SCREENING OF OIL CONTAMINATED SOIL FOR ISOLATION OF LIPIDS DEGRADING BACTERIA

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ABSTRACT: *Soil offers a rich environment for the growth of various types of bacteria including lipase producing bacteria. The study at hand was performed to isolate and identify lipids degrading bacteria from soil contaminated with oil from different areas of Peshawar. The lipolytic bacteria were isolated and screened from these collected samples by serial dilution methods on Luria bertani (LB) agar medium and following Congo red assay. A total of 30 oil contaminated soil samples were collected from which 56 bacterial isolates were obtained. The isolate was further examined for lipids degrading activity qualitatively using Congo red assay. Out of these 56 lipolytic isolates, 4 isolates showed significant activity, 7 showed high lipolytic activity while 11 showed moderate lipolytic activity. The left over 34 isolates were grouped in low lipolytic activity range as they showed low lipolytic activity. These 22 efficient isolates were identified by Gram staining and various biochemical tests including oxidase test, catalase test, endospore staining, glucose fermentation, starch hydrolysis, motility test, indole test and mannitol fermentation test according to Bergey's manual of determinative bacteriology (8th Edition). The identified strains belonged to Bacillus, Pseudomonas, Staphylococcus, Lactobacillus, Streptococcus, Aeromonas, Serratia, Enterococcus and Yersinia species. The results of the present study may provide basis to deal with lipids present in municipal solid waste and in waste water to decrease pollution as well as to purify waste water.*

Key words: Soil, lipolytic bacteria, Serial dilution methods, Luria bertani, Biochemical tests.

INTRODUCTION

Despite the fact that microorganisms are very tiny, their significance cannot be neglected. The basis of biosphere is microorganisms. They are the organism that first populates the earth. All other organisms including humans, animals and plants depend upon them, without microorganisms no life can exist on earth [1]. Microorganisms are present in all types of environment. They can be seen in any environment that is wide range of temperature, pressure, they can be found in oceans and also in polluted environment [2].

Through the production of variety of enzymes microbes break down various carbon and energy sources into their components for example: fatty acids, carbohydrates, vitamins, amino acids and nucleotides. These enzymes produced are not only essential for metabolism of these microorganisms but can also have practical and industrial applications. Nowadays microorganisms are of great interest as small biofactories because of their capability to tolerate wide range of conditions [1]. Hydrolases are the most important enzymes used in industries. Hydrolases include lipases, proteases, pectinases and carbohydrases [3].

Lipases are fat splitting enzymes which required excess water to catalyze the formation of glycerol and fatty acids from long-chain triacylglycerols by hydrolysis [4, 5]. Bacteria (both gram positive and gram negative) can produce lipases but most preferable source is gram negative bacteria. Among these bacteria, most efficient lipase production is done by the genus *Pseudomonas* [5, 6]. In biotechnology and organic chemistry bacterial and fungal lipases are mostly used. Commercially bacterial lipases are important as they can be produced in large amount in shorter time. There is large number of bacteria which can produce lipases but commercially only few are available which are either wild strains or genetically modified [5, 7].

About a century ago microbiologist C Eijkmann stated that many bacteria have the ability to make and secrete lipid degrading enzymes or lipases [8]. Claude Bernard in 1856 discovered lipase for the first time while examining pancreas and its function during digestion of fats [9]. In 1936 Holwerda et al and after that in 1945 Schonheyder and Volqwarts examined interfacial activation in lipases. Two scientists Sarda and Desnuelle in 1958 explained lipases by using the phenomenon of interfacial activation and stated that a boundary or interface is formed in the middle of substrates of lipases which are insoluble in water and water and the activity of lipases to degrade lipids depend upon this interface formation [10].

MATERIALS AND METHOD

This study was carried on from March to May 2013, at the Centre of Biotechnology and Microbiology at University of Peshawar.

