha Beet Bazar, M.A. Natham oil mills, store of J.K. Tiffins, store of Kurshith Biryani Point soils supported maximum number of lipase production with the percentage of lipase produces of 100%. A least percentage of lipase producers were recorded from Vinayaka Traders, Patha Beet Bazar (83.63%).

In the present investigations, the occurrence of lipase producers in soils enriched with different oils and was also determined and precised table 3. A critical perusal of table 3 reveals that the distribution of lipase producers varied in soils enriched with different oils. Kakatiya University soil enriched with palm oil harboured maximum percentage of lipolytic colonies i.e. 98.22%. A least percentage of lipase producers were recorded from labour colony soils enriched with palm oil (61.33%).

It is evident from the tables 1, 2 & 3 that oil soaked soil had harboured maximum number of lipase producers, next to these soils enriched with oils harboured good number of lipase producers compared to normal soils of Warangal districts. Two reasons can be attributed for this phenomenon. Firstly, the lipolytic population which was insignificant earlier becomes dominant with the availability of substrate. Secondly, some bacteria because of their adaptive nature used lipid substrates as carbon sources in the absence of other carbon sources. Screening of 969 microbial strains isolated from soil sample for lipolytic activity (Cardenass *et al.*, 2001). Isolation of lipolytic microorganisms from palm oil mill wastes, garbage disposal sites and also from normal soil samples (Razak *et al.*, 1997).

Primary screening

The screening programs for the isolation of lipolytic bacteria are still continuing, as they may provide novel isolates or high yielding strains, which can be exploited for industrial purposes. The most widely employed primary screening method for the detection of lipolytic activity is the zone of hydrolysis in

tributyrin agar medium (Bora Limpon *et al.,* 2006). A total of 13 bacterial cultures were isolated by primary screening on tributyrin agar medium. The results are presented in table 4 and selection of efficient strains for extracellular lipolytic activity was based on the lipolytic potential (R/r) of the isolates.

The isolates which have shown lipolytic potential (R/r) of 0.6 and above 0.6 were selected for further screening. Based on their lipolytic potential (R/r > 0.5), a total of 13 bacterial isolates i.e. LP1, LP2, LP3, LP4, LP5, LP6, LP7, LP8, LP9, LP10, LP11, LP12, LP13 were selected. The lipolytic potential (R/r of above of these isolates were found to be 1.5, 1.4, 2.6, 2.0, 2.6, 5.0, 0.6, 2.0, 0.75, 1.75, 3.33, 5.0 respectively were further screened under submerged fermentation conditions for the production of lipase activity. The statistical analysis (mean and SD) for the above data was mentioned in the table 4.

Identification

Most of the bacterial cultures were isolated by plate dilution method from soil samples. The isolates which exhibited a clear zone on tributyrin agar medium were treated as positive organisms for lipase production. These isolates were subjected to preliminary identification. These positive bacterial isolates were identified with the help of Bergey's Manual of Determinative Bactieriology. Depending upon morphological characteristics and biochemical test the bacterial isolates were tentatively identified and were assigned to various genera. The isolates designated as LP1, LP5, LP7, LP10, LP13 were identified as Bacillus sp. (designated as BC). The isolate LP3, LP6, LP8, LP11 were identified to be *Pseudomonas* sp (designated as PS). The isolates LP4, LP12 belonged to *Staphylococcus* sp (designated as SH) and LP9 as *Proteus* sp and designated as PT.

Secondary screening

The submerged fermentation process for production of lipases using various media has been reported by many researchers. Lipases of *Pseudomonas* sp. strain S5 shows great promise for the production of lipases by employing an economical medium (Roy Nityananda *et al.,* 2004).

