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Screening identification and characterization of lipase producing soil bacteria from upper dir and Mardan Khyber Pakhtunkhwa, Pakistan

Nadeem Ullah[2,1*], Muhammad Daud[3], Hidayatullah[3], Muhammad Shabir[5], Ayşegül Taylan Özkan[1], Sami ullah[5], Muhammad Qasim[2]

[1]*Faculty of Medicine, Department of Microbiology, Near East University, Turkish Republic of Northern Cyprus*

[2]*Department of Microbiology, Kohat University of Science and Technology Kohat, Khyber Pakhtunkhwa, Pakistan*

[3]*Department of Microbiology, Hazara University Manshera, Dhodial, Khyber Pakhtunkhwa, Pakistan*

[4]*Department of Biosciences, Comsat Institute of Information Technology, Islamabad, Pakistan*

[5]*Center of Biotechnology and Microbiology, University of Peshawar KhyberPakhtoonkhwa, Pakistan*

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Abstract

Microbial lipases are one of the most important extracellular metabolites which have been the main focus of scientific research due to its huge biotechnological usage over the years. Although there are many sources of lipases but microbes are the best producers. In this regards, the present work was conducted to isolate and screen out lipolytic bacteria from soil of Upper Dir and Mardan areas. Later on the lipolytic activity was observed through optimization of cultural physico-chemical parameters. Screening of lipolytic bacteria was achieved by inoculation of sample on selective medium that is tributyrine agar. Out of thirty (30) soil samples, Twenty one (21) were lipase producers. Fifteen (15) were gram positive and six (6) were gram negative. *Bacillus* spp were further explored for growth and lipase production optimization. *Bacillus* spp produce optimum growth at 37°C and pH 7. Furthermore, the optimum enzyme production for *Bacillus* spp was observed at pH 7 temperature 37°C and at 48hours time duration.

* **Corresponding Author:** Nadeem Ullah ✉ nadeemullah178@gmail.com

Introduction

Human demand to variety of products gradually increases as the process of civilization and industrialization increases (Kumar *et al.,* 2012). Natural products pulled more human attention as compared to industrial synthetics because they are easily degradable and do not deteriorate our environment (Akanbi etal 2010).The major group of biocatalyst are Lipases that catalase the hydrolysis of insoluble triacylglycerols to glycerol mono, diacylglycerol, and free fatty acids and subsequent synthesis of esters. Lipases do not require co-factor for catalytic activity and remain active in organic solvent (Araviandan etal 2006).

Lipases have cosmic presence in nature such as soil, industrial effluents, oil contaminated areas etc and originated mostly from plants, animals, fungi, yeast and bacteria (Kumar *et al.,* 2012. Microbial lipases attracted more attention due to its easy isolation,ease of genetic manipulation, high yield possible, systematic amount due to absence of seasonal variations and quick growth of micro-organisms or low-priced media. Bacterial lipases are glycoproteins in nature but some extracellular lipases are lipoprotein (Bhavani G etal 2012).

Microbial lipases have high biotechnological applications (Chirajyoti D etal 2006). It becomes important biocatalyst in various industrial sectors such as dairy and food industries for cheese ripening flavor enhancement and hydrolysis of milk fat, and lipolysis of cream and butter fat (feng etal 2010) Other applications include paper, pharmaceutical, cosmetics, detergent, leather, single cell protein production of fine chemicals, waste water treatment, bakery products, and biofuels industries (Bhavani G etal 2012).

Microbial lipases are high in demand due to their specificity in stereo chemistry and less energy utilization (Sharma etal 2001). Several bacterial lipase producing genera have been reported but *pseudomonas* and *bacillus* are the prominent producers (jaegar etal 1999) .The aim of the study is to identify and characterize lipase producing bacteria isolated from soil of Upper Dir and Mardan KhyberPakhtoonkhwa.

Materials and methods

Collection of soil samples

A total of 30 samples from two districts (Dir and Mardan) were collected from different sites such as garages, workshops, garbage hills, rivers, flour mills, sugar mills, marble industry, gardens, and slaughter houses(feng etal 2010). The soil samples were taken in appropriately labeled pre-sterilized bottles with the help of sterile spatula from the depth of 0.5 to 1.0 cm surface and subsurface. Samples were then immediately transferred to the laboratory of Microbiology, Kohat University of Science and Technology, Kohat Pakistan and stored at 4°C till analysis.

