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Isolation and Screening of Lipase Enzyme from Bacterial Strains Isolated from Sambhar Lake

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Abstract
Sambhar Salt Lake Rajasthan (26°58'N 75°05'E) is a unique solar saltern and is a heaven for halophiles that belong to the extremophiles group. The potential of using bacteria as source of industrially important and relevant enzymes has stimulated interest in exploration of enzymatic activities. Lipase is one of the most important naturally occurring polymers and appears to be the cheapest future raw material of detergent industry. The current study was taken up mainly to study moderate halophiles of the lake. The halophilic bacteria are able to survive on 5 to 15% NaCl. This paper proposes to discuss some recent work on the isolation and screening of lipase enzyme. Bacterial strains were selected for screening onto tributyrin agar plates and clear zones around the colonies indicated the production of extracellular lipase and p-Npp estimation was done to confirm lipase production.

Keywords
Lipases, bacterial strain, submerged fermentation, primary and secondary screening.

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INTRODUCTION:
Lipase is an economically essential hydrolytic enzyme which leads to the conversion of triacylglycerol into simple glycerol and free fatty acids. Lipase extensively found in bacteria, fungus, plants, animals and actinomycetes (joshi and kuila. 2018) and mainly those produced from bacteria are more stable than others. In commercial terms, bacteria lipases are more important because their cultivation is ease and optimized to increase the production (joshi et al., 2019). Industrial application of new origin of lipase different catalytic features will increases their demands for new strains.

Various sources of lipase producing microorganism are such as vegetable oil factories (Watanabe et al., 2000), agricultural waste (Salihu et al., 2012), soil contaminated with oils (Sirisha et al., 2010), dairy waste (Sorhaug and Stepaniak, 1997) and natural oils for example petroleum oil, olive oil, vegetable oil and coconut oil are used to increase the lipase production.

From the industrial perspective, lipase enzyme considers very essential because they can have

produced on large scale. Currently, investigation report confirms that submerged fermentation is extremely adequate in large scale production of industrially significant enzymes for example celluloses, lipase and pectinases (Bharathi et al., 2018). In the field of biotechnology, submerged fermentation is an optimistic tool for the production of microbial enzymes and in developing countries it is the most suitable production methods (Tolan and Foody, 1999).

Submerged fermentation has several benefits over conventional solid state fermentation, including resembling the environmental surrounding for different microorganisms, higher productivity, less operational problems, decrease energy and cost requirements, compression of fermentation vessel, poor capital and recurring expenditure, better oxygen circulation and less effect in downstream processing, (Singhania et al.,2010).

Lipase production was depending upon the type o bacterial strains, selection of the culture medium, temperature, culture condition, pH and the type of carbon sources (Thakur, 2012). The number of journals are available that deals with the optimization of the culture medium components and the culture conditions for extracellular lipase production by bacteria (Mahmoud *et al.,* 2015), but only few examined the probabilities for improving the enzymes production by strain improvement.

The present research has focused on production of lipase enzyme from the bacterial strain, which is isolated from Sambhar Lake, Rajasthan. We have demonstrated the isolation and screening of lipase producing bacteria.

MATERIALS AND METHODS:

Microorganism culture: The bacterial strain was isolated from water samples which was collected from Sambhar Lake near Jaipur and grown in nutrient agar for the further use. It was stored at 4°c in refrigerator.

Submerged culture and screening of lipase: 100 ml of submerged culture was prepared in 250ml of Erlenmeyer flask which contain sodium nitrate, potassium dihydrogen phosphate, magnesium sulphate, potassium chloride and wheat bran was used as a carbon source. 0.5ml of wild strain and mutant strain was inoculated in respective flask to study the enzyme production. Flasks were incubated at 37°c under shaking condition for 2-3 days. After certain incubation time, the clear liquid supernatant was used for qualitative screening for identification of lipase on tributyrin agar (TBA) (Kumar et al., 2012) and quantitative estimation using pNPP (*p*-nitrophenyl palmitate) as a substrate (Winkler and Stuckmann, 1979).

