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Natural substrates for extracellular lipase production from *Pseudomonas aeruginosa* HE05 isolated from diesel oil contaminated soil

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ABSTRACT
The present work focused on the production of extracellular lipase from *Pseudomonas aeruginosa* HE05 emplying natural oils of plants as substrat. Nine isolates (HE01-HE09) were isolated from the desiel oil contaminated soil and screened for lipase activity on nutrient agar medium supplemented with 1% olive oil and tween 80. *Pseudomonas aeruginosa* HE05 was identified as the hyperproducer of lipase. Maximum lipase production (3123U/ml) was recorded when 1% olive oil and 1% tween 80 added in nutrient broth used as a substrate in submerged fermentation. The optimal production was achevied in 96 hrs at 37^{o}C at 100 rpm. Quantification of lipase units were performed by turbidimetric assay. Supplementation of nutrient broth with natural oils was found as inducer as well as substrate for the production of lipase. Among the natural oils, mustard oil, corn oil, almond oil are better substrate for extracellular lipase production.

Key Words: lipase, *Pseudomonas aeruginosa*, mesophile, olive oil, Tween 80.

1. INTRODUCTION:
Lipases are universal, extracellular enzymes delivered from plants, animals and most importantly from microorganisms. Lipases are triacyl glycerol acyl hydrolases (EC 3.1.1.3) that catalyze the hydrolysis and production of esters from glycerols and long-chain unsaturated fats[1, 2]. Lipases have extensive physiological importance and industrial potential. These enzymesshow a high level of specificity and enatioselectivity for esterification and transesterification responses [3]. Some imperative lipase generating bacterial genera consists of *Bacillus*, *Pseudomonas* and *Burkholderia*[4,5,6].Lipase production by microbes is greatly influenced by growth conditions which include carbon and nitrogen sources, presence of inducers and inhibitors, incubation temperature, pH, inoculum size and oxygen concentration [5].As lipases are inducible, carbon source has been accounted as the main variable that influence lipase expression. Inducers such as olive oil or other inducers including triacylglycerols, fatty acids and tweens are required for production of these enzymes [7].
Lipases are considered as enzymes of interest which demand is growing rapidly due too their vast applicationsin edibles,cleanser,medicine, textile,cosmetic,pulp industry, oil biodegradation and waste treatment industries [8,9]. The use of lipases in bioremediation procedure is becoming the latest trendnow a days [10].

This study was aimed to select the suitable substrate to achieved hyperproduction of extracellular lipase from anewley isolated *Pseudomonas aeruginosa* HE05 under submerged fermentation.

2. MATERIALS AND METHODS:
2.1 Collection of soil sample
The collection of desiel oil contaminated soil sample, for the present study wasdone from the automobile workshop near University of Karachi road. The soil has been exposed to desiel oil for long period. The sample was collected from a depth of 10cm using a sterile spatula in plastic zipper bags and were immediately transferred to the lab for subsequent isolation. The soil sample was stored at room temperature.

2.2 Screening of Lipase producing bacteria
Bacterial cultures were isolated by serial dilution technique from desiel oil contaminated soil on nutrient agar plates. Primary screening for the lipase production was done on Nutrient agar plates that were supplemented with1% olive oil and 1% tween 80 and incubated at 37^{o}C for 48 hrs. Lipase producers were identified through the formation of precipitated zones around the colonies.

2.3 Taxonomic identification
Morphological characterization of the bacterial isolates was performed by Gram staining. Cultural characterization of the lipase producing strain was done by growing it on differential media such as Brilliant Green Agar, Eosin Methylene Blue agar and Cetrimide Agar. For Biochemical characterization, Catalase, Citrate, Oxidase, H_2S and Motility tests were performed and identification of isolated strainswas done according to the guidelines of Bergey's manual of Determinative Bacteriology[11].

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2.4 Production of Lipase in submerged fermentation

The selected bacteria was inoculated in the media composed of Nutrient Broth supplemented with olive oil (1%) and tween 80(1%) pH 7, 10% inoculum was used in fermentation as starter culture. Fermentation was carried out in shaking water bath at speed of 100 rpm at 37℃ for 72 hrs. The resulting cells were separated by centrifugation from supernatant at 5000 rpm for 10 min and cell free filtrate(CFF) was utilized to determine lipase activity.