Pure culture isolation

Samples of soil were diluted serially from 10^{-1} up to 10^{-6} in sterile distilled water each dilution were cultured on nutrient agar plates by pour plate method to obtain isolate colonies after 24 hour of incubation (Kumar *et al.,* 2012).

Screening of Lipase producing bacteria

Pure bacterial isolates were screened for lipase production as described by Kumar *et al.* (2012). Bacterial colonies were streaked on tributyrin agar and incubated at 37°C for 48 hour. Clear zone around the colonies on tributyrin agar were observed indicating lipase producing bacteria.

Identification of lipase producing isolates

Lipase producing bacterial colony was observed on the basis of shape, size, consistency, margin, elevation, opacity, pigmentation and Gram reaction as described in Bergey's manual of determinative bacteriology (kalyana and lakshmi 2013) Biochemical tests include catalase test, indole test, urease test, motility test, fermentation of sugars and Vogel Proskauer test(selya etal 2008).

Temperature and pH optimization for growth of

selected best lipase producing bacteria

Nutrient broth containing 1% (v/v) tributyrine was prepared and autoclaved at 121°C and 15 psi for 15min. Those isolates of bacteria which produce lipase were inoculated in the tributyrine broth and incubated in a shaker incubator at 150 rpm speed and in different temperature (4°C, 37°C, 50°C and 60°C) for 18,24,48,72 hours. OD was measured at 600 nm for growth optimization of selected best lipase producing bacterial isolates.

Preparation of crude lipase enzyme from broth

2 ml Broth were taken from broth culture incubated at different temperature and pH Culture was then centrifuged at 10,000 g at 4°C for 10min. Supernatant (crude lipase enzyme) was then collected in eppendorf tube, mixed properly and OD was measured at 600nm.

Assay of crude lipase enzyme by agar well diffusion method (cup well method)

To form a well of 4mm diameter the tributyrine agar plates were punched aseptically with sterile cork borer. Then crude lipase enzyme of 50µlwas loaded in each well and incubates at 37°C for 48 hours. After incubation the clear zones around the wells were measured in millimeter.

Lipase Assay by Titrimetric Method

The lipase activity was measured by titrimetric method and olive oil using as a substrate at pH 7.0. The reaction cocktail was prepared by 5% (W/V) gum acacia in 100Mm sodium phosphate buffer, pH7.0 and 1ml of each crude lipase enzyme was added to the reaction.

Cocktail of 10ml separately at their respective culturing temperatures and incubated for 15 min. at 100 rpm in a shaker incubator. The reaction was quenched and fatty acids were extracted by adding 1ml of acetone: ethanol solution (1:1) and swirling the contents swiftly. Phenolphthalein indicator of 2-3 drops was added to each of the reaction mixture with respect to different crude lipase enzymes of isolates and the control. The contents of each reaction mixture were titrated with 0.05M NaOH solution to the end point of pink color at pH 10.0. Lipase activity was calculated as micro moles of free fatty acids formed from olive oil per ml of crude lipase enzyme as per equation:

$$Activity = \frac{(VS - VB).N.1000}{S}$$

Where, *VS* is the volume of 0.05M NaOH solution consumed by the enzyme-substrate cocktail (ml); *VB* is the volume of 0.05M NaOH solution consumed in the titration by the substrate (Control) cocktail (ml); *N* is the molar strength of the NaOH solution used for titration (0.05M); *S* is the volume of substrate cocktail solution (10ml). One unit (U) of lipase enzyme is defined as the amount of enzyme required to liberate 1µmol of fatty acids from triglycerides (Bhavani *et al.*, 2012).

Statistical analysis

SPSS version 14.0 for windows was used for the statistical analysis of the data.

Results and discussion

Thirty soil samples from different sites of Upper Dir and Mardan were considered in the study. Out of 52 bacterial isolates only 21 isolates were lipase producing. A diversified lipolytic activity was observed ranges from a large clear zone (strong lipolytic activity) to small zone (weak lipolytic activity). Two best lipase producing bacterial isolates were identified as *Bacillus spp.* and Pseudomonas spp. bases on their culture, microscopic and biochemical characteristics. Bacillus spp was further analyzed which produced optimum growth at 37°C, 50°C and 7,9 pH. These were ideal conditions for lipase production and optimization of *Bacillus* spp. as performed by Bhavani *et al.*, (2012). , E.Sirisha *et al*. (2010), Kumar *et al.* (2012).

