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Isolation of Lipolytic Bacteria and Production of Lipase from Agro-By Products

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Abstract:

A total of hundred bacterial cultures were isolated from soils collected from oil contaminated sites. They were screened for lipolytic activity and some of these lipolytic bacteria were tested for antibiotic susceptibility, heavy metal tolerance and plasmid curring. Among the total heterotrophs 70 to 80%bacteria were lipolytic. The selected lipolytic bacteria exhibited different resistance pattern for various antibiotics and heay metals. It was found that among these isolates Pseudomonas aeruginosa LB-2 was the most efficient isolate. In the next part of the investigation linseed and mustard oil cakes were evaluated as fermentation substrate for lipase production using Pseudomonas aeruginosa LB-2. The lipase production with oil cake was compared with those of synthetic media. It was found that both linseed and mustard oil cakes were found useful for lipase production and have their own specificity. Linseed oil cake does not require nutrient supplement for lipase production but produces comparatively low lipase. Mustard oil cake in contrast requires nutrient supplement and inducers but produces significantly high lipase.

Key words: *Pseudomonas aeruginosa* LB-2, lipase production, linseed oil cake, mustard oil cake.

Introduction

Lipases (E.C. 3.1.1.3.) are one of the most important classes of hydrolytic enzyme catalyzing both the hydrolysis and

the synthesis of esters. It has got wide industrial applications as in detergent, dairy product, bakery foods, pharmaceuticals, cosmetics, leather industries and paper pulp industries. In addition to these, there are several promising fields for lipases like in biodegradation of plastics, *viz.* polyhydroxyalkanoates and polycaprolactane (Jaeger *et al*. 1995), and resolution of recemic mixtures to produce optically active compounds (Rao *et al*. 1993). Development of lipase based technologies for the synthesis of novel compounds is rapidly expanding the uses of these enzymes. One limiting factor of commercial lipase is the shortage of lipase having specific processing characteristics required by the industries and the second limiting factor is its production cost, which eventually influences the cost of lipase processed products. Several agro byproducts including wheat bran, rice bran, dexrin, sugarcane baggase, coconut oil cake, olive oil cake and gingili oil cake have been found as potential substrate lipase for production (Benjamin and Pandey 1997; Lin *et al*. 1996; Riveramunoz *et al*. 1991; Christen *et al*. 1993, Kamini *et al*. 1998).

Being an agricultural country, billion tons of agricultural by-products like oil cake different baggase and barns are being produced every year. Only some of these are utilized as fuel and animal feed, rest are discarded as waste in the environment. Utilization such wastes may be an effective alternative of high cost synthetic production media.

In the present investigation lipolytic bacterial cultures were isolated from oil contaminated soils. The isolates were characterized for resistance towards different antibiotics and heavy metals. On the basis of overall characteristics, the most efficient lipolytic bacteria *Pseudomonas aeruginosa* LB-2 was selected. Using this isolate some of the abundantly and cheaply available oil cakes were evaluated for lipase production in large scale.

Materials and methods

Sample collection:

Soil samples were collected from various oil-contaminated sites, such as waste disposal area of oil stores, oil mills and commercial caterers. Approximately 100 grams of each soil samples were collected in sterile poly bags, brought to the laboratory tested within 36 hrs. for bacterial isolation.

Enumeration and Isolation of Bacteria:

For enumeration of the bacterial load in the soil samples, 10 gram of sample was dissolved in 90 ml of sterilized double distilled water and this suspension was serially diluted up to 10^{-7} according to the standard method of Nakayama (1981). A 0.1 ml of above diluted suspension was then spreaded over the solid nutrient agar plates and incubated for 24 hrs at 37 ^{o}C. The colonies grown on the plates were counted and the bacterial load was recorded as CFU/gram sample.

Approximately 100 total cultures were randomly collected from all the samples and stored in to nutrient agar slants for screening.

