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ISOLATION OF A NOVEL HALOTOLERANT LIPASE PRODUCING STRAIN OF *PSEUDOMONAS STUTZERI*

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ABSTRACT

Soil sample from commercial kitchen was examined for the presence of lipolytic bacteria. Lipolytic activity was screened by using Rhodamine B and tween 80 as lipid substrates. Twenty strains were isolated from the sample, of which twelve strains (SN-1, SN-2, SN-3, SN-7, SN-9, SN-10, SN-12, SN-14, SN-15, SN-17, SN-19, & SN-20) showed lipolytic potential. SN-12 and SN-20 showed low lipolytic potential on two different screening media. Submerged fermentation was carried out to collect cell free filtrate as source of crude lipase and to quantitatively monitor its lipolytic activity. The strain SN-3 showed highest lipase activity i.e. 14.91 $\pm$ 0.09 U/mL and therefore it was selected as a potential lipase producing bacteria to carry out further studies. The isolate SN-3 showed large, wrinkled and opaque colonies. Microscopic observation revealed the isolate as gram negative, rod shaped, non-spore former, and non-pigmented bacteria. According to biochemical analysis, the isolated strain is a motile, hemolytic bacterium. It is able to hydrolyze starch and fix nitrogen. The isolate is halotolerant and sustain up to 7% of NaCl and can grow in pH 4.5. In the light of these findings, the strain SN-3 has been characterized as *Pseudomonas stutzeri.* This is a potential lipolytic bacterial strain and can be further identified through molecular technologies.

Key words: *Pseudomonas stutzeri*, Lipolytic activity, Tween 80, Rhodamine B

INTRODUCTION

Lipases (EC 3.1.1.3) are the enzymes that catalyze the breakdown of triglycerides into free fatty acids and glycerol (Muthumari *et al*., 2016). Lipases occur abundantly in nature from prokaryotes to eukaryotes as the lipid hydrolysis is the vital need of all living creatures, and has been emerged as key enzymes in rapidly growing field of biotechnology. They are being exploited as versatile and inexpensive catalysts to degrade lipids to cater the needs of white and green biotechnology. Researchers has focused on interfacial dependence; and chemo-,regio-, and enantio-specific properties of lipases for their application in food, textile, detergent, leather, oleo-chemical, pharmaceutical, cosmetic, and paper industries. Most substantial areas of lipases scope are food, detergent, and pharmaceutics (Navvabi *et al*., 2018). Lipases, being able to catalyze transesterification reactions, can be utilized for the formation of value added products such as biodiesel. Lipolytic activity is a potential attribute in bioremediation procedure of oil spill and waste management from oil processing factories. These implementations of lipases made them chief elements in green technology and in advancement of sustainable development (Liu and Cokare, 2017). The industrial applications of lipases need a continuous and economical supply which could only be fulfilled by microbial lipases. Microorganisms can easily be maintained and directed to produce lipase by using low cost materials. Additionally high yield and ease of genetic manipulation in microorganisms make them the most favorable resources for industrial lipases (Berhanu and Amare, 2012). Microbial lipases are produced by bacteria, fungi, yeasts and actinomyces. Lipases from all of these microorganisms are varying in nature by virtue of their chemical and physical properties (Ugo *et al*., 2017). Among all microorganisms, bacterial lipases are of great interests. Bacterial lipases are usually extracellular and can be produced by submerged fermentation (Gupta *et al*., 2004). Increasing demand of bacterial lipases is due to their stability at high temperatures and tolerance to wide pH ranges. Bacteria commercially exploited as lipase sources are *Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Chromobacterium* and *Pseudomonas* (Beisson *et al*., 2004). *Pseudomonas* lipase is the most extensively used bacterial lipase for biotechnological applications because of its temperature stability, high enantio-selectivity and activity under broader range of pH (Ramani *et al*., 2010). Nonetheless, there is also a constant urge to explore new and novel lipases to further expand their industrial usage. Therefore, the present study was designed to isolate new bacterial resource of lipase and to determine its lipolytic potential for continuous degradation of lipids.

MATERIALS AND METHODS

Sample Collection

Greasy soil was collected from a commercial kitchen (Gurumandir, Karachi) at the depth of 6-10 cm using a sterilized spatula. The sample was transported aseptically to the laboratory and processed immediately.

