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Isolation and identification of lipase producing Bacillus pumilus GN9.

Vishnu Priya Kalapatapu	Dept. of Biochemistry, GIS, GITAM University, Visakhapatnam-530045, AP, India.
Rajesh Battina	Dept. of Biochemistry, GIS, GITAM University, Visakhapatnam-530045, AP, India.
Bhaskar Reddy Iska	Dept. of Biochemistry, GIS, GITAM University, Visakhapatnam-530045, AP, India.

ABSTRACT

A total of 65 bacterial cultures obtained from four different samples were screened on Tributyrin agar medium. Based on their zone of hydrolysis, 19 cultures were selected for further screening under submerged fermentation. Among the selected cultures, GN9 exhibited maximum lipolytic zone (2.8 R/r) and maximum lipase activity (7.2 U/ml) in production medium. Hence the culture GN9 was selected for taxonomical identification. Based on phenotypic and molecular characterization, the culture GN9 was identified as Bacillus pumilus. The 16S rRNA gene sequence of culture GN9 was deposited in GenBank with the following accession number KT624198 and the isolate GN9 was deposited in MCC under the accession number 2887.

KEYWORDS : 16S rRNA, Bacillus pumilus, identification, lipase, tributyrin agar.

INTRODUCTION

Lipases (EC.3.1.1.3, triacylglycerol acylhydrolases) are a group of enzymes, which have the ability to hydrolyze triacylglycerols at an oil-water interface to release free fatty acids and glycerol. Lipases are present in microorganisms, plants and animals [1]. Lipases catalyze a wide range of reactions, including hydrolysis, alcoholysis, acidolysis, esterification and aminolysis [2]. Lipases especially of microbial origin, have great potential in commercial applications such as detergents, fine chemicals, additives in food, waste water treatment, cosmetics, leather and pharmaceuticals [3]. The development of lipase with novel properties by searching the present biological diversity play a pivotal role in future enzyme technology [4].

Primary screening involves the requirement of some elementary tests to detect and isolate microorganisms possessing the desired property from a large microbial population and secondary screening involves the detection of useful microorganisms in a fermentation process and is essential in any systematic screening program. Screening methods either directly use microorganisms under study or quantify lipase activity in the crude enzyme [5]. Taxonomical identification of the organism has a dual purpose first to name the organisms according to some internally accepted system so that, with the least possible confusion, bacteriologists may communicate to each other their findings concerning a certain bacteria and also to indicate the current concept of the relationships of bacteria to each other and to other living organisms. Several reports have been made on lipase production by different microorganisms [6].

This chapter deals with the screening of different samples for the isolation of lipase producing bacteria and taxonomical identification of the best lipolytic bacteria.

MATERIALS AND METHODS

Materials

Nutrient broth, nutrient agar, olive oil, tributyrin agar and all other chemicals used in the present study were of analytical grade and procured from HiMedia Laboratories (Mumbai, India).

Samples Collection

Four different oil seed cakes (groundnut, coconut, soyabean, mustard) obtained from local oil mills were aseptically collected into sterile plastic bags and were processed within 24 hrs under laboratory conditions. To process the samples, 1.0 g of each collected sample was suspended in sterile distilled water to a final volume of 10 ml and vortexed for 10 min on a vortex machine. The suspension was allowed to settle and the clear supernatant was used for isolation of microorganisms.

Isolation of Bacteria from Collected Samples

Isolation of bacteria was performed by serial dilution [7] and spread plate method on nutrient agar medium. The medium was sterilized by autoclaving at 15 lb pressure (121°C) for 15 min. Nystatin (20 µg/ml), an antifungal agent was also added to the medium to control fungal contamination. After incubation, well isolated colonies were picked and further purified by repeated streaking on nutrient agar plates. The pure bacterial cultures obtained were stored at 4°C for further studies.