Antibiotic Susceptibility Testing:

Susceptibility to different antibiotics for both heterotroph and coliforms were determined on Muller-Hinton media (Hi-media), containing antibiotics (µg ml^{-1}): Gentamycin 10, chloramphenicol 30, kanamycin 30, tetracycline 30, bacitracin 10, streptomycin 25, carbenicillin 100, nalidixic acid 30, vancomycin 30 and ampicillin 30.. The test cultures were spot inoculated over the plate and incubated at 37±1^{o}C for 24 hours. The isolates were classified as resistant or susceptible by observing their growth pattern. The antibiotic resistance index ARI was calculated as described by Hinton et al, (1985).

Multiple antibiotic resistance (MAR) was calculated as described by Krumperman (1983).

Heavy metal tolerance among bacterial isolates:

The minimal inhibitory concentration (MIC) for heavy metal was determined by agar dilution method (Lulli *et al*. 1983). Nutrient agar plates supplemented with different concentrations of heavy metal were inoculated with test cultures in their exponential growth phase. The plates were incubated at 37ºC for 36 to 48 hrs. the metal salts used were $MnCl_2$, $CuSO_4$, $ZnSO_4$, $Co(NO_3)_2$, $NiCl_2$, $K_2Cr_2O_7$, $PbNO_3$, $FeCl_3$, $AgNO_3$, As_2O_3, $NiCl_2$. The metal ion concentrations ranged from 25-500µg ml^{-1}. The isolates exhibiting growth after overnight incubation were considered tolerant to the metal.

Plasmid curing:

Curing of plasmid was performed by ethidium bromide, an inhibitor of DNA synthesis. Overnight broth cultures (0.5ml) of lipolytic strains were inoculated to 20µl ethedium bromide containing peptone water. After overnight incubation the cultures were spreaded over nutrient agar plates and again plates were incubated for24 hrs at 37º C (Ramteke 1993). The colonies developed in the plates were randomly collected in 50 numbers and these all individual colonies were tested for lipolytic activity, antibiotic resistance and heavy metal tolerance by the standard screening method as described earlier. The curing effect was assessed on the basis of presence and absence of particular characteristics. Absence of activity was considered as complete elimination of plasmid. The percentage cured or elimination of plasmid was calculated by dividing the total number of completely cured cultures by the total number of resistant strains and multiplied with 100. Similarly, curing effect was calculated for antibiotic susceptibility and heavy metal tolerance.

$$\% \text{ Cured} = \frac{\text{No. of organism Cured}}{\text{No. of resistant organism}} \times 100$$

Fermentation of oil cake:

Linseed and mustard oil cake obtained from oil mills were air dried, crushed and a semisolid suspension was prepared by suspending 20grams of crushed oil cake in minimal volume of distilled water. The suspension thus obtained was considered as 100% oil cake suspension. It was further diluted to 75, 50 and 25% with synthetic media in 250 ml conical flasks. Fermentation mixtures were inoculated with 1.0 ml of overnight grown *Pseudomonas aeruginosa* LB-2. The flasks were incubated at 37°C shaking (9120 rpm) up to 36hrs.

Enzyme extraction:

At regular intervals of six hours, 10 ml fermentation substrate was withdrawn from the flask and were centrifuged at 8000rpm for 20 min at 4°C. The supernatant was tested for lipase assay and protein content.

Lipase assay:

The lipase activity measured in the enzyme extract by the method described by Winkler and Stuckmann (1979) using *p*- nitrophenyl palmitate as substrate, where one unit of lipase activity was defined as the amount of enzyme releasing 1µmol of *p*-nitrophenol per minute under the assay condition.

Protein estimation:

The protein content in the enzyme solution was determined by the method of Bradford, (1976) using bovine serum albumin as standard.

Results and discussion

Microorganisms such bacteria, yeast and fungi are known to secrete lipase during their growth on hydrophobic substances and are also involved in recycling of insoluble organic material like oils and lipids in the nature. This is called 'clean technology' in which materials are systematically used and reused to bring about the drastic increase in resources productivity needed to make human activity sustainable (Habba *et al*. 2000). On the other hand microorganisms involved in such activities have been identified as potent lipase producers (Suzuki *et al*. 2001 and Kim *et al*. 2002).