TBA plate assay

Bacterial isolates were tested for lipolytic activity using tributyrin agar (TBA) medium. The composition of TBA medium (g l-1) is as follows: peptone, 5; yeast extract, 3; agar, 15; tributyrin (glycerol tributyrate), 10 and pH was adjusted to 7.5. The isolates were individually point inoculated on TBA plates and incubated at 37 ˚C for 2-3 days. Zone of hydrolysis were detected around bacterial colonies at the end of incubation due to hydrolysis of glycerol tributyrate (Pandey *et al.,* 2015).

p-NPP Analysis:

The stock solution of *p*-nitrophenyl palmitate (*p*NPP; 20 mM) was prepared in isopropanol. The reaction mixture containing 75 µl of *p*NPP stock solution in Tris-HCl buffer (0.05 M, pH-8.0, final volume 3 ml) was preincubated at 70 °C in a water bath shaker (160 rpm) for 10 minutes. Then, 25 µl enzyme (Crude protein extract) was added and reaction was allowed to complete at 35 °C for 30 minutes in a water bath shaker. After 30 minutes, further enzymatic reaction was stopped by the addition of 1 ml of stopping reagent (chilled acetone-ethanol). In control (blank) set the reaction mixture was incubated without the addition of enzyme and 25 µl of enzyme was added after addition of stop reagent (acetone and ethanol, 1:1 v/v) and 410 nm absorbance was recorded (Winkler and Stuckmann, 1979).

RESULTS AND DISCUSSION:

Isolation and primary screening of lipolytic bacterial strains

The brine water is collected from brine and soil sample from Sambhar Lake of Rajasthan were used for the isolation of bacteria on NA plates. From pond water B has maximum bacterial were isolated, whereas least were from A. this indicates that water having highest number of bacteria whereas soil has minimum number of bacteria. Highest bacterial growth in the water of pond A may be due to the presence of brine which changes its color in every season.

Bacteria with different colony characteristics (shape, texture, surface and elevation) appeared in NA plates shown in figure 1. The majority of the isolated bacterial colonies were circular in shape while others were irregular. The texture of the isolated bacteria was circular and irregular. Most of the colonies are creamy in color. Other colonies were white. In some bacteria poor to moderate and in a few heavy growth was observed.

To the present findings, Kumar *et al.* (2012) documented that maximum number of bacteria was isolated from the brine water samples sambhar lake Rajasthan. Gaur et al., (2015) investigated the influence of various culture media (NA and Halophilic agar plate) on colony characters and brine collected study of different bacterial isolated from different color water sample. Gaur and Mohan (2014) studied on moderate Halophilic bacteria on month May and June when brine watercolor change red to orange and yellow to green they are have seen maximum growth Halophilic agar plate. Rohban et al 2008 found different species of bacteria such as halovibrio, halobacillus, salicola, oceanobacillus, gracilibaclus, thalassobacillis and salinicoccus which were isolate haloiphilic bacteria and most DNase and lipase producing genera are halomonas and gracilibacillus. Bharathi et al., 2018 found bacterial isolates of soil samples from petrol spilled soil which corroborated with the findings Mobarak-Qamsari et al., 2011 isolated lipase from oily environment which were growth in NA plates. Similarly, Amoozegar et al., 2008 isolate thermophilic lipase from moderately halophilic bacterium from soil samples. Baati et al., 2010 earlier reported the isolation of strains of *Alteromonadaceae* (10%), *Alteromonadaceae* (10%), *Halomonadaceae* (52.5%), *Idiomarinaceae* (7.5%) and *Vibrionaceae* (15%), from the water samples which Isolation and characterization of moderately halophilic bacteria. The recommended for isolation of lipolytic bacteria such as *Geomicrobium halophilum* and Pseudomonas *aeruginosa (*Echigo et al., 2010; Gaur et al., 2008)

In the current study, primary screening of lipolytic bacterial isolates was performed in TBA medium plates. Lipolytic activity was expressed as formation of zone of hydrolysis (clear zone or halo zone) around bacterial colonies shown in figure 2. Development of clear zones around the colonies indicated the production of extracellular lipase, which hydrolyzed the tributyrin, therefore the opacity of the medium around such colonies could not be retained (Figure 2). Among 10 bacterial isolates tested for extracellular lipase production in TBA plates, 2 isolates (38%) exhibited zone of hydrolysis. 8 isolated bacterial strains did not exhibit any growth in TBA plates, which showed that they were unable to utilize any ingredients of TBA medium. Remaining isolates could grow but did not show the zone of lipolytic activity suggesting that they did not produce any extracellular enzyme for tributyrin degradation. In contrast, Kumar et al., (2012) found only 7% bacterial strains isolated from different areas of Haryana exhibiting zone of hydrolysis. In the present investigations, one of the isolates Bacillus safensis (WA1) demonstrated a greater halo zone than other isolates (Fig.2). Among all the positive isolates, 2 were from pond A, 2 were from pond B, 3 were from soil sample C, 2 from soil sample pond D and remaining 1 from water sample pond D. Lipolytic activities of these isolates were further confirmed by quantitative screening in SmF. Tributyrin is considered as a gold standard for identification and isolation of lipolytic microorganisms. Primary screening of lipolytic bacterial isolates of oily soil samples using TBA medium has been performed by several investigators (singh et al., 2017; Mobarak-Qamsari et al., 2011, Feng et al., 2011, Wadia and jain 2017; Choudhary. 2017; Zheng 2017)