It is evident from table 5 that maximum lipase production using production medium was found at 48 hours of incubation period by all the selected isolates. Of all the isolates, LP5 exhibited maximum lipase (8.50 EU/ml) having a growth of O.D 0.545. The minimum lipase was produced by LP7 (3.50 EU/ml) exhibiting the growth OD of 0.331. The statistical analysis by ANOVA showed that the influence of medium on growth and production of lipase among the isolates and incubation period was statistically significant at $p<0.0001$, $p<0.05$ respectively. The data regarding two way ANOVA analysis with p and f-values are represented in table at 5% levels.

An attempt was made to compare the two assay methods viz., gel diffusion assay (enzyme activity and zone diameter) and titrimetry. The results are presented in table 6. The results presented in table 6 reveal that the best reported media showing the lipase production at regular intervals of time and zone of hydrolysis using agar diffusion assay.

The hydrolysis zone diameter was maximum when the assay was carried out

at 48 hours of incubation and at 72 hours assay it declined. The highest hydrolytic zone was observed by LP8 (25mm) at 48 hours incubation and at 72 hours it has 22mm of hydrolytic zone and least was exhibited by LP13 (10mm) at 48 hours and at 72 hours least was exhibited by LP2 and LP12 (9mm).

Table.1 Distribution of lipase producers in different soils of Warangal

SL.NO	Place of sample collection	No. of colonies (10^6 CFU/g)		Percentage of lipolytic colonies
		Total No. of colonies	Lipolytic colonies	
1	Kakatiya University	25	5	20.00
2	Green house KU	50	20	40.00
3	Hanamkonda, Chowrastha	109	15	13.76
4	Nayeemnagar	95	28	29.47
5	Autonagar	63	48	76.19
6	Bheemaram	15	5	33.33
7	Hasanparthy	22	9	40.90
8	Mandibazar	31	18	58.06
9	Pochamma Maidan	46	12	26.08
10	Under bridge	19	10	52.63
11	Girmajipet	22	10	45.45
12	Labour Colony	15	5	33.33
13	Warangal Chowrastha	22	12	54.54
14	Patha Beet Bazar, Wgl	36	13	36.11
15	Kothawada	12	6	50.00
16	Yellam Bazar	48	21	43.75