Microbial resistance to antibiotics and metal ions is a potential health hazards because the traits are generally associated with the transmissible plasmid. The increased incidence of multiple antibiotic resistance (MAR) organisms has attracted attentions of many workers to the phenomenon of infectious nature of the transferable drug-resistant plasmids. R-plasmids are of great importance in human hygiene and have created many problems in practical medicine. They can severely impede the quick and easy treatment of bacterial infections. The process of gene transfer through bacterial conjugation has been suggested to play an important role in gene spread in natural environment.

Although, resistance to metal ions is of less concern than resistance to antibiotics, however, such an association is significant as knowledge of resistance to metals may provide useful information in mechanism of antibiotic resistance. Such bacterial population, resistance to antibiotics and heavy metal are generally useful in biological monitoring and may also be used as genetic markers.

The isolate no, BL-2 shown resistance against antibiotics, bacitracin and nalidixic acid and heavy metals Hg (50µg/ml) and lead (200µg/ml) (Table 3).

Table: 3. Antibiotic Resistance pattern of Bacterial lipase

Bacterial Isolates	TE 23	VA 30	GM 10	K 30	ST 25	B 10	C 30	CB 100	NA 30	AM
LB-2	-	-	-	-	±	+	-	-	+	-
LB-4	+	+	±	±	-	+	-	±	-	±
LB-5	+	+	-	-	-	+	+	-	-	-
LB-6	+	+	±	+	-	+	-	±	±	±
LB-7	-	-	-	-	±	+	-	±	±	±

Note: 1. TE = Tetracycline, VA = Vancomycin, GM = Gentamycin, ST = Streptomycin. B= Bacitracin, C= Chloramphinicol, CB = Carbenicillin, Na = Nalidixic Acid, AM = Ampicillin

2 (-) = Sensitive, (+) = Resistant, (±) = Intermediate.

3. All antibiotic concentrations are in µg/ml.

Hence was 2R organism. Isolate no LB-2 showed MAR pattern against tetracycline, vancomycin, kanamycin, bacitracin and partial resistant against gentamycin, carbenicillin, nalidixic acid and ampicillin. Isolate no LB-7 was resistant towards bacitracin only. The MAR pattern was also shown by the isolate no. LB-4, which was resistant to tetracycline, vancomycin and bacitracin, with moderate activity towards gentamycin, kanamycin, carbenicillin and was resistant against most of the heavy metals used at it all test concentrations except for Zn^{2+} and Ag^{2+} (Table 4). Isolate no. LB-5 shown MAR pattern towards tetracycline, vancomycin, bacitracin and carbenicillin. It also showed resistant towards many metals ions like nickel, arsenic, iron, copper, mercury, zinc, cadmium and lead. The overall studies with these isolates indicated that the isolate no. LB-2 and LB-7 had a low resistance potency for antibiotic as well as heavy metals in compared to the isolates no, LB-6 and LB-4, which showed high resistance potency towards both antibiotics and heavy metals. The isolate LB-5, showed moderate resistance for antibiotic and heavy metals tested.

Table: 4. Heavy metal tolerance among bacterial isolates

Bacterial Isolates	Ni	As	Fe	Cr	Cu	Hg	Co	Zn	Cd	Ag	Pb
	200	200	200	25	50	25	50	100	100	100	200
LB-2	-	-	-	-	-	+	-	-	-	-	-
LB-4	+	+	+	+	+	+	+	-	+	-	-
LB-5	+	+	+	-	+	+	-	+	+	-	-
LB-6	+	+	+	+	+	+	+	+	+	+	+
LB-7	-	-	-	+	-	-	-	-	_	-	-

Note: 1. All concentrations are in µg/ml.
2 (+) = Resistant, (-) = Sensitive

On plasmid curing, LB-2 showed 10% and 24% curing for antibiotic bacitracin and nalidixic acid respectively, while for heavy metals, it was 4% and 6% for mercury and lead respectively (Table 5).

