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Extracellular Lipolytic Enzyme Production by a Novel Extremely Halophilic Bacterium

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Abstract:

Halophilic bacteria live in extreme salt conditions and can produce enzymes which are highly stable. A study was conducted to isolate lipolytic enzyme producing halophilic bacteria from salted fish. As lipolytic enzymes from halophiles can tolerate extreme salt conditions, it is highly stable and can tolerate harsh industrial conditions and thus has got potential biotechnological applications. In the present work 17 halophilic isolates were obtained from salted fish samples on Seghals Gibbons Complex (SGC) medium. The lipolytic activity of the strains was tested by Tween hydrolysis on SGC medium containing 1% Tween 80 or Tween 40. A strain SH1 which showed the highest relative enzyme activity was identified by 16 S rDNA sequencing as a novel strain belonging to the class *Gamma proteobacteria*. The strain was found to grow in a salt range, 2 to 5 M and thus confirmed as an extreme halophile. It showed lipolytic activity at salt concentration in the range 2 to 4 M and pH range 6 to 11. Thus it forms a promising strain for the production of halophilic lipolytic enzymes and thus can have industrial applications.

Keywords: Extreme halophiles, Salted fish, Lipolytic enzymes, Tween 80

1.0 Introduction:

Halophiles are extremophiles that grow in environments with very high concentrations of salt. Microorganisms able to grow in presence of salt are found in all three domains of life: Archea, Bacteria, and Eukarya (Oren, 2002b; Oren 2006). Halophiles can be divided into slight halophiles that grow optimally in 3% (w/v) total salt, moderate halophiles with optimal growth at 3 - 15% (w/v) salt and extreme halophiles that grow at 15 – 30% (w/v) salt (Ventosa *et al.*, 1998). Among the different halophiles extreme halophiles are of great importance as they require very high salt concentration for growth. They are found in solar saltern, on the surface of dried-salted fish, fish sauce fermentation tanks, rock salt, in dehydrated kitchen salt and even in purified salt (Gutierrez and Gonzalez, 1972; Larzen, 1984).

Specific adaptation to the halophilic life exhibited by these groups of microorganisms makes them interesting source of enzymes with industrial potential (Ventosa *et al.*, 1998). Among the various enzymes of halophilic origin, lipolytic enzymes are those with potential applications in a variety of biotechnological fields (Ghasemi *et al*., 2011).

Lipolytic enzymes of bacterial origin mainly include carboxyl esterases, true lipases and various types of phospholipases (Arpigny and Jaeger, 1999). Though the major function of lipases is hydrolysis of triglyserides, it can also catalyse esterification, interesterification and transesterification reactions. Thus lipases are versatile enzymes that have potential applications in food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries (Houde *et al.,*2004). Many microbial lipolytic enzymes are available now and some of them are already being used in various industries. Since industrial processes are commonly carried out under harsh conditions, it would be of great importance to identify enzymes which retain their optimal activity at extremes of pH, temperature and different concentrations of salts. Since halophilic lipolytic enzymes are adapted to extreme environments, they are unusually stable and therefore they could serve as suitable candidates for industrial processes. The current work was designed to identify halophilic microorganisms with potential to produce lipolytic enzymes.

2.0 Materials and Methods:

2.1 Isolation of Halophilic Bacteria and Screening for Lipolytic Enzyme Production:

Salted fish samples were collected from local fish markets of Kottayam, Kerala. The samples used were Indian mackerel (*Rostrelliger kanagurata*), Shark (*Rhincodon typus*), Sole fish (*Cynoglossus semifasciatus*) and Butter fish (*Rachycentron canadus*). Small pieces of samples of about 1gm were inoculated into 50 ml of Seghals Gibbons Complex (SGC) medium, in 250ml conical flask, containing bacto vitamin-free casamino acids — 0.75% , bacto-yeast extract- 1.00%, sodium citrate- 0.3%, potassium chloride- 0.2%, $MgSO_4.7H_2O$- 2%, NaCl- 20%, and $FeCl_2.H_2O$- 2.30 mg (Sehgal and Gibbons, 1960). The flasks were incubated on rotary shaker at 200 rpm at 37˚C until turbidity appeared. The turbid cultures were streaked on SGC medium containing 1.8% (w/v) agar and incubated at 37˚C for seven days. Diverse colonies selected on basis of colony characteristics were screened for production of lipolytic enzymes on SGC agar medium containing 1% Tween 80 or Tween 40. The bacterial isolates were spotted onto the agar plates and were incubated at 37˚C for 10 days. A positive reaction for the lipolytic enzyme was indicated by the formation of opaque zone around the colony. The diameter of the colony and zone of opaqueness around colony were measured. Relative enzyme activity (REA) was calculated using the formula:

REA = Diameter of the opaque zone/ Diameter of colony (Kanlayakrit and Boonpan, 2007).