Identification of selected strains of bacteria

Out of fifty two isolates only twenty one isolates were characterized for while *Bacillus spp* showed optimum growth at 37°C, 50°C and 7,9 pH morphological cultural and biochemical characteristics and were identified at the genus level.

Colonies zone clearing of Bacillus spp on tributyrine agar.

From the above figures it is clear that bacterial isolate *bacillus spp* has maximum lipase activity at a range of temperatures 4 °C to 60 °C after 24 to 96 hrs of incubation.

Table 1. Effect of pH on growth of *Bacillus* spp at different incubation time.

Isolates	pH	24hours	48hours	72hours	96hours
Bacillus spp	3	0.02	0.04	0.05	0.07
Bacillus spp	5	0.12	0.19	0.25	0.29
Bacillus spp	7	0.16	0.24	0.28	0.35
Bacillus spp	9	0.19	0.26	0.34	0.38

The above table demonstrate that the bacterial isolates *Bacillus* have maximum lipase producion at 37 °C and 50 °C tempetatures and pH 7,9 after different incubation periods.

Table 2. Effect of pH on lipase production (zone in mm) of *Bacillus* spp. at different incubation time.

Isolates	pH	24hours	48hours	72hours	96hours
Bacillus spp	3	0.2	0.3	0.5	0.8
Bacillus spp	5	0.3	0.4	0.7	10
Bacillus spp	7	0.9	10	12	12
Bacillus spp	9	0.6	0.8	0.9	0.9

Table 3. Effect of pH on lipase activity (µmol/ml) of *Bacillus* ssp. at different incubation time.

Isolates	pH	24hours	48hours	72hours	96hours
Bacillus spp	3	4	7	8	9
Bacillus spp	5	7	9	10	11
Bacillus spp	7	6	8	12	14
Bacillus spp	9	5	7	10	12.5

The above table shows that the bacterial isolates *Bacillus* have maximum lipase activity at 37 °C and 50 °C tempetatures and pH 7,9 after different incubation periods.

Table 4. Biochemical characteristics Lipase Producing bacterial isolates.

S.No	Code of Isolates Coa		Cat	OX	Vp	U	Ind	Gramreaction	Motilty	Spore
1	FM1D (Flour Mill Dir)	-	+	+	-	-	-	+	+	+
2	GB1D (Garbage 1 Dir)	-	+	+	-	-	-	-	-	-
3	RPD (River Panchkora Dir)	-	+	+	-	-	-	+	+	+
4	WS1 D (Workshop 1 Dir)	-	+	-	-	-	-	+	+	+
5	SMM (Sugar Mill Mardan)	+	+	+	+	-	+	+	-	-
6	H3D (Hill 3 Dir)	+	+	-	-	-	-	+	-	-
7	OPM (Oil Pump Mardan)	+	+	-	-	+	-	-	-	-
8	KRM (Kalpani River Mardan)	-	+	-	-	+	-	+	+	-
9	MIM (Marbal Industry Mardan)	+	+	-	-	+	-	+	-	-
10	H1M (Hill 1Mardan)	+	+	+	-	-	-	+	+	-
11	SH1 D (Slaughter House Dir)	-	+	-	-	-	+	+	+	+
12	GD1D (Garden 1 Dir)	+	+	+	-	-	+	+	-	-
13	(GDIM Garden 1 Mardan)	+	+	-	-	-	+	+	-	-
14	CID (Chips Industry Dir)	+	+	+	-	-	+	-	-	-
15	GBM (Garbage Mardan)	+	+	+	-	-	+	-	-	-
16	H2d (Hill 2 Dir)									
17	WS2 D (Worskshop 2 Dir)	+	+	-	-	-	-	-	-	-
18	SH2M (Slaughter House 2 Mardan)	+	+	-	-	-	-	+	+	+
19	NSD (Nursery Dir)	-	+	+	-	-	-	+	+	+
20	GB2M (Garbage 2 Mardan)	+	+	-	-	-	-	+	+	+
21	SH1 D (Slaughter 1 Dir)	+	+	+	-	-	-	+	+	+