Table: 5. Curing of antibiotic R- plasmid of Bacterial isolates

Bacterial Isolates	No. of Colonies Tested	Percentage Cured			
		Bacitracin (10)	Nalidixic Acid (30)	Tetracycline (30)	Chloramphenicol (30)
LB-2	50	5 (10)	12 (24)	-	-
LB-4	50	7 (14)	-	9 (18)	-
LB-5	50	6 (12)	-	10 (20)	8 (16)
LB-6	50	7 (14)	-	10 (20)	-
LB-7	50	3 (6)	-	-	-

Note: All concentrations are in µg/ml.
Calculated Value $x^2 = 76.909$
Tab value = 21.0244 at 5%, Prob. 12 df

LB-4 showed 14% curing for bacitracin and 18% for tetracycline, whereas it was 6% for mercury 4% for lead, 8% for iron and 4% for chromium. LB-5 showed 12%, 20% and 16% curing for antibiotics, bacitracin, tetracycline and chloramphenicol respectively, whereas it was 10%, 8%, 8%, 4% and 4% for heavy metals, mercury, lead, iron and cadmium respectively (Table 6).

Table: 6. Curing of Heavy metal R-Plasmid of Bacterial isolates

Bacterial Isolates	Total colonies Tested	Percentage of Curing									
		Hg		Pb	Fe		Ag		Cr		Cd
		25	50	200	100	200	50	100	25	50	100
LB-2	50	-	2(4)	3(6)	6(12)	-	1(2)	-	1(2)	-	-
LB-4	50	-	3 (6)	2(4)	-	4(8)	2(4)	-	-	2(4)	-
LB-5	50	-	5(10)	4(8)	-	4(8)	2(4)	-	-	-	2(4)
LB-6	50	-	1(2)	4(8)	-	7(14)	-	2(4)	-	1(2)	-
LB-7	50	3(6)	-	5(10)	3 (6)	-	1(2)	-	-	2(4)	-

NOTE: All concentrations are in µg/ml.

LB-6 showed 14% and 20% curing for antibiotics bacitracin and tetracycline respectively, whereas it was 2%, 8%, 14%, 4% and 2% for heavy metals mercury, lead, iron, silver and chromium respectively. The isolate LB-7 showed 6% curing for antibiotic bacitracin and 10% and 45% for heavy metals lead and chromium, respectively. On plasmid curing no isolate showed loss lipolytic activity, indicating chromosomal mediated lypolysis of test culture.

Almost all soil samples of kitchen waste shows rich flora of lipolytic bacteria. Lipolytic bacteria ranged 70% to 80% of total organisms isolated (Table 1) from the sample.

Table 1. Lipolytic Bacterial Percentage in various sample

S. No	Sampling Site	Sample Type	CFU/ml	% Lipolytic Bacteria
1	Site-1	Oil Store Waste	$2.3X10^6$	80%
2	Site-2	Do	$2.2X10^6$	80%
3	Site-3	Do	$2.35X10^6$	75%
4	Site-4	Kitchen Waste	$2.2X10^6$	70.0%
5	Site-5	Do	$3.0X10^6$	74.0%
6	Site-6	Do	$3.1X10^6$	72.0%

Among the entire sample analyzed, it seems that the presence of lipolytic bacteria was independent to the heterotrophic bacterial percentage in a particular sample. However, it is somewhat dependent on the nature of samples. It has been found that the native oil containing soil samples (i.e. form oil mills and stores) have greater lipolytic bacterial

percentage as compared to the used oil containing kitchen waste soil samples. This may be due to less available percentage of fatty acid and lipids in used oil (Habba *et al*. 2000) and thus contain low carbon source for lipolytic bacteria to act on. Similar observation was made by Zaccone *et al*. (2002), who studied qualitative and quantitative distribution of heterotrophic bacterial community in the Antarctic sea. They found more than 60% lipolytic bacteria among the total heterotrophs studied.

Total 10 bacterial cultures, which exhibited good ZCI (zone of clearance index) on tributyrin/olive oil/ tween 80 agar plates indicating their lipolytic potency (Figure 1), were selected for detailed investigation.

Figure:1. Screening of cell free extract on tributyrin agar plate for lipolysis

These 10 selected isolates were also screened on rhodamine B olive oil agar plates (Figure 2).