2.5 Lipase estimation by Turbidimetric Assay

Turbidimetric Assay was carried out using Tween 20 as a substrate to determine lipase activity [12]. The reaction mixturecontaine 20mM Tris-HCl (pH 8.5), 1.8% tween 20 and 3mM $CaCl_2$. A volume of 0.3 ml of enzyme source was added to 3.7 ml of reaction mixture and incubated at 37℃ for 1 hour. A sample of 1 ml was removed after 30 min intervals to determine OD at 500nm. The reaction mixture with heat denatured enzyme was used as a control and reaction mixture without enzyme was used as a blank. The amount of enzyme that caused an absorbance increased equal to 0.01 OD per min at A_{500} nm is considered as one lipase unit [13].

2.6 Influence of incubation time on lipase production

The effect of fermentation time on extracellular lipase production was examined by incubating the flasks in shaking water bath for 24, 48, 72, 96 and 120 days at 37^{o}C. The CFF was collected and assayed for lipase after completion of designated time interval for fermentation.

2.7 Influence of natural oils on Lipase production

The selected bacteria were grown on different media prepared using different oils as a substrate for highest lipase production, the nutrient broth (Oxoid) was used as control and different natural oil media were prepared in nutrient broth with 1% concentration i.e. corn oil, olive oil, almond oil, mustard oil, castor oil, coconut oil and tween 80 and olive oil in combination with tween 80. The fermentation media was incubated in shaking water bath (100rpm) at 37℃ for 96hrs. The resulting cells were separated by centrifugation from supernatant at 5000 rpm for 10 min and cell free supernatant was utilized to determine lipase activity.

3. RESULTS AND DISCUSION:

Extracelluar lipase producing bacteria were isolated from desiel oil contaminated soil as it could be a great source of habitat of lipid-degrading bacteria due to the presence of a lipid rich environment [3,14,15].

3.1 Primary Screening of soil isolates for lipase producers

Nine different bacterial isolates were isolated by serial dilution technique, maintained on agar slants and for future use and all isolates were preserved in 80% glycerol at -20℃. All of the isolates were then screened for lipase production on nutrient agar medium containing olive oil (as an inducing agent) and tween 80. Positive strains were identified on the basis of white precipitates around the colonies indicating lipolytic activity.

Among isolated strains, HE05 showed highest lipolytic activity indicated by largest precipitated zone approximately 17mm in diameter, while HE09 displayed moderate activity with 9mm zone dimmeter and HE06 displayed low with 6mm zone, while rest of the cultures does not show any activity on activity plates. In literature, olive oil with tween 80 are being utilized in primary screening methods for the identification of lipase producers and formation of calcium crystals in agar make it visible and direct interpretation of result is possible [16,17].

3.2 Identification of the Bacterial Strains

Morphological and cultural character are studies for the identification of all the isolates. The characteristics of different isolates and their hydrolytic zone dimmeter are presented in Table 1. The bacterial strain HE05 showed maximum zone of hydrolysis and its morphology showed that it is Gram negative rods, scattered and motile. When grown on Nutrient Agar produce small, irregular, shiny colonies with pink pigmentation. On Eosin Methylene Blue agar, purple smooth colonies were observed without a metallic sheen, indicating that it is a gram negative, lactose non-fermentor (Figure 1a) and on Brilliant Green Agar it produced yellow colonies surrounded by moderate yellow zones, showing that it is a slow fermentor of lactose (Figure 1b). The strain showed positive catalase, oxidase and citrate utilization test but no H_2S production.

On the basis of above results, HE05 was identified as *Pseudomonas aeruginosa*. For further confirmation, it was grown on Cetrimide agar which is a selective medium for *Pseudomonas aeruginosa* and inhibitory to other gram-negative

bacteria. It resulted in the production of a blue-green pigmentation on this agar; whereas *E.coli* was usedas control which show no growth (Figure 2).

3.3 Lipase estimation in submerged fermentation
In turbidimetric method, it was observd that highest lipase activity of 1,036U/ml was obtained in the culture medium supplemented with olive oil and tween 80.

3.4 Influence of incubation time on lipase production
The lipase activity increased with the production time until it reached its maxima in 96hrs i.e.2492 units /ml and after there is a sharp decline in lipase production. The production of lipase was reported to be late in stationay phase supported the extended fermentation time (Figure 3).

3.5 Influence of natural oils on lipase production
Lipid as carbon sources usually appear to be fundamental for getting a high lipase yield [18]. Eight different natural oil used as supplements in nutrient broth to find out the best substrate for the production of lipase in submerged fermentation. Lipase production was enhanced and supported by the addition of natural oils as compare to the control which does not contain oil. Mustard oil (2083U/ml), corn oil (1763U/ml), almond oil (1003U/ml)were found to be superior to olive oil (996U/ml) in lipase production (Figure 4).