Sample collection

Soil samples were collected from various vicinities of Peshawar including Peshawar University, Hayatabad, UET Peshawar, Awami shopping centre etc by the following procedure. First the soil surface was stabbed 10-15 cm deep by using sterile spatula. Then it was transferred to polyethylene bag, and then the soil was thoroughly cleaned by withdrawing all the extra particles like rocks etc. Then about 10 g of sample was taken into zip lock bag. After accumulating all the soil samples, these were then tagged with proper number, location and date. All the soil samples

were stored under suitable conditions i.e. 4°c in the laboratory till further utilize.

Sample inoculation

Luria bertani (LB) media was used for soil sample inoculation and Congo red assay was used for selection and screening of lipolytic bacteria. First soil sample was diluted by serial dilution procedure and then by using direct plating method spread on LB media plates.

Serial dilution

For every sample 1 gm soil was taken by using weighing balance. 9 ml aqueous solution of Nacl (0.85%) was taken in sterile test tubes under sterile condition of LFH. This 1 gm soil was added to test tube containing saline solution and solution was made by proper mixing the soil. 1 ml from this soil solution was shifted to another test tube having saline solution by using sterile micropipette. This solution was properly mingled and labelled as 10^{-2}. Then with the help of micropipette 1ml from 10^{-2} concentration test tube was shifted to next test tube having 9 ml saline solution and was labelled as 10^{-3}. Then 1ml was transferred from 10^{-3} to next test tube and marked as 10^{-4} concentration. Similarly 1ml was transferred from 10^{-4} to 10^{-5} and from 10^{-5} to 10^{-6} .Then sample was taken from last three concentration i.e. 10^{-4}, 10^{-5} and 10^{-6} because it provides separated and distinct colonies.

Direct plating method

For sample inoculation petri plates having LB media previously kept in incubator for sterility test were taken and kept inside LFH for sample pouring. With the help of sterile micropipette 0.1ml sample was taken from 10^{-5} and 10^{-6} concentrations and was spread on LB agar plates by using sterile spreader. Plates were incubated at 37°C in incubator for 48 hours and then growth was observed.

Screening of lipolytic bacteria by Congo red assay

Agar medium containing chromogenic substrates (Congo red) was prepared and was autoclaved, poured and then allowed to solidify. The plates were spot inoculated with bacterial isolates which were indiscriminately picked from LB agar plate and then incubated at 37°c in incubator and lipolytic activity was checked by clear zone formation around the colonies. Colonies having clear zone formation were cautiously noticed and labelled carefully.

Identification of bacterial isolates

Bacterial colonies obtained were further streaked on LB agar plates and streaked plates were kept in incubator at 37°C for 24 hours. Pure bacterial isolates were selected and checked through Gram staining and growth characteristics. Further identification was done by performing different biochemical test according to Bergey's manual of Determinative bacteriology.

Gram staining procedure

Lipolytic bacterial isolates were further determined through Gram staining procedure and by examining morphological characteristics. For Gram staining a smear was prepared on the surface of slide by spreading one loop of the culture in a drop of distilled water. The slides were air dried and then heat fixed. 2-3 drops of crystal violet dye were added so that it covered the film and then washed after 30 seconds. Then 2-3 drops of Gram's iodine were added to smear for 60 seconds and then washed with water. The smear was covered with 95% ethyl alcohol and instantly washed with water. Then safranine was spread over the smear for 30 seconds and washed off with water. The slides were blot dried and were observed under microscope.

Preservation and maintenance

For preservation of culture, slants were made, and bacterial isolates were streaked, then placed in incubator overnight, and stored the slants at 4°c.

Biochemical tests

Further pure culture identification was done by performing various biochemical tests according to Bergey's manual of Determinative Bacteriology (8th edition) which is specific for Gram positive cocci and rods and Gram negative cocci and rods including oxidase test, endospore staining, catalase test, starch hydrolysis, glucose fermentation and motility test.