Enrichment of the sample and Isolation of Lipolytic Bacteria

Lipolytic bacterial strain was isolated by enrichment culture technique with slight modifications (Kumar *et al*., 2012). The soil sample was mixed (1:50 w/v ratio) with enriched nutrient medium (Yeast Extract $2gL^{-1}$, Peptone $5gL^{-1}$, Sodium chloride $5gL^{-1}$, beef extract $1gL^{-1}$, olive oil 10 mLL^{-1} prepared in deionized water with proper mixing) and agitated well at 37^0 C for 30 minutes on a rotary shaker at 120 rpm. Thereafter, serial dilutions were prepared in normal saline and 200µL from last dilution was poured on nutrient agar plates for quantification of bacteria. The plates were incubated at 37^0 C for 72 hours. Emerged colonies were further transferred to screening media for lipase production.

Screening of Bacterial Isolates for Lipolytic Activity

Qualitative Screening

Lipolytic ability of bacterial isolates was screened by using two screening media; Tween 80 agar (Media 1 or M1) and Rhodamine B agar (Media 2 or M2). Tween 80 agar was prepared with nutrient broth ($1.30gL^{-1}$), Olive oil (10.0 mLL^{-1}), Tween 80 (1.0 mLL^{-1}), and agar (20.0 gL^{-1}) in 1L of deionized water and the pH was adjusted to 7.0. Whereas, composition of Rhodamine B agar was similar with Tween 80 agar except with the addition of Rhodamine B solution ($1.0gL^{-1}$). Rhodamine B was filtered through nitrocellulose and transferred to the sterile media. Colonies from nutrient agar were first grown on tween 80 agar and those which showed larger zone of lipolysis were further screened on Rhodamine B agar.

Quantitative Screening

For quantitative assessment of lipolytic potential, isolated colonies were further subjected to fermentation in nutrient broth supplemented with 1% olive oil. This submerged fermentation process was carried out in 25mL Erlenmeyer flask at 37^0 C for 48 hours. Cells were centrifuged at 10,000 rpm at 0^0 C for 10 minutes. Cell free filtrate was assayed for lipase activity.

Enzyme Assay

Lipase activity was measured titrimetrically by using buffered substrate (Kumar *et al*., 2017). Buffered substrate was prepared by homogenizing olive oil in gum acacia. To assessed lipolytic potential 1mL enzyme was incubated with 1mL of buffered substrate for 15 minutes. Reaction was ceased with 1mL chilled acetone: ether (1:1) and then the liberated fatty acids were estimated by titrating the mixture with 0.1N NaOH. Lipolytic activity was calculated by the formula given below;

$$\text{Lipase activity} = \frac{\text{Vol. of NaOH consumed (mL)} \times \text{Molarity of NaOH}}{\text{Vol. of Lipase (mL)}} \times \text{Reaction Time (min)}$$

One unit of lipase was defined as the amount of enzyme that liberates 1µM free fatty acids in one minute under assay conditions.

Culture Purification

The strain with maximum lipase activity was selected and purified to obtain isolated colonies. The culture was maintained on nutrient agar slants and subcultured fortnightly. The strain was further identified on morphological, and biochemical bases.

Culture Characteristics

Colonial characteristics were observed such as color, shape, margin, elevation and surface of colonies on the nutrient agar medium.

Morphological Identification

Gram staining and endospore staining of the selected strain was performed to observe shape, and arrangement of cells and the observations were recorded under phase contrast light microscope (AmScope M150C)

Biochemical Tests

Biochemical tests were carried out by following Bergey's Manual of systematic Bacteriology (Holt *et al*., 1994). Bacterial cultures in late-logarithmic phase (in Luria broth and on Luria agar) were used to perform all the

tests. Production of catalase, oxidase and urease was tested. Nitrate reduction and tryptophan, starch utilization tests were performed. Organism was also evaluated for its aptitude of growth at pH 4.5 and in presence of 7% sodium chloride. Glucose, Sucrose and Lactose fermentation capability tests were also performed. Growth on blood agar, MacConkey agar, motility test, methyl red (MR) test, and Voges Proskauer (VP) tests were also conducted.

RESULTS AND DISCUSSION

In present study, twenty bacterial strains were isolated from kitchen grease as shown in Table 1. All the isolates were screened qualitatively for lipase activity on tween 80 agar medium (M1) and on Rhodamine B agar medium (M2). Twelve strains exhibited lipolytic potential as they produce large zone of hydrolysis in the lipid containing media. Ten isolates (SN-1, SN-2, SN-3, SN-7, SN-9, SN-10, SN-14, SN-15, SN-17, SN-19) exhibited zones on both the screening media i.e. M1 and M2. Whereas, two bacterial cultures (SN-12 & SN-20) showed lipolytic activity on M2 only. This was further resolved after the quantitative determination of lipolytic activity. The strains were grown on lipase induction medium for 48 hours and the quantitative lipolytic activity was measured titrimetrically on cell free filtrate.