The olive oil, among natural oils has been extensively used for screening, as a sole substrate and inducer for lipase production [19]. Lipase synthesis from *Bacillus* sp. was recorded on addition of 1% olive oil, however, very little activity was noted without the addition of olive oil even after incubation for an extended time [20]. Gilbert and co-workersused olive oil as the sole carbon source for lipase production by *Pseudomonas aeruginosa*[21]. However, lipase reported from *Pseudomonas aeruginosa* HE05 has substrate preference to mustrad oil, 2.09 fold higher production as compare to olive oil, and it is in accordance with the previous reports of lipase production from *Pseudomonas* sp where 3.75 fold increased [22].Mustard oil for lipase induction in *Pseudomonas aeruginosa* BN-1 was also reported in another study [23].Coconut oil showed moderate activity as compared to other oils. while coconut oil has been reported to be the best substrate for lipase from *P. aeruginosa* LST-03 [24].Complete inhibition of lipase production was monitor when medium was supplemented with castor oil.

The potential of Tween 80 (fatty acyl esters) was also descussedin primary screening the lipase producing organism as strong inducing agents of lipase.The significant induction in the production of lipase (776 U/ml) was observed when Tween 80 was used as sole substrate in fermentation medium. Tween 80 as nonionic detergent in media also contribute to increase the substrate availability to lipase.Olive oil in combination with tween 80 showed the highest lipase activity (3213U/ml) as it has been suggested that lipase activity has reliance on adequate surface tension and viscosity [16,17].

4. CONCLUSION
It was concluded that *Pseudomonas aeruginosa* HE05 isolated from a soil contaminated by deseloil,which is a mixture of alkanes and aromatic compound, could be used as a potent lipase producer. The enzume production is enhanced by addition of locally available natural oils in medium.

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CONFLICT OF INTEREST STATEMENT
All authors declare no conflict of interest, financial or other, exists. Authors declare that they respect the journal's ethics requirements.

REFERENCES
[1] Maruyama T, Nakajima M, Ichikawa S, Nabetani H, Furusaki S, Seki M. (2000). Oil–water interfacial activation of lipase for inter esterification of triglyceride and fatty acid. *J. Am. Oil. Chem. Soc.,* **77**:1121–1127.

[2] Verma ML, Azmi W, Kanwar SS. (2008). Microbial lipases: at the interface of aqueous and non-aqueous media. A review. *ActaMicrobiol. Immunol. Hung.*, **55**:265–294.

[3] Soberon-Chavez, G. and Palmeros, B. (1994). Pseudomonas Lipases: Molecular Genetics and Potential Industrial Applications. *Crit. Rev. Microbiol.*,**20**: 95-105.

[4] Ahmed EH, Raghavendra T, Madamwar D. (2010). A thermostable alkaline lipase from a local isolate *Bacillus subtilis* EH 37: characterization, partial purification, and application in organic synthesis. *ApplBiochem Biotechnol*;**160**:2102–2113.

[5] Gupta, R., N. Gupta, and P. Rathi. (2004). Bacterial lipases: an overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.*,**64**: 763- 781.

[6] Belfrage P, Fredrikson G, Stralfors P, Tornquist H. In: Borgstrom B, Brockman HL (1984). eds. Lipases. Amsterdam: *Elsevier Science*; 365–416.

[7] Treichel H, Oliveira D, Mazutti MA, Di Luccio M, Oliveira JV. (2010). A review on microbial lipases production. *Food Bioproc. Technol.*, **3**:182–196.

[8] Houde A, Kademi A, Leblanc D. (2004). Lipases and their industrial applications: an overview. *Appl. Biochem. Biotechnol.*,**118**(1–3):155–170.

[9] Hasan F, Shah AA, Hameed A. (2014). Industrial applications of microbial lipases. *Enzyme and Microb. Technol.*, **39:** 235– 251.

[10] Pandey A, Benjamin S, Soccol CR, Nigam P, et al. (1999). The realm of microbial lipases in biotechnology. *Biotechnol. Appl. Biochem.*,**29**: 119-131.

[11] Brenner DJ, Krieg NR, Staley JT, Garrity GM. (2005). Editors. Bergey's manual of systematic bacteriology. Springer; New York.

[12] Von Tigerstrom RG and Stelmaschuk S. (1989).The use of Tween 20 in a sensitive turbidimetric assay of lipolytic enzymes.*Can. J. Microbiol.* , **35**: 511–514.

[13] Schlemmer AF, Ware CF, Keen NT. (1987). Purification and characterization of a pectin lyase produced by *Pseudomonas fluorescens* W51. *J. Bacteriol.*,**169**(10):4493-4498.