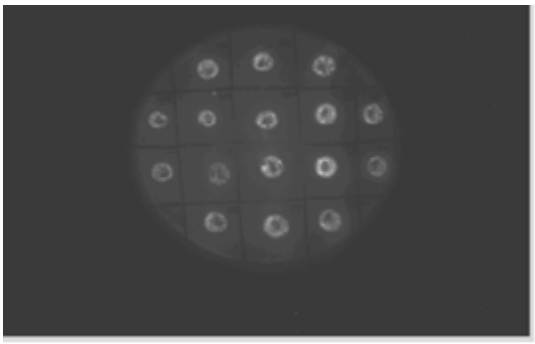

Figure: 2. Screening of lipolytic bacteria showing fluorescence on rhodamin B olive oil plate

All the bacterial cultures tested produced orange fluorescence when irradiated with 350nm UV light. Lipolysis among these cultures was quantitatively estimated in culture supernatant spectrophotometrically (Table -2). It was revealed that the isolates no. LB-2 produced 11.86 U/ml lipase with 2.16 U/mg specific activity, which was followed by LB-6 with 10.21U/ml activity and 1.90U/mg sp. activity.

Table: 2. Lipolytic Activity selected Bacterial Isolates

S.No.	Isolates No.	Zone of clearance Index* (cm)	Lipase Activity U/ml	Specific Activity U/mg
1	LB-1	1.59	10.01	1.45
2	LB-2	2.22	11.86	2.16
3	LB-3	1.25	6.01	1.11
4	LB-4	1.89	9.08	1.85
5	LB-5	1.50	8.11	1.50
6	LB-6	2.11	10.21	1.90
7	LB-7	1.90	10.55	1.88
8	LB-8	1.35	9.54	1.36
9	LB-9	1.33	6.50	1.30
10	LB-10	1.43	8.59	1.40

The highest specific activity produced by the isolate LB-2 identified as *Pseudomonas sp* showed greater specific activity than the values reported earlier in crude extracts by several

lipolytic microorganisms *viz.* 0.61 U/ml from *Bacillus strain* (Zhang *et al*., 2002). Similarly, Abdou (2003) extracted 0.103u/mg specific activity in culture supernatant of *Serratia mrsescens*. However, the specific activity obtained in these cultures were lower than the activity of *Cryptococcus* sp. S-2 lipase *i.e.* 50.52U/mg (Kamini *et al*. 2000).

Lipase, being an industrially important enzyme has got a good market demand. Several workers are now days engaged to find out the way to produce lipase with a simple and cost effective process. Substrates utilization for nutrient supply for microbial growth is a major cost influencing factor. Alternative substrates are being explored for producing lipase. Agricultural waste such as oil cake, bagasse and bran are found to be good substrate for lipase production (Rao *et al*. 1993; Benjamin and Pandey 1997; Kamini *et al*., 1998; Gombert *et al*. 1999), which are cheap as well as easily available.

Linseed and mustard Oil cake was used as substrate for lipase production using *Pseudomonas* aeruginosa LB-2 by submerged fermentation (Smf). Smf fermentation of pure linseed oil cake produced lipase 36.56 U/g oc, when pure oil cake was used (Figure 3, Table 7). Supplementing the oil cake with synthetic lipase production media (LPM) at 25% conc. decreased lipase production, where, only 29.10U/g oc lipase was produced. However addition of LPM at 50% and 75% concentration increased it to 35.68 U/g oc. and 42.08 U/g oc. respectively. So no pronounced influence of additional C/N supplier LPM was recorded on lipase production with linseed oil cake.

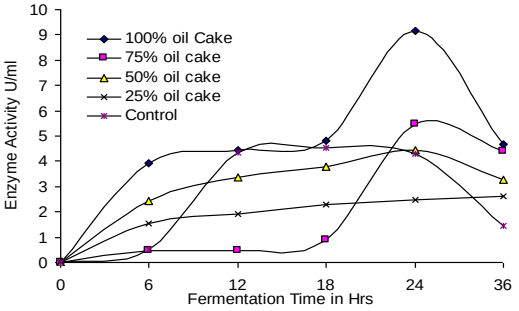

Figure 3. Lipase production through fermentation of linseed oil cake

Table: 7. Production of lipase using oil cake

Oil cake %	Linseed oil cake		Mustard oil cake	
	U/ml Suspension	U/g Cake	U/ml Suspension	U/g Cake
100%	9.14	36.56	2.94	11.76
75%	5.47	29.10	22.8	121.6
50%	4.46	35.68	17.87	142.96
25%	≥2.63	42.08	≥1.82	29.12
Control	4.55	-	4.55	-