Table 1. Screening of bacterial isolates for lipolytic potential on M1 (tween 80 agar) and M2 (rhodamine B agar).

Isolated Bacterial Strains	Lipase Screening on M1	Lipase Screening on M2	Lipase Activity U/mL
SN-1	+	+	7.29 ± 0.23
SN-2	+	+	10.33 ± 0.24
SN-3	+	+	14.91 ± 0.09
SN-4	–	–	–
SN-5	–	–	–
SN-6	–	–	–
SN-7	+	+	5.55 ± 0.31
SN-8	–	–	–
SN-9	+	+	3.39 ± 0.55
SN-10	+	+	4.76 ± 0.19
SN-11	–	–	–
SN-12	–	+	2.33 ± 0.18
SN-13	–	–	–
SN-14	+	+	9.29 ± 0.13
SN-15	+	+	8.69 ± 0.33
SN-16	–	–	–
SN-17	+	+	9.83 ± 0.24
SN-18	–	–	–
SN-19	+	+	6.56 ± 0.32
SN-20	–	+	5.20 ± 0.18

Lipolytic activity of isolated bacterial strains; + denotes presence of lipolytic activity whereas – denotes absence . Results are expressed as Mean ± SD and n=3)

The results in Table 1 present that some strains has low lipolytic potential (2.33-4.76 U/ml; SN-9, SN-10 & SN-11), some showed moderate lipase production (5.20-7.29 U/ml; SN-12, SN-7, SN-8, SN-1) while few exhibited high lipase activity (8.69-14.91 U/ml; SN-2, SN-3, SN-4, SN-5, SN-6). The two strains (SN-12 & SN-20) showed low lipase activity (2.33 + 0.18 & 5.20 + 0.18) which could be linked to their negative activity on tween 80 agar (M1). The strain SN-3 was selected as the potential lipase producer as it exhibited highest lipase activity 14.91± 0.09 U/mL. It was evaluated for its colonial features, identified morphologically and biochemically. Colonial features were recorded and summarized in Table 2. It showed that the organism give large, wrinkled and opaque colonies

(Fig. 1). According to the morphological findings isolate SN-3 is a gram negative non spore former, rod shaped bacteria. The gram negative nature provides the clue that it is not a *Bacillus* (Fritze, 2004). The negative gram reaction (Fig. 2), absence of spores, motile nature and aerobic mode of respiration suggested that it could belong to the genus *Pseudomonas* (Palleroni and Norberto, 2008). The organism does not produce any pigment thus belongs to non-pigmented group of the genus *Pseudomonas* (Spiers *et al*., 2000). It exhibited diffused growth which reflects presence of flagella. It showed β-hemolysis on blood agar whereas no growth on Macconkey agar (Fig. 2). It can hydrolyze starch and have the ability to produce catalase and oxidase. It can also utilize citrate and glucose (Table **2**), this chemoorganotrophic mode of nutrition is the characteristic of genus *Pseudomonas* (Lysenko, 1961).