[14] Mobarak-Qamsaari, E., R. Kasra- kermanshahi and Moosavi-nejad, Z. (2011). Isolation and identification of a novel, lipase-producing bacterium *Pseudomonas aeruginosa* KM110.*Iran.J.Microbiol*. **3**: 92-98.

[15] Sanchez, S. (2005). Ecology and industrial microbiology Microbial diversity-the bright and promising future of microbial manufacturing.*Cur.Opin.Microbiol.*,**8:** 229-233.

[16] Shelley AW, Deeth HC, and MacRae IC. (1987). Review of methods of enumeration, detection and isolation of lipolytic microorganisms with special reference to dairy applications.*J. Microbiol. Metho.*,**6**:123-137.

[17] Thomson CA, Delaquis PJ, and Mazza G. (1999). Detection and Measurement of Microbial Lipase Activity: A Review. *Crit. Rev. Food Sci. Nutri.*,**39**(2): 165-187.

[18] Sharma R, Chisti Y, Banerjee UC. (2001). Production, purification, characterization, and applications of lipases. *Biotechnol.Advan.*,**19**: 627-662.

[19] Bornscheuer UT. (2002) Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiology Reviews*, **26**(1):73-81.

[20] Sugihara A, Tani T, Tominaga Y. (1991). Purification and characterization of a novel thermostable lipase from *Bacillus* sp. *J. Biochem.*,**109**:211–216.

[21] Gilbert EJ, Cornish A, and Jones CW. (1991). Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2.*J. Gen.Microbiol.*,**137**: 2223-2229.

[22] Rathi, P, Bradoo, S, Saxena, RK, Gupta, R. (2000). A hyper-thermostable, alkaline lipase from *Pseudomonas* sp. with the property of thermal activation.*Biotechnol.Lett*.,**22:** 495-498.

[23] Noman MS, Iqbal S, Bano S, Khan AB, ul-Qader SA, Azhar A. (2010). Purification and characterization of 60kD lipase linked with chaperonin from *Pseudomonas aeroginosa* BN-1. *Afric. J. Biotec.*,**45**: 7724-7732.

[24] Ogino H, Nakagawa S, Shinya K, Muto T, Fujimura N, Yasuda M, Ishukawa H (2000). Purification and characterization of organic solvent-stable lipase from organic solvent –tolerant *Pseudomonas aeruginosa* LST-03.*J. Ferment. Bioeng*.**89:**451-457

Table 1. Characteristics of Bacterial Isolates

Culture Code	Gram Reaction	Colony Pigmentation	Colonial Form	Optical Property	Colony Size	Hydrolytic Zone Diameter
HE01	G +ve	White	Irregular	Opaque	Small	—
HE02	G +ve	White	Filamentous	Opaque	Medium	—
HE03	G +ve	White	Filamentous	Opaque	Medium	—
HE04	G +ve	White	Irregular	Opaque	Medium	—
HE05	**G - ve**	**Pink**	**Irregular**	**Transparent**	**Small**	**17mm**
HE06	G +ve	White	Irregular	Opaque	Small	5mm
HE07	G +ve	White	Filamentous	Translucent	Large	—
HE08	G +ve	White	Filamentous	Translucent	Large	—
HE09	G +ve	Cream	Irregular	Opaque	Large	9mm

(a) (b)

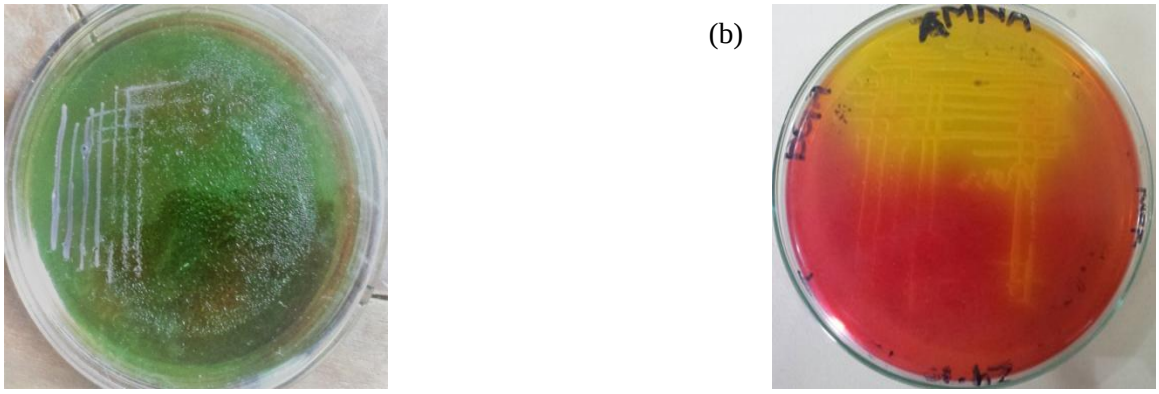

Figure 1.Cultivation of *Pseudomonas aeruginosa* HE05 on (a) EMB agar and (b) Brilliant Green Agar