Primary Screening for Lipolytic Bacteria

The pure bacterial cultures were screened for lipase activity on tributyrin agar medium [8]. The tributyrin agar medium pH 7.5 was prepared with 5 g/L peptone, 3 g/L yeast extract, 15 g/L agar, 10 ml/L tributyrin in distilled water, sterilized by autoclaving for 20 min with 15 lb pressure at 121°C and cooled to 60°C. Aliquots of 20 ml were transferred to petridishes and allowed to solidify. A loopful of each pure culture was streaked onto the tributyrin agar plates separately and the inoculated plates were incubated at 37°C for 24 hrs. After incubation, the clear zone of hydrolysis around the colonies indicated the lipase production. The positive cultures which showed maximum zone of hydrolysis were chosen for further studies.

Secondary Screening for Lipase Production

All the selected lipase producing bacterial cultures were screened for production of lipase. The production medium (pH 7.0) was prepared with 5.0 g/L $(NH_4)_2SO_4$, 6.0 g/L Na_2HPO_4, 2.0 g/L KH_2PO_4, 3.0 g/L $MgSO_4$, 3.0 g/L $CaCl_2$ and 10 ml/L olive oil in distilled water, autoclaved at 15 lb pressure (121°C) for 20 min and cooled to about 60°C. The production medium was inoculated with 1.0 ml of overnight grown selected lipase producing bacterial cultures separately and incubated in an orbital shaking incubator (100 rpm) at 35°C for 24 hrs. After incubation, the culture was centrifuged at 10,000 rpm and 4°C for 15 min and the supernatant was used for assay of lipase.

Lipase Assay

The lipase activity was assayed with emulsified olive oil (1.25 ml olive oil and 3.75 ml of 2% polyvinyl alcohol) as a substrate by titrimetric method [9]. The lipase activity was determined by titration of the liberated free fatty acids from olive oil emulsion against standard alkali solution. The reaction mixture consists of 5.0 ml of emulsified olive oil, 4.0 ml of phosphate buffer (0.05 M, pH 8.0) and 1.0 ml of enzyme was incubated for 20 min at 37°C. After incubation, the reaction was terminated by adding 20 ml of ethanol (95%) and the released free fatty

acids were titrated with 0.05 M NaOH in presence of phenolphthalein indicator. One unit of lipase activity is defined as the amount of lipase required to liberate one µmole free fatty acid equivalent per minute under the assay conditions.

Identification of Selected Bacterial Culture

Phenotypic Characterization

Phenotypic characterization was studied based on different morphological, cultural and biochemical characteristics which were carried out as recommended in the Laboratory Manual of Microbiology [10]. The results were compared with a standard description given in Bergey's Manual of Determinative Bacteriology, 9th edition [11].

Molecular Characterization

The genomic DNA was isolated using the protocol of Weisburg *et al*., [12] After the isolation, qualitative and quantitative analysis, about 50 ng of DNA was used for sequencing by PCR using Big Dye® Terminator v3.1 Cycle Sequencing Kits [Applied Bio systems]. PCR products were purified by using the gel elution kit, and product obtained is sequenced on ABI 3730xls Genetic Analyzer (Applied Biosystems). The nucleotide sequence obtained was subjected to homology search using BLAST program [13]. Representative sequences of 10 similar neighbors in BLAST analysis were retrieved and aligned using multiple alignment program CLUSTAL W. The multiple alignment file was then used to create neighbor-joining tree using MEGA version 4 software [14].

Microbial Culture Collection (MCC) Accession Number

The identified bacterial culture GN9 was deposited in the Microbial Culture Collection (MCC), Pune, India for authenticity and accession number.

Statistical Analysis

All assays in this study were carried out in triplicates and the results are presented as the mean of three replicates ± SD. The data of estimates of lipase activity were subjected to one way analysis of variance (ANOVA) using Microsoft Excel 2007 to access the significance.

RESULTS AND DISCUSSION

Isolation of Bacteria from Collected Samples

A total of 65 bacterial cultures was obtained from the enriched samples by dilution technique using nutrient agar medium (Table 1). These bacterial cultures were repeatedly sub-cultured to obtain pure cultures and were maintained on nutrient agar slants at 4°C.