Fig.1 Phylogenetic tree showing evolutionary relationships of 11 taxa

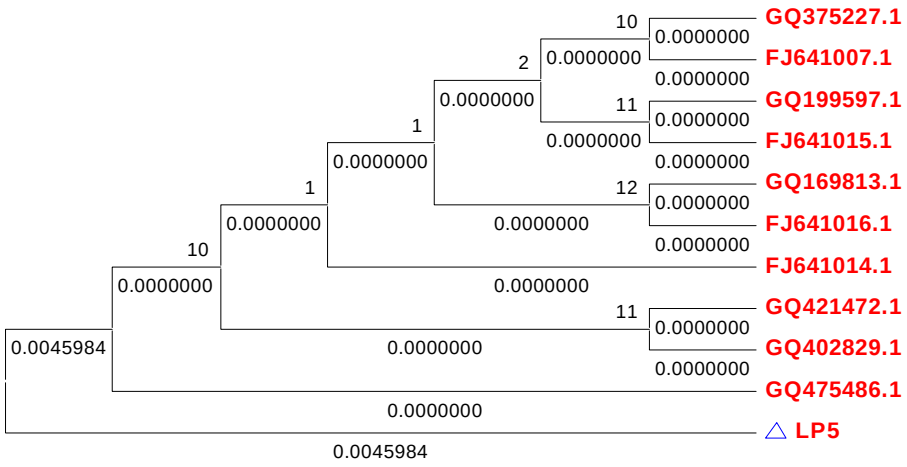

Table.2 Distribution of lipase producing bacteria in soils soaked with oil

Sl. No.	Place of sample collection	No. of colonies (10^6 CFU/g)		Percentage of lipolytic colonies
		Total No. of colonies	Lipolytic colonies	
1	Oil Mill under bridge	57	57	100.00
2	Arun Kiranam & General, Wgl.	90	85	94.44
3	Oil Stores-1, Wgl.	50	49	98.00
4	Oil Stores, Patha Beet Bazar, Warangal	90	87	96.00
5	Oil Traders, Patha Beet Bazar, Warangal	40	36	90.00
6	Oil Stores-2, Kothawada, Warangal	32	28	87.50
7	Oil Traders, Patha Beet Bazar	24	24	100.00
8	Kiranam, Kothawada, Wgl.	40	40	100.00
9	Oil Traders-2, Patha Beet Bazar	110	92	83.63
10	Oil Stores-3, Yellam Bazar, Warangal	62	60	96.77
11	Oil Mills, Wgl.	54	54	100.00
12	Oil Stories-4, Chowrastha, Hnk.	46	42	91.30
13	Oil Store-5 Vilas, Wgl.	96	95	98.95
14	Oil Store-6 Tiffins, Wgl.	85	85	100.00
15	Oil Store-7, K.U.	76	73	96.05
16	Oil Store-8	105	105	100.00

Table.3 Occurrence of lipase producing bacteria in soils enriched with different oils

Sl. No.	Sample	Olive oil			Palm oil			Groundnut oil			Coconut oil		
		No. of colonies (10^6CFU/g)			No.ofcolonies (10^6CFU/g)			No.of colonies (10^6CFU/g)			No. of colonies (10^6CFU/g)		
		Total	Lipolytic	%	Total	Lipolytic	%	Total	Lipolytic	%	Total	Lipolytic	%
1	Kakatiya University	31	28	90.32	58	57	98.22	79	62	78.48	104	85	81.73
2	Hanamkonda Chowrastha	15	14	93.30	32	30	93.75	9	7	77.77	92	88	95.65
3	Pochamma Maidan	46	44	95.65	12	10	83.33	36	33	91.66	73	71	97.28
4	Nayeemnagar	51	46	90.19	33	30	90.90	47	41	87.23	65	53	81.53
5	Bheemaram	49	44	89.79	21	18	85.71	35	33	94.28	22	18	81.81
6	Labour Colony	24	21	87.50	13	8	61.33	21	17	80.95	42	38	90.48

Table.4 Evaluation of lipolytic potential of selected bacterial strains on tributyrin agar

Isolate	Colony+Zone diameter (mm) (A)	Colonydiameter (mm) (B)	Zonediameter (mm) (C)	Lipolyticpotential D = (C/B)
LP1	10	4.00	6.00	1.50
LP2	12	5.00	7.00	1.40
LP3	11	3.00	8.00	2.60
LP4	12	4.00	8.00	2.00
LP5	11	3.00	8.00	2.60
LP6	12	2.00	10.00	5.00
LP7	10	6.00	4.00	0.60
LP8	18	6.00	12.00	2.00
LP9	12	4.00	8.00	2.00
LP10	14	8.00	6.00	0.75
LP11	11	4.00	7.00	1.75
LP12	13	3.00	10.00	3.33
LP13	12	2.00	10.00	5.00
Mean	**12.15**	**4.15**	**8.00**	**2.35**
SD	**2.08**	**1.72**	**2.12**	**1.39**

Table.5 Production of lipases on various reported media by selected lipolytic bacterial isolates

Isolates	24 hours		48 hours		72 hours	
	Growth	Eu/ml	Growth	Eu/ml	Growth	Eu/ml
LP1	0.288	4.25	0.344	5.00	0.469	1.00
LP2	0.127	4.50	0.324	5.00	0.479	2.00
LP3	0.384	4.00	0.609	4.75	0.518	1.00
LP4	0.390	3.50	0.487	4.50	0.464	0.75
LP5	0.305	2.20	0.545	8.50	0.545	1.00
LP6	0.262	1.75	0.308	3.60	0.458	2.00
LP7	0.101	1.00	0.331	3.50	0.547	0.75
LP8	0.164	4.00	0.502	5.50	0.456	1.25
LP9	0.337	0.75	0.544	3.50	0.467	0.25
LP10	0.300	3.50	0.580	6.00	0.732	1.25
LP11	0.216	0.75	0.484	2.50	0.602	0.50
LP12	0.161	1.75	0.200	4.00	0.605	1.75
LP13	0.105	1.00	0.351	4.50	0.527	1.75