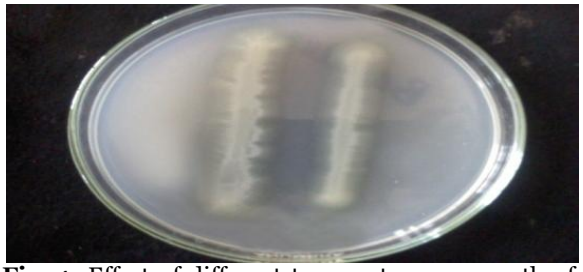


Fig. 1. Effect of different temperature on growth of *Bacillus spp* at 24 hours of incubation.

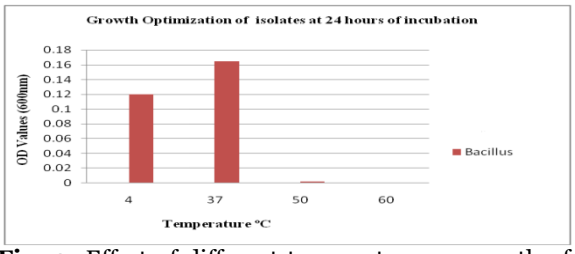


Fig. 2. Effect of different temperature on growth of *Bacillus* spp. at 48 hours of incubation.

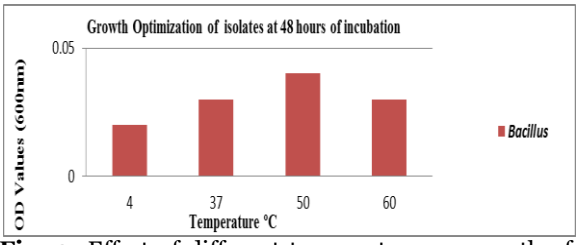


Fig. 3. Effect of different temperature on growth of Bacillus spp. at 72 hours of incubation.

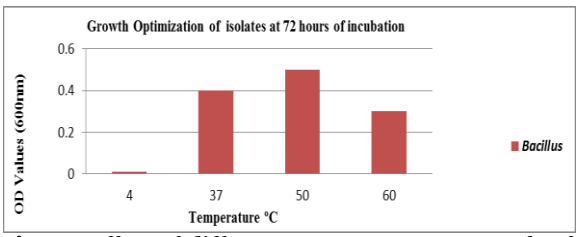
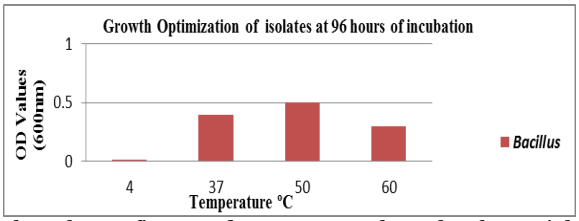


Fig. 4. Effect of different temperature on growth of *Bacillus* spp. at 96 hours of incubation.



The above figures demonstrate that the bacterial isolates *bacillus* have maximum lipase producion at 37 ºC and 50 ºC tempetatures after different incubation periods.

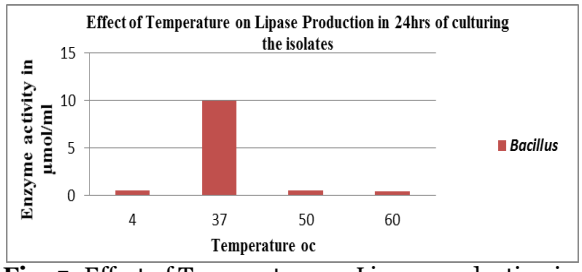


Fig. 5. Effect of Temperature on Lipase production in 24hrs of culturing the isolates.

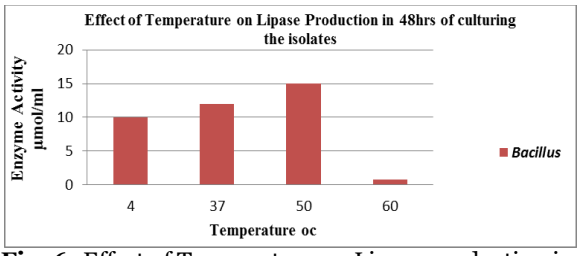


Fig. 6. Effect of Temperature on Lipase production in 48hrs of culturing the isolates.