Table 1: Bacterial Cultures obtained from Different Samples

Samples	Sample code	Total no. of bacterial cultures
Groundnut	GN	22
Coconut	CN	19
Soyabean	SB	13
Mustard	MT	11

Primary Screening for Lipase Producing Bacteria

The results on primary screening of bacterial cultures indicates that out of 65 isolates, 22 isolates (GN-3, 7, 9, 12, 15 and 21; CN- 3, 6, 13, 18 and 19; SB- 3, 5, 9 and 12; MT- 1, 6, 8 and 11) were selected for further screening of lipase production in liquid medium based on their zone of hydrolysis (≥1.5 R/r) and macro morphological characteristics.

Secondary Screening for Lipase Production

The ability of all the selected 19 bacterial cultures for the production of lipase was measured using production medium and the results of lipase activity are presented in Table 2. Among the selected bacterial cultures, maximum lipase activity (7.2 U/ml) was observed with bacterial culture GN9 and it was selected for further studies.

Table 2: Lipase Activity of the Bacterial Cultures under Submerged Fermentation Conditions

So. No	Bacterial cultures	Lipase activity (U/ml)
1	GN3	4.8 ± 0.17
2	GN7	2.6 ± 0.08
3	**GN9**	**7.2 ± 0.29**
4	GN12	1.4 ± 0.02
5	GN15	2.3 ± 0.16
6	GN21	6.6 ± 0.14
7	CN3	3.8 ± 0.17
8	CN6	5.4 ± 0.12
9	CN13	6.0 ± 0.19
10	CN18	4.8 ± 0.14
11	CN19	6.9 ± 0.19
12	SB3	5.8 ± 0.15
13	SB5	2.6 ± 0.11
14	SB9	4.0 ± 0.18
15	SB12	4.9 ± 0.16
16	MT1	4.1 ± 0.12
17	MT6	6.8 ± 0.09
18	MT8	2.8 ± 0.15
19	MT11	6.2 ± 0.16

The values represent the mean of three replicates ± SD ($P<0.001$, ANOVA)

Identification of Selected Bacterial Culture GN9

Phenotypic Characterization

A) Morphological characteristics

The results on colony morphology and microscopic observation of the selected bacterial culture GN9 are presented in Table 3. The results showed that the colony morphology of the bacterial culture GN9 appeared to be circular, dull, rough, opaque, flat and entire on nutrient agar medium. The microscopic observation of differentially stained bacterial culture GN9 showed that the bacterium is rod shaped, gram negative, spore forming and arranged in 2-3 chains.

Table 3: Morphological Characteristics of Bacterial Culture GN9

Characteristics	Result
Colony morphology	
Size	Small
Color	Opaque
Form	Circular
Surface	Rough
Texture	Dry
Elevation	Flat
Margin	Entire
Microscopic observation	
Gram stain	Gram negative
Shape	Rods
Size	Small
Arrangement	2-3 chains
Spore formation	Positive

B) Cultural characteristics

The results on cultural characteristics of the selected bacterial culture GN9 are presented in Table 4. The results showed that the culture GN9 did not grow at temperatures below 20°C and above 50°C but showed growth between 20 and 50°C. Further, growth was observed in media having initial pH ranging 6.0–10 and also in media having 3% concentration of NaCl. In addition the culture was grown in the presence of oxygen, indicating that the bacterium is aerobic.