Oxidase test

A clean slide was taken and a small piece of filter paper was placed on it. Few drops of freshly prepared oxidase reagent were added on the filter paper. Pure colony of the test organism was smeared on the filter paper with the help of sterile wooden stick. In about 30-60 seconds, the colour change was observed.

Catalase test

30% Hydrogen peroxide drop was taken on a glass slide. 24 hours fresh culture was used as inoculums. Bacterial smear was made in the H_2O_2 drop by using sterile loop. Bubble formation on the slide showed catalase positive strains.

Starch Hydrolysis

In a sterile environment starch agar medium was poured to petri plates after autoclaving. Sterility of the plates was checked by incubation for 24 hours at 37°c. Then fresh culture of bacteria was inoculated into these plates and incubated for 24-48 hours at 37°c. After this, Gram iodine solution was prepared, poured on these incubated plates and left for 1 minute (30 seconds). Then these were properly rinsed off. Starch hydrolysing isolates showed the formation of clear zone around the colony, while non-hydrolysing isolates showed no clear zone formation.

Endospore test

On a clean glass slide a drop of distilled water was added to the slide. A smear was prepared on this droplet by using fresh culture of bacteria and was then air dried and heat fixed. The smear was flooded with malachite green (0.5% aqueous solution). The slide was heated so that malachite green was steamed for 5 min but was not allowed to evaporate. More malachite was added by heating the slide over again. Then the slide was cooled and washed under running water. Then flooded the smear with counter stain i.e. safranine for 30 second. Then the slide was washed with distilled water to remove extra stain. The slide was examined under microscope, spores appeared green inside the bacteria while absence of green colour showed non-endospore forming bacteria.

Indole test

Test tubes were taken and about 1.0 mL aliquot of the Indole broth was dispensed in it. It was then autoclaved and was allowed to cool. The tubes were lightly inoculated with a fresh pure culture of 18-24 hours. These tubes were incubated at 35-37°c for 24-48 hours. Then 10-12 drops of

kovac' s reagent was added to the broth, and then it was Stir substantially. Results were observed within 3-5 minutes. Development of red to pink colour showed positive result, while no colour change showed negative result.

Gelatin hydrolysis test

Falcon tubes were taken and about 5.0 ml nutrient gelatin broth was poured into it. The falcon tubes containing the media were autoclaved and the left for some time to cool. The tubes were then inoculated with 24 hours fresh pure culture. One of the tubes was left uninoculated to serve as control. Then the tubes were cooled in the ice bath. Hydrolysis is detected at low temperature by phase transition. Then the results were observed. Gelatin hydrolysing isolates showed liquid appearance on gelatin, while no liquid appearance on gelatin showed negative result.

Glucose fermentation

Falcon tubes were taken and 5.0 ml glucose broth was dispensed into these tubes and autoclaved. Then inoculation was done in the broth with fresh pure culture. The tubes were kept in incubator for 48 hours at 37^{o}c. Then the results were observed, yellow colour appearance showed positive result while the red color showed negative result.

Motility tests

Test tubes were taken and about 5.0 ml media were poured to each test tube and then autoclaved. Then the media was inoculated with 24 hours fresh culture by stabbing a sterile inoculating loop in the centre of the medium. After inoculation, the test tubes were incubated in incubator at 37^{o}c for 24-48 hours. Results were observed.

RESULTS AND DISCUSSIONS

Collection of soil samples

A total of 30 oil contaminated soil samples were collected from different areas of Peshawar. These soil samples were screened for isolation of lipase producing bacteria.

Screening and isolation of lipolytic bacteria

Microorganisms isolated from oil contaminated soil were screened for lipase producing activity. Screening and isolation procedure resulted in 56 lipolytic isolates from different soil samples which on further sub culturing on Luria-Bertani (LB) media gave isolated pure colonies.