Primary screening of lipase producing filamentous bacterial was performed in Petri plates containing agar medium supplemented with tributyrin or olive oil by Yamada et al. 1962 and the development of halo zones around the bacterial colonies indicated the production of lipase in these plates. Thus, the bacterial can be sorted and selected for the production of extracellular lipase both in the liquid (SmF) and solid medium (SSF) (Cardenas). Ramesh et al., (2014) showed that highest zone of lipolytic activity was observed in screening medium by Bacillus sp with highest lipase activity 8.75 U/ml) at 36 hours.

Quantitative screening of bacterial isolates for extracellular lipase production in SmF

Initial screening of lipase production by ten bacterial isolates was further confirmed by the hydrolysis of natural triacylglycerol in SmF. The production of lipase ranged from 2.41 ± 0.02 U ml-1 min-1 to 82.21 ± 0.90 U ml-1 min-1. Maximum lipase activity (82.21 ± 0.90 U ml-1 min-1) was achieved from the isolate Bacillus safensis WA1 after 48 h of incubation. Therefore, it was selected for further studies. This Bacillus safensis WA1 strain was isolated from pond water B of sambhar lake, Rajasthan. Although, the isolate WA2 demonstrated higher activity in primary screening (Fig. 4.2) but in secondary screening it could not match with the isolate WA2, indicating that confirmative quantitative screening is required to point out the real commercial value of a strain. Several reports are there on lipase assay using different methods (Chaturvedi et al., 2010, Kumar and Kanwar 2012; Toscano et al., 2013, Ramesh et al., 2014)

The majority of the techniques are intended to detect the product of hydrolytic reactions of lipase catalysis. These methods of lipase assay are as follows:

(i) titrimetry; (ii) colorimetric (spectrophotometer) (Shukla and Desai, 2016). Most of the investigators have used spectrophotometric assay using *p*-NPP as a substrate (Joshi *et al.,* 2006; Pera *et al.,* 2006; Karanam and Medicherla, 2008; Hosseinpour *et al.,* 2011; Xia *et al.,* 2011; Jayaprakash and Ebenezer, 2012; Niaz *et al.,* 2013; Shukla and Desai, 2016) due to its easy handling and short reaction time, therefore the same has been followed for the present study.

Two fermentation processes viz. SSF and SmF have been used by various workers (Pera *et al.*, 2006; Bindiya and Ramana, 2012; Thota *et al.*, 2012; Sundar and Kumaresapillai, 2013) but we utilized SmF because all process parameters can be simply regulated to optimize the enzyme production, biomass can be easily determined by centrifugation or filtration, area requirement is very less, high amount of enzyme can be extracted, and recovery of enzyme is very easier in this fermentation method shown in figure 3. The majority (more than 85%) of the industrial enzymes are produced by SmF (verma and Sharma 2014)). Similar to the present study, Rajesh Kumar et al., (2013) reported that in qualitative screening on TBA plates, diameter of halo zones for Enterococcus faecium MTCC 5659. Were observed as 6.0, 5.0 and 1.0 mm, it indicates that primary screening must be followed by secondary screening in order to identify the isolates of real industrial importance.

Besides, in most of the studies species of *bacillus* have been found the most potent lipase producers. Alabras et al., *(*2017) reported screening of lipolytic fungi from diesel oil contaminated soil sample using SmF and among the tested bacteria, highest lipase activity was achieved by the isolate of G.stearothermophilus and the maximum lipase enzyme production of (71 U/ml).

Extracellular lipase producer in SmF when compared with other isolates. The other bacterial species with remarkable activity are Bacillus subtilis (Mazhar et al., 2017), Pseudomonas aeruginosa (Izrael-Zivkovic et al., 2009), *Pseudomonas fluorescence* (Fernandez et al., 1990) and Streptomyces Sp (Yuan et al., 2017). Sarkar and Laha (2013), Verma and Sharma (2014) and Veerapagu *et al.* (2014) are some other examples cited in literature that used SmF in contrast to SSF as taken by us.