Table 4: Cultural Characteristics of Bacterial Culture GN9

Characteristics	Result
Growth at different temperature	
<20°C	-
>20°C to 50°C	+
>50°C	-
Growth at different pH	
pH<6.0	-
pH>6.0 to 10	+
pH>10	-
Growth at different NaCl concentrations (%)	
3%	-
6%	+
8.5%	-
10%	-
12%	-

C) Biochemical characteristics

The results on biochemical characteristics of the selected bacterial culture GN9 are presented in Table 5. The bacterial culture GN9 is positive for catalase, oxidase, nitrate and citrate tests. The culture had hydrolyzed tributyrin, tween 80, casein, gelatin and starch. In case of carbohydrate fermentation, the culture GN9 has shown acid production with starch, dextrose, galactose, fructose, aldonitol, manitol, sorbitol, and dulcitol. Based on morphological, cultural and biochemical characteristics the bacterial culture GN9 showed typical characteristics of genus *Bacillus.*

Table 5: Biochemical Characteristics of the Bacterial Culture GN9

Characteristics	Result	Characteristics	Result
Biochemical characteristics		**Carbohydrate utilization**	
Oxidase test	+	Starch	+
Catalase test	+	Raffinose	-
Nitrate test	+	Sucrose	-
Methyl red test	-	Lactose	-
Indole production test	-	Maltose	-
Urease test	-	Ribose	-
Citrate utilization test	+	Arabinose	-
		Xylose	-
Hydrolysis of		Rhamnose	-
Tributyrin	+	Dextrose	+
Tween 80	+	Mannose	-
Gelatin	+	Galactose	+
Casein	+	Fructose	+
Starch	+	Aldonitol	+
		Mannitol	+
Amino acid and their derivatives Utilization		Sorbitol	+
		Dulcitol	+
		Inositol	-
Arginine	+	Myo-Inositol	-
Lysine	-	Salicin	-
Ornithine	-		

Molecular Characterization

A) 16S rRNA gene sequencing

The 16S rRNA gene was amplified and the resultant gene product was found to be 1525 bp. The PCR product of 16S rRNA gene was sequenced and the obtained sequence was deposited in GenBank with the following accession number KT624198**.** For the past few decades, sequencing of 16S rRNA genes are serving as tools for bacterial detection and identification; and the identity of the selected bacterial culture GN9 was confirmed by sequencing of 16S rRNA gene.

B) BLAST search analysis

The 16S rRNA gene sequence of selected bacterial culture GN9 showed identity of 99% with *Bacillus pumilus*, *Bacillus safensis, Bacillus stratosphericus*, *Bacillus altitudinis*, *Bacillus aerius*; 97% identity with *Bacillus atrophaeus, Bacillus subtilis,* and *Bacillus vallismortis.*

C) Phylogenetic tree construction

To identify the bacterial isolate, representative sequences (97–99% identity) from BLAST were retrieved and aligned using CLUSTAL W. From the multiple sequence alignment data, length of the sequence was identified as 1525 nucleotides with 1391 conserved and 128 variable nucleotides. Among the ten representatives used in the analysis, bacterial culture GN9 showed 99% similarity towards *Bacillus pumilus* with a bootstrap confidence 69% (Figure 1). Hence, the culture under study with following accession number MCC 2887 was conclusively confirmed as *Bacillus pumilus.* Lipase produced from *Bacillus pumilus* was also reported by Saranya *et al*., [15] and Rakesh Kumar *et al*., [16].

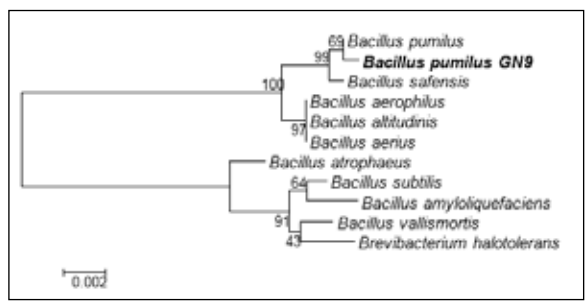

Figure 1: Phylogenetic Relationship of *Bacillus pumilus* GN9 (Highlighted)

CONCLUSION

In the present study a lipase producing bacteria was isolated from groundnut oil seed cake using tributyrin agar medium. Based on phenotypic and molecular characterization, the bacterial culture GN9 was identified as *Bacillus pumilus*. The result of the present study indicated scope for utilizing lipase producing bacteria for further enhanced production of lipase, purification, characterization and various industrial applications.

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