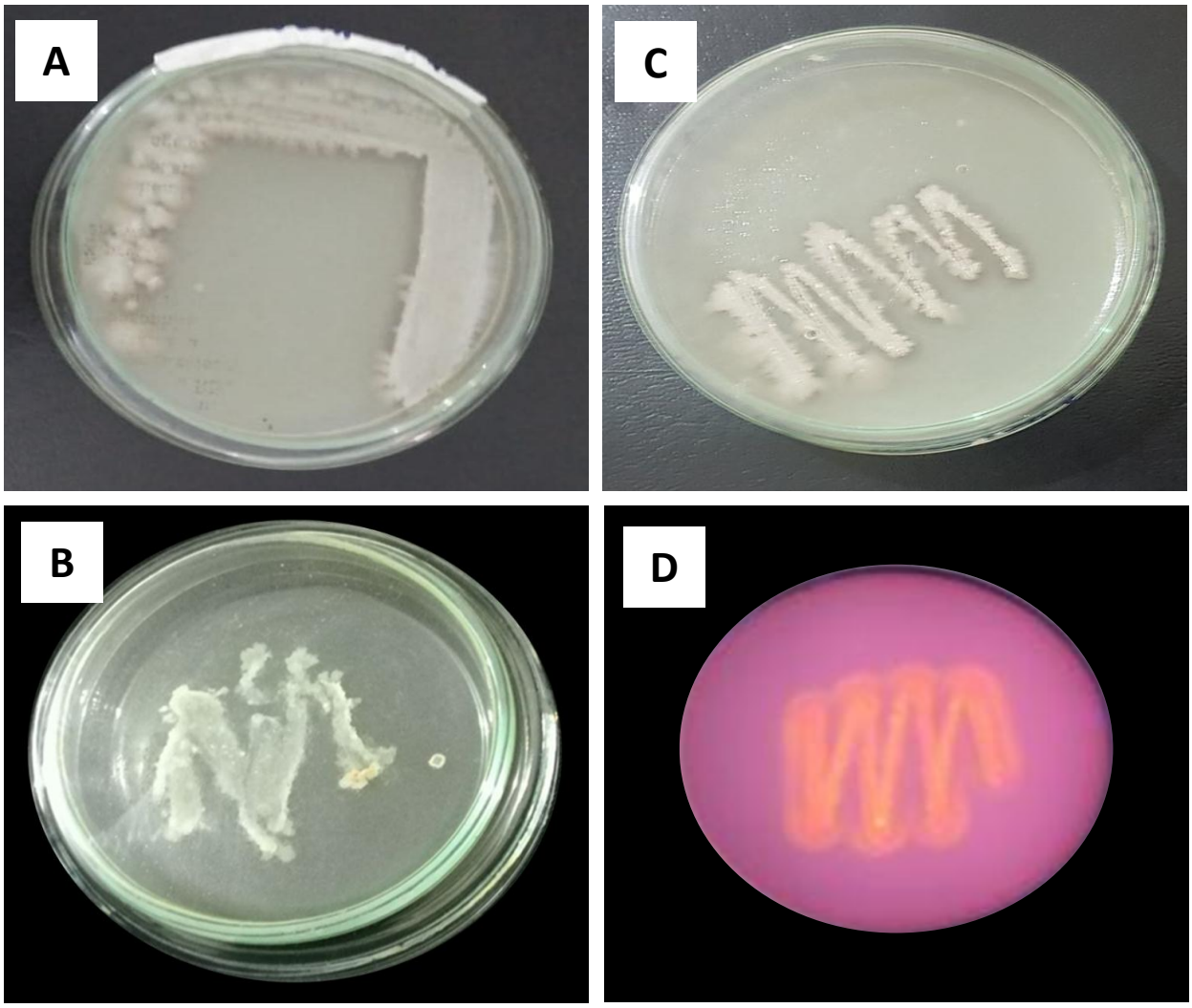

Fig. 1. Pure Culture of isolate SN-3 and growth on nutrient agar (A,B); on tween 80 agar medium or M1 (C); Rhodamine b agar medium or M2 (D).

The selected strain is a denitrifying pseudomonad as it has the ability to fix nitrogen. The denitrifying nature, utilization of starch and glucose as carbon sources, typical wrinkled colony appearance suggested the isolate as *Pseudomonas stutzeri* group (Palleroni *et al*., 1970; Rainey *et al*., 1994) Additionally it has been found that the isolate is not able to reproduce below pH 4.5; a characteristic of *Pseudomonas stutzeri*. The strain SN-3 possessed halotolerant nature and showed growth in the presence of up to 7% NaCl; these observations are in accordance with the reported literature on *Pseudomonas stutzeri* group (Lalucat *et al*., 2006). On the basis of these colonial, morphological and biochemical observations, the selected strain has been referred as *Pseudomonas stutzeri*. The strain confirmation requires further molecular tests.

Table 2. Colonial, Morphological and Biochemical profile of isolate SN-3.

Characteristics	**Observations**
A) Colonial Features	
Shape	Coral, Filamentous
Margin	Lobate
Elevation	Flat
Size	Large
Texture	Rugose (Wrinkeld)
Appearance	Dull
Pigmentation	Non pigmented, tan
Optical property	Opaque
B) Morphology	
Gram Staining	Gram -ve rods
Spore formation	Negative
Motility	Positive
C) Biochemical Tests	
Flourescent Pigment	Absent
Ctalase	Positive
Oxidase	Positive
Urease	Positive
Nitrate Reduction	Positive
Indole Test	Negative
Citrate Utilization	Positive
Starch Hydrolysis	Positive
Methyl Red Test	Positive
Voges Proskauer Test	Negative
H_2S Production	Positive
Growth on blood agar	β-hemolysis
Fermentation Reactions	
Glucose	Acid
Sucrose	Negative
Lactose	Negative
Salt Tolerance	
Growth in 6.5% NaCl	Positive
Growth in 7 % NaCl	Positive
Acidic pH Tolerance	
Growth in 4.5 pH	Positive
Growth in 4.o pH	Negative