Qualitative analysis of lipolytic isolates

Qualitative analysis of these 56 bacterial isolates from different soil samples showed that these isolates can produce lipases in different levels. Out of these 56 isolates only 22 isolates were selected for further study. The lipolytic activity of these isolates was determined by zone diameter to colony diameter ratio on Congo red plate. Out of these 22 isolates, 4 isolates showed significant lipolytic activity (range 4.2-5.6), 11 isolates showed moderate activity (range 2.2-2.8) and 7 isolates showed high activity (range 2.9-4) given in (table 1).

Table 1: Lipolytic bacteria (zone to colony ratio=>2.5)

Lipolytic bacteria	Moderate Lipolytic activity (Z/C) (Ratio 2.2-	High Lipolytic activity (Z/C) (Ratio 2.9-4)	Significant Lipolytic activity (Z/C) (Ratio 4.2-5.6)
22	11	7	4

Where Z= zone, C= colony

Biochemical Identification of Isolates

The identification of these 22 efficient isolates was done on the basis of various biochemical tests, including Gram staining, catalase test, oxidase test, glucose fermentation, starch hydrolysis, endospore staining, motility test etc.Different bacterial identification methods were used given in Bergey's manual of determinative bacteriology (8^{th} Edition), which showed that the bacterial isolates were Gram positive rods, Gram positive cocci and Gram negative rods. Out of these 22 isolates, 6 isolates were Gram positive rods that turned out to be *Bacillus* and *Lactobacillus species*, while 7 were Gram positive cocci belonging to *Staphylococcus, Streptococcus* and *Enterococcus* genus. The rest of the 9 isolates were Gram negative rods such as *Pseudomonas aeruginosa, Yersinia, Serratia* and *Aeromonas hydrophila* (Tables 2-4).

So far work has been done on Lipolytic bacteria isolated from different sources. These bacteria belong to different genera *Achromobacter* [11], *Bacillus sp* [12,13], *Burkholderia glumae* [4], *Cryptocoocus laurentii* [14], *Pseudomonas* [15,12], *Acinetobacter sp, Arthrobacter sp, Brevibacterium sp, Staphylococcus sp* [16].

Our results were slightly different from previously studied lipolytic bacterial species. The bacterial specie we isolated from oil contaminated soil were dominantly from genus *Bacillus* and *Pseudomonas*, some were Gram positive cocci belonging to genus *Staphylococcus, Enterococcus* and *Streptococcus* while the rest belongs to *Lactobacillus, Aeromonas, serratia* and *Yersinia spp.* (Figure 1) shows the percentage of occurrence of different bacteria in our samples. *Bacillus spp.* Has highest percentage of occurrence that is 23%, followed in order by *Pseudomonas spp.* 18%, *Aeromonas spp.* 14%, *Staphyloccous spp.* 14%, *Streptococcus spp.* 9%, *Enterococcus spp.* 9%, *Lactobacillus spp.* 5%, *Serratia spp.* 4% and *Yersinia spp*. 4%. While highest Lipolytic activity was shown by *Pseudomonas aeruginosa*. Similar bacterial isolates have been reported by Haliru *et al* [16] and Jaeger et al [17]. Research study carried out in 1999 by Jaeger et al shows that *Pseudomonas aeruginosa* is efficient in production of lipase enzyme [17].

Table 2: Biochemical tests for identification of Gram positive rods

S.No	Sample code	Gram staining	Endospore staining	Motility	Oxidae	Catalase	Starch hydrolysis	Aerobic	Remarks
1	SR2	G+ (small thin rods)	+	+	+	+	+	+	*Bacillus spp.*
2	SR8	G+ (small thin rods)	+	+	_	+	+	+	*Bacillus spp.*
3	SR5	G+ (small thin rods)	+	+	+	+	+	+	*Bacillus spp.*
4	SRA	G+	+	_	_	_	_	_	*Lactobacillus spp.*
5	SRC	G+ (small thin rods)	+	+	+	+	+	+	*Bacillus spp.*
6	SR9	G+ (small thin rods)	+	+	+	+	+	+	*Bacillus spp.*