Note: Value given is a mean of 3 replicates
C.D. Substrate = 0.033, Concentration = 0.54

In case of mustard oil cake, 11.76 U/g oc. enzyme was produced, which increased more than tenfold with addition of synthetic LPM at 25% conc. (Figure 4, Table 7.). Further addition of LPM at 50% conc. also increased lipase production at (i.e. >12 fold). However, lipase production decreased up to 19.12 U/g oc. with addition of 75% LPM. This is probably because the optimum fermentation time was increased with the addition of 75% LPM. The overall production of lipase was significantly increased in mustard oil cake, when it was supplemented with C/N supplied LPM.

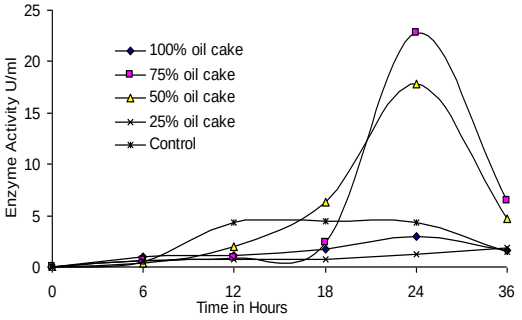

Figure 4. Lipase production through fermentation of mustard oil cake

There are several reports available describing lipase production utilizing agricultural byproducts, but most of the work has been executed through solid-state fermentation (SSF), which was done with fungal or yeast cultures (Abbas *et al*. 2002; Haq *et al*. 2002; Mahadic *et al*. 2002). Although several bacteria have been identified as potent lipase producers, prospects creation is required for utilization of such potential organism for lipase production using agro-byproducts, as fermentation substrates. Such work has not been yet reported, there for, present results lack direct supporting data. However, utilization of oilcake, bran, and bagasse for lipase through microbial cultures are described. Lipase production U/g substrate is comparable with the present findings. Kamini *et al*. (1998) described lipase production using gingili oil cake by *Aspergillus niger* and they found 363.6 U/g oc. lipase. They also found that addition of various nitrogen sources and carbohydrates did not influence lipase production that supports the present finding with linseed oil cake. Cardova *et al*. (1998) used sugarcane bagasse and olive oil cake for lipase production by *Rhizomucor pusillus* and *Rhizopus rhizopodifarunis*. They described that using sugarcane bagasse alone, *Rhizomucor pusillus* and *Rhizopus rhizopodifarunis* produced 4.99U/g and 2.67U/g enzyme respectively, which increased to 20.24U/g and 79.60U/g lipase respectively when bagasse was supplemented

with olive oil cake. Similar finding was shown by Gombert *et al.* (1999), who found 27.8 U/g oc lipase using babassu oil cake as substrate for *Penicillium restrictum*. Addition of 2% olive oil cake in fermentation medium increased it to 303U/g lipase. The highest lipase 630U/g was produced using wheat bran supplemented with olive oil cake by *Aspergillus niger* (Mahadik *et al*., 2002). Haq *et al.* (2002) employed several substrates for lipase production by *Rhizopus oligosporus* and found lipase unit 48.0U/g almond meal, 35.0U/g form coconut meal, 28.0U/g from soybean meal, 26.04U/g from rice husk and 46.oU/g from wheat bran. The highest lipase production of 142.96U/g mustard oil cake is higher than many reports described above. Lipase production 42.08U/g linseed oil cake is also higher than many reports.

Conclusion

Both linseed and mustard oil cakes were found useful for lipase production and have their own specificity. Linseed oil cake do not require nutrient supplement for lipase production but produce comparatively low lipase. Mustard oil cake in contrast requires nutrient supplement and inducers but produce significantly high lipase.

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