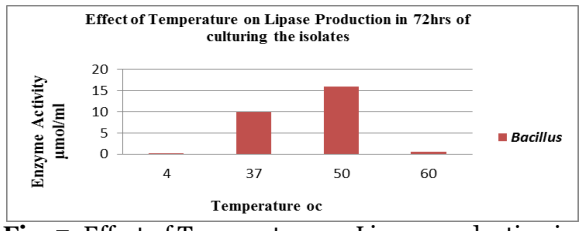
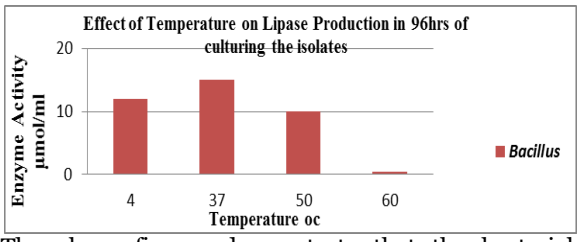


Fig. 7. Effect of Temperature on Lipase production in 72hrs of culturing the isolates.



The above figures demonstrate that the bacterial isolates *bacillus* have maximum lipase producion at 37 ºC , 4 ºC and 50 ºC tempetatures after different incubation periods.

Fig. 8. Effect of Temperature on Lipase production in 96hrs of culturing the isolates.

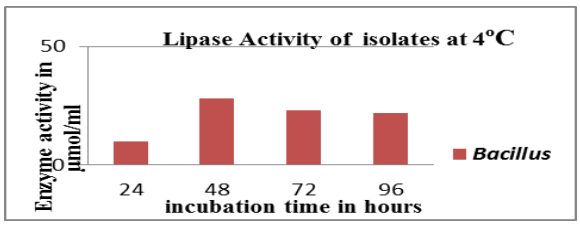


Fig. 9. Lipase activity (µmol/ml) of *Bacillus* at 4°C.

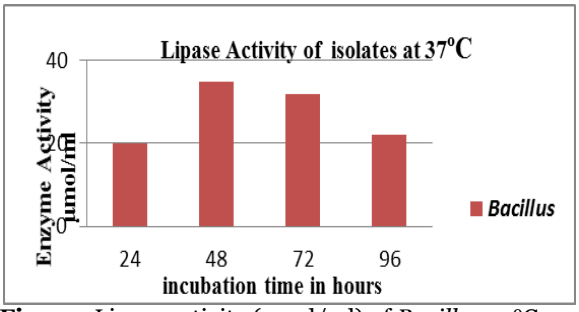


Fig. 10. Lipase activity (µmol/ml) of *Bacillus* 37°C.

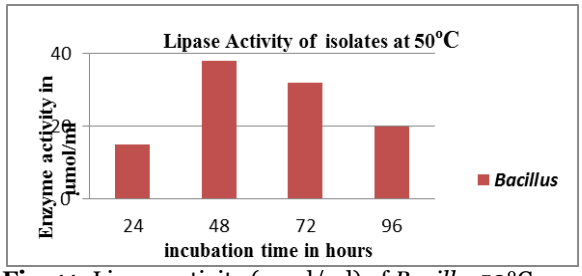


Fig. 11. Lipase activity (µmol/ml) of *Bacillus*50°C.

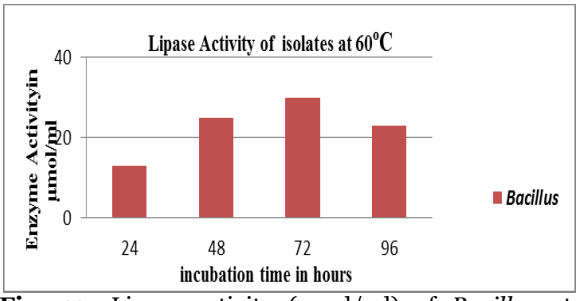


Fig. 12. Lipase activity (µmol/ml) of *Bacillus* at 60°C.

Conclusion

The present study

highlighted that soil of Upper Dir and Mardan are rich in lipase producing bacteria which showed diversified lipase production/activity. Further study on molecular characterization of lipases and lipases producing indigenous bacterial flora is needed for novel discovery which can be further used in many newer areas where they can serve as potential biocatalysts.

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