Table 3: Biochemical tests for identification of Gram positive cocci

S. No	Sample code	Gram staining	Catalase	Mannitol fermentation	Glucose fermentation	Starch hydrolysis	Aerobic	Motility	Remarks
1	SRK	G+ (clusters)	+	+	+	_	_	_	*Staphylococcus spp.*
2	SR1	G+ (diplococcus)	_	+	+	_	_	_	*Enterococcus faecalis*
3	SR4	G+ (chains or pairs)	_	+	+	_	_	_	*Streptococcus spp.*
4	SRH	G+ (clusters)	_	+	+	_	_	_	*Staphylococcus spp.*
5	SR10	G+ (clusters)	_	+	+	_	_	_	*Staphylococcus spp.*
6	SRE	G+ (diplococcus)	_	+	+	_	_	_	*Enterococcus faecalis*
7	SR12	G+ (chains or pairs)	_	+	+	_	_	_	*Streptococcus spp.*

Table 4: Biochemical tests for identification of Gram negative rods

S. No	Sample code	Gram Staining	Oxidase	Glucose fermentation	Motility	Catalase	Indole	Gelatinase	Starch hydrolysis	Remarks
1	SR3	G- (rods)	+	_	+	+	_	+	_	*Pseudomonas aeruginosa*
2	SR7	G- (rods)	+	_	+	+	_	+	_	*Pseudomonas aeruginosa*
3	SRB	G-(rods with rounded ends	+	+	+	+	+	+	+	*Aeromonas hydrophila*
4	SRF	G- (rods)	+	_	+	+	_	+	_	*Pseudomonas aeruginosa*

5	SR11	G- (short rods)	_	+	+	+	_	+	_	*Serratia spp.*
6	SRI	G- (rods)	+	_	+	+	_	+	_	*Pseudomonas aeruginosa*
7	SR6	G-(rods with rounded ends	+	+	+	+	+	+	+	*Aeromonas hydrophila*
8	SRG	G- (rods with rounded ends	+	+	+	+	+	+	+	*Aeromonas hydrophila*
9	SRJ	G- (small rounded rods)	_	+	_	+	_	+	+	*Yersinia spp.*

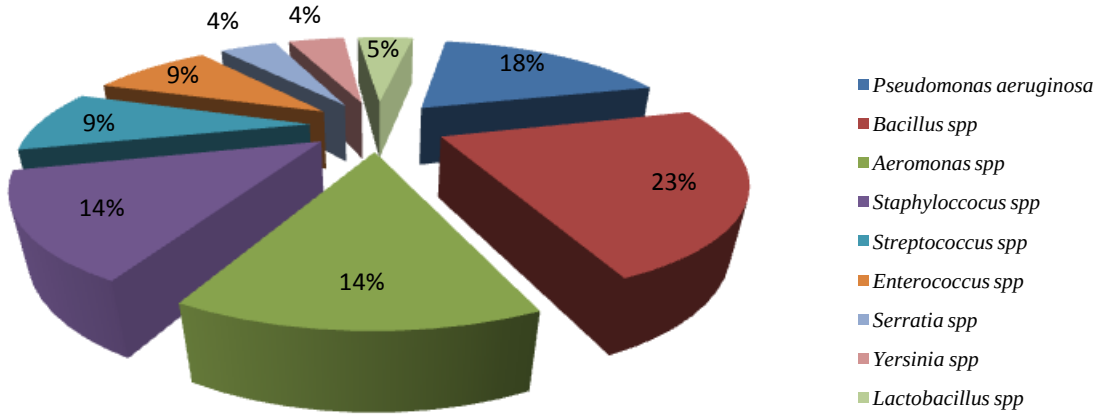

Figure 1: Occurrence of different Lipolytic bacteria in soil samples

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