Table.6 Comparison of two assay methods for production of lipases on best reported medium

Isolates	Enzyme activity (Eu/ml)		Zone diameter (mm)	
	48 hours	72 hours	48 hours	72 hours
LP1	6.00	0.75	24	13
LP2	7.40	1.00	19	9
LP3	6.40	1.25	16	14
LP4	5.00	1.75	16	14
LP5	9.00	0.75	11	10
LP6	4.00	1.00	17	16
LP7	4.50	1.00	16	15
LP8	7.80	1.25	25	22
LP9	4.50	2.25	11	10
LP10	6.25	0.75	21	19
LP11	3.00	1.00	22	20
LP12	4.00	0.75	11	9
LP13	5.50	1.50	10	12
Mean			**16.84**	**14.076**
SD			**5.145**	**4.25**
t-value			**1.49**	
p-value			**>0.05(Not Significant)**	

BLAST data: (Alignment view using combination of NCBI GenBank and RDP databases).

Alignment View	ID	Alignment Result	Description
	Consensus	**0.96**	Sample LP5 16S rDNA
	GQ475486.1	**1.00**	*Bacillus subtilis* strain Y-IVI 16S ribosomal RNA gene
	GQ421472.1	**0.99**	*Bacillus subtilis* strain L4 16S ribosomal RNA gene
	GQ402829.1	**1.00**	*Bacillus sp.* G3(2009) 16S ribosomal RNA gene
	GQ375227.1	**0.99**	*Bacillus subtilis subsp. subtilis* strain CICC 10076 16S ribosomal RNA gene
	GQ199597.1	**0.99**	*Bacillus subtilis* strain I527 16S ribosomal RNA gene
	GQ169813.1	**1.00**	*Bacillus subtilis* strain B107 16S ribosomal RNA gene
	FJ641016.1	**1.00**	*Bacillus subtilis* strain IMAUB1036 16S ribosomal RNA gene
	FJ641015.1	**1.00**	*Bacillus subtilis* strain IMAUB1035 16S ribosomal RNA gene
	FJ641014.1	**1.00**	*Bacillus subtilis* strain IMAUB1031 16S ribosomal RNA gene
	FJ641007.1	**1.00**	*Bacillus subtilis* strain IMAUB1018 16S ribosomal RNA gene

From the above table it is evident that both the methods were ideal for assay but there is a slight ambiguity in assaying the lipase using the gel diffusion assay but the both show that at 48 hours the production of lipase by all the selected strains is maximum.

Agar diffusion assay zone diameters among the isolates and incubation period were statically not significant ($p>0.05$) according two way ANOVA analysis at 5% levels.

16S rDNA identification and phylogenetic tree analysis

Strain Lp5 was identified as *Bacillus subtilis* Y-IVI sp. 16S ribosomal RNA was employed for identification of the Lp5 strain. The 16S rDNA nucleotide sequence obtained for Lp5. The phylogenetic tree analysis of Lp5 strain was constructed on the basis of comparison of the 16S rDNA sequence of this strain with other Bacillus sp. Strains available in the NCBI Genebank database. The phylogenetic tree analysis of strain Lp5 was compared with 10 other bacillus sp. Sequences. It evidenced a high degree of homology with *Bacillus subtilis* Y-IVI. The phylogenetic relationship of closely related Bacillus sp. is depicted in figure 1. On the basis of its morphological, cultural, biochemical characteristics, 16S rDNA strain Lp5 was identified as *Bacillus subtilis* strain Y-IVI with Gene Bank accession number KJ872590.

Acknowledgements

Thanks are due to Head, Department of Microbiology, Kakatiya University, Warangal for encouragement and facilities.

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