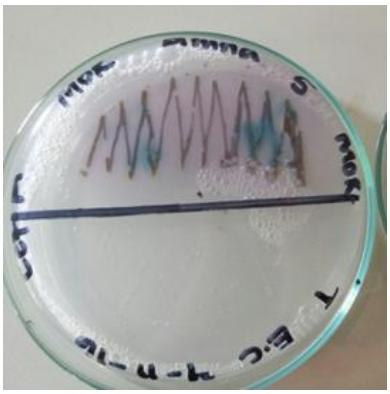

Figure 2. Blue green pigment produced by *Pseudomonas aeruginosa* HE05 on Cetrimide agar, while no growth is observed with *E.coli* used as a control

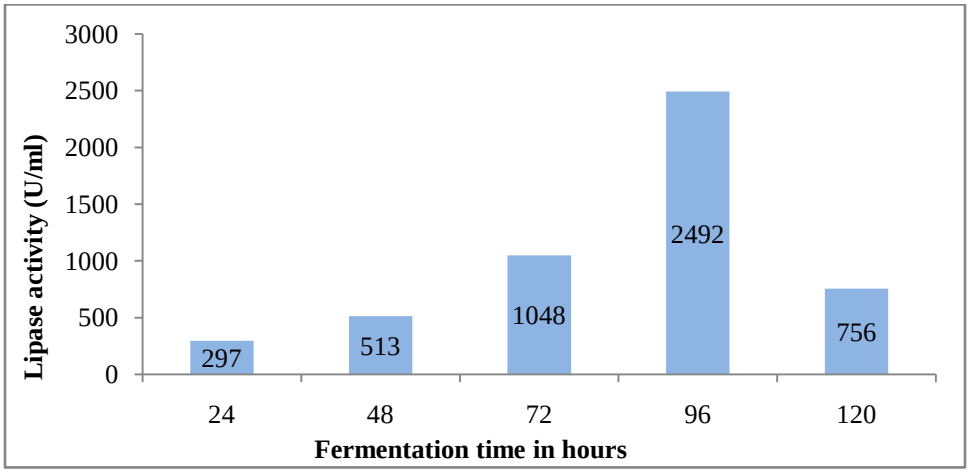

Figure 3. Influence of fermentation time on lipase production from *Pseudomonas aeruginosa* HE05.The assay was carried out in triplicates and average was plotted.

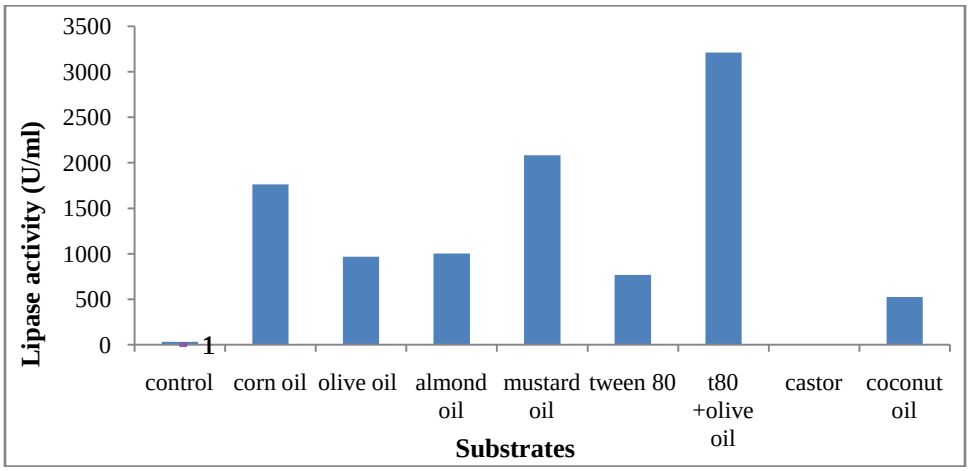

Figure 4: Influence of different substrate on lipase production from *Pseudomonas aeruginosa* HE05. The assay was carried out in triplicates and average was plotted.