2.2 Activity on different Tweens:

To find the activity of the lipolytic enzyme towards different tweens, the isolates which showed positive lipolytic enzyme activity were spotted on SGC agar medium supplemented with different Tweens like Tween 20, 40, 60 and 80 (polyoxyethylenesorbitan monolaurate, monopalmitate, monostearate, and monooleate, respectively) and incubated at 37˚C for 10 days. The presence of lipolytic enzyme was demonstrated by the formation of conspicuous halos. This is due to the formation of precipitates of calcium laurate, palmitate, stearate, or oleate around the zones of bacterial growth (Gutierrez and Gonzalez, 1972). The isolate showing maximum REA was selected for further studies.

2.2 Optimization of Conditions for Production of Lipolytic Enzyme:

Optimization of salt concentration

For optimization of salt concentration for lipolytic enzyme production, the SH1 isolate was grown on SGC agar having different salt concentrations and supplemented with 1% Tween 40 for 10 days at 37˚C.

Optimization of pH

For optimization of pH for lipolytic enzyme production, the SH1 isolate was grown on SGC agar having different pH ranging from 6 to 11 and supplemented with 1% Tween 40 for 10 days at 37˚C.

2.4 Identification of Halophilic Isolate:

Morphological and physiological characterization of the strain

The isolate was gram stained according to Dussault (1955) and also its motility test was performed. Biochemical tests were performed for the physiological characterization of the strain.

Salt tolerance of the halophilic isolate

The salt tolerance of the isolate was studied by growing it on SGC agar media supplemented with salt at concentration ranging from 1M to 5M. This was then incubated at 37˚C for 10 days.

2.5 Molecular Identification of Halophilic Isolate:

The molecular identification of the isolate was carried out by 16S rDNA sequence based method. For this, the genomic DNA was isolated from the cultured cells and the quality was analyzed by electrophoresis using 0.8% agarose gel. Isolated DNA was used as template for PCR. PCR amplification of 16S rDNA of the isolate was carried out using primers specific to bacterial 16S rDNA sequence. Five microliters of the extracted DNA was amplified with primers 27F 5'-AgA gTT TgA TCM Tgg CTC-3’ and 1525R (5'-AAg gAg gTg WTC CAR CC-3’). All reactions were carried out in reaction mixture containing 20 pmol of each primer, 200 mM of each deoxyribonucleoside triphosphate, 5 µl of 10X PCR buffer (100 mM Tris-HCl, 15 mM MgCl2, 500 mM KCl; pH 8.3), and 0.5 U of *Taq* DNA polymerase, the volume was increased to 50 µl with sterile MilliQ water. PCR was performed with the following thermocycling program: 3 minutes denaturation at 94°C, followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 58°C,

2 minutes extension at 72°C, and a final extension step of 7 minutes at 72°C. Ten microliters of PCR products was visualized by electrophoresis in 1.5% (wt/vol) agarose gel incorporated with 2µl of ethidium bromide (10 mg/ml) for confirmation of amplification. Concentration of the amplicon was checked in a Nanodrop ND 2000. The amplicon was purified using Nucleospin purification column. Sequencing of the amplicon was done with forward and reverse primers in ABI 3730xl cycle sequencer. The sequence data was further subjected to BLAST analysis. This was also used for phylogenetic analysis by neighbor joining method using MEGA 5.

3.0 Results and Discussion:

3.1 Isolation of Halophilic Bacteria and Screening for Lipolytic Enzyme Production:

Based on colony characteristics 17 isolates of halophilic bacteria were obtained from four different fish samples. To screen for lipolytic enzyme production, the bacterial isolates were spotted on SGC agar medium containing 1% Tween 80 or Tween 40 and incubated at 37˚C for 10 days. Formation of opaque zones around the colonies indicated positive result. Tween 80 is considered as an intermediate substrate for lipases and esterases (Plou *et al.*, 1999). Among the different bacterial isolates screened for the lipolytic activity, five strains showed positive result. The isolates were M1, M2 and M3 isolated from Indian mackerel and SH1 and SH2 isolated from shark.

3.2 Activity on different Tweens:

The bacterial isolate SHI seemed to hydrolyze all tweens and showed maximum REA (Table 1). This shows that the strain can have lipase or esterase activity. So the strain SH1 was selected for further studies.