There are some studies in which both SSF and SmF fermentation processes were compared for the lipase production by bacteria (Colla *et al.,* 2015) and found SmF as better method. Colla *et al.* (2015) recently documented that lipase produced by SmF was found more temperature tolerant and pH stable than lipase obtained by SSF.

Figure 1. A and B showing pure culture of bacterial isolates.

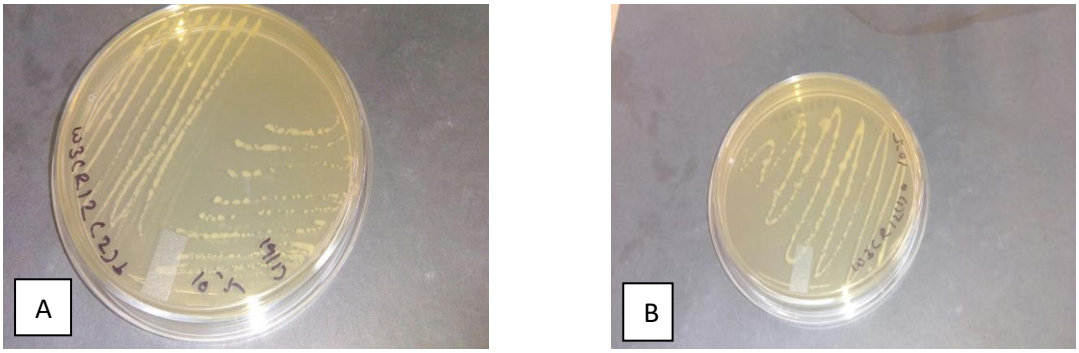
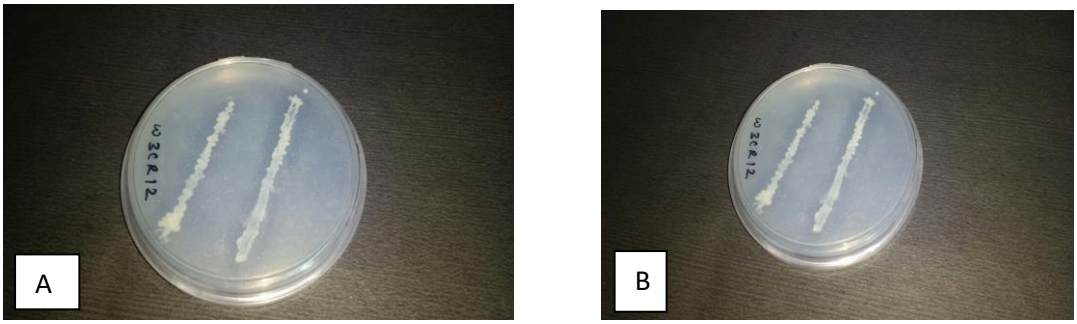


Figure 2. A and B Formation of zone of hydrolysis on Tributyrin agar plates by bacteria.



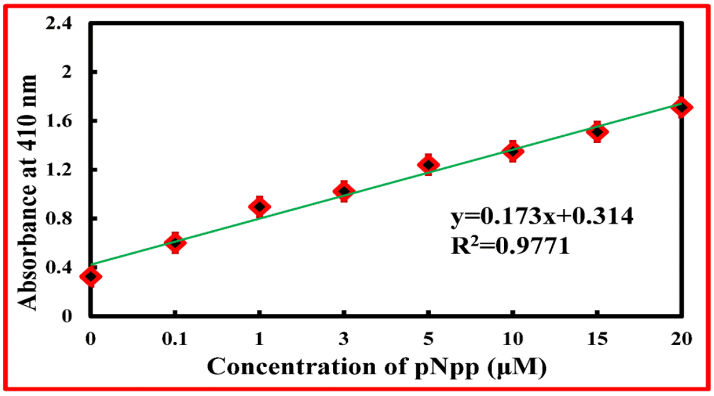


Figure 3. graph showing increase in lipase production after p-npp analysis

CONCLUSION:

In the current experimental design, isolated halophilic bacteria will be used for production lipases. The above proposed work can be useful for production of halophilic lipases from potential, low cost and highly available substrates. Bacillus safensis is used for the screening of lipase enzyme.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest in the publication.

ETHICAL STATEMENT:

Not applicable

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