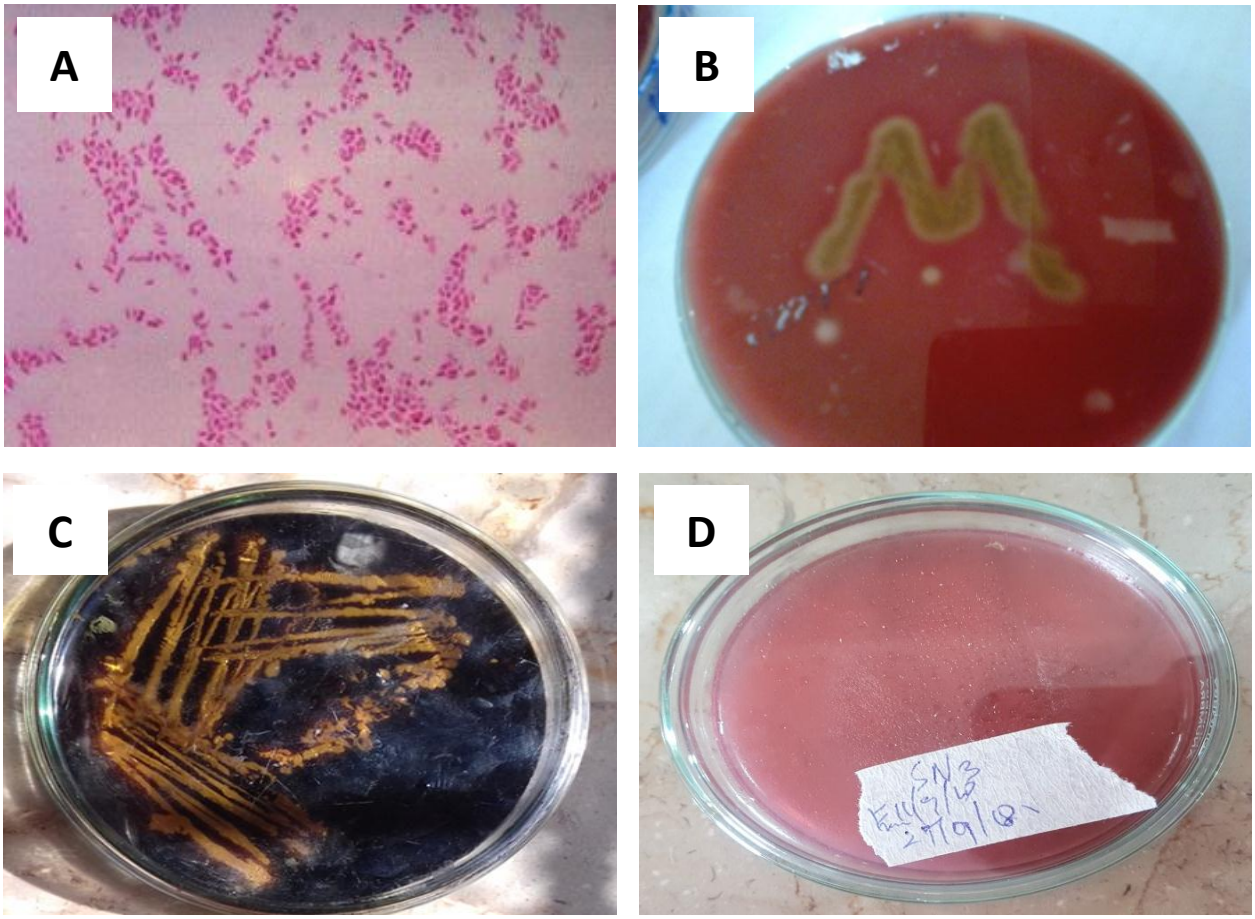

Fig. 2. Gram staining of the isolate SN-3 (A) showing gram negative rods under 10,000 magnification of light microscope ; Strain SN-3 exhibit β- hemolysis on blood agar(B); starch utilization reaction (C); No growth on MacConkey agar (D).

CONCLUSION

In the present study, a novel lipolytic strain has been isolated; identified as gram negative, non-spore forming bacteria and characterized as *Pseudomonas stutzeri* SN-3. This isolated strain is a salt tolerant, acidic bacterium and showed encouraging lipolytic potential. This organism would be a promising candidate for bioremediation purpose in local areas as it is isolated from the indigenous source.

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