Table 1: Hydrolysis of different Tweens by the strain SH1

Tweens used in the media	REA
Tween 80	2.1
Tween 40	5.5
Tween 60	3.5
Tween 20	2.57

3.3 Optimization of Conditions for Production of Lipolytic Enzyme:

Optimization of Salt Concentration:

For optimization of salt concentration for lipolytic enzyme production, the SH1 isolate was grown on SGC agar having different salt concentrations and supplemented with 1% Tween 40 for 10 days at 37˚C. The SH1 isolate showed lipolytic enzyme production at various concentration of salt used in the study ranging from 2M to 4M (Fig 1). This shows that the enzyme can tolerate high salt concentration and so can have potential industrial applications.

Optimisation of pH

For optimization of pH for lipolytic enzyme production, the SH1 isolate was grown on SGC agar having different pH ranging from 6 to 11 and supplemented with 1% Tween 40 for 10 days at 37˚C.The SH1 isolate showed lipolytic enzyme activity at a pH ranging from 6 to 11 (Fig 2). The production of lipolytic enzyme at high pH shows that the enzyme can tolerate high alkaline conditions. Several of the halophilic enzymes display polyextremophilic properties. These enzymes not only remain active and stable in high salt environment but are often also thermotolerant and alkaliphilic (Moreno *et al.*, 2009). These properties make halophilic enzymes attractive for various biotechnological applications as they would be able to catalyze reactions under harsh conditions typical of many industrial processes.

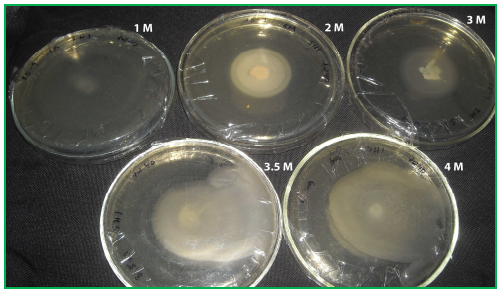

Fig 1 Production of lipolytic enzyme by strain SH1 at different salt concentrations

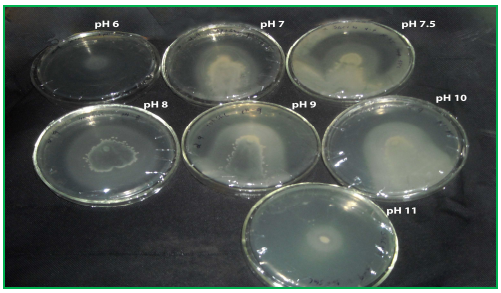

Fig 2 Production of lipolytic enzyme by strain SH1 at different pH

Table 2: Morphological and physiological characteristics of the strain

Name of the test	Observation
Gram's stain	Negative
Cell shape	Rods
Colour	Beige
Motility	Motile
Catalase	Positive
Oxidase	Positive
Starch hydrolysis	Negative
Tween hydrolysis	Positive
Casein hydrolysis	Negative
Carbohydrate fermentation	
Glucose	Negative
Galactose	Negative
Lactose	Negative
Growth in : 0 M NaCl	_
1 M NaCl	_
2M NaCl	+
3M NaCl	+
4M NaCl	+
5M NaCl	+

3.4 Identification of Halophilic Isolate Morphological and Physiological Characterization of the Strain

The isolate was gram stained and also its motility test was done. The results showed that the SH1 isolates is gram negative and motile. The biochemical tests done and the results are shown in Table 2.

Salt tolerance of the halophilic isolate:

The salt tolerance of the isolate was studied by growing it on SGC agar media supplemented with salt at concentration ranging from 1M to 5M. This was then incubated at 37˚C for 10 days. The result showed that the isolate SH1 could grow at salt concentration ranging from 2M to 5M (Fig 3). As the isolate could grow only at salt concentration above 2M and could grow up to 5M salt concentration, it was found to be an extreme halophile.

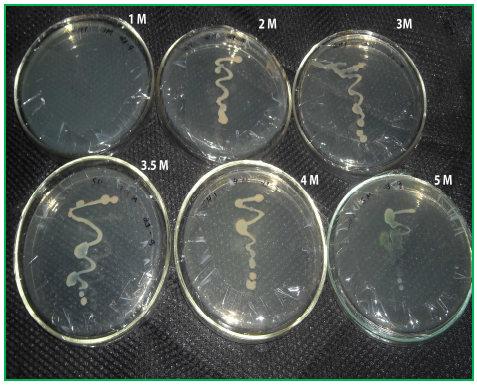

Fig. 3: Growth of SH1 at different salt concentrations

3.5 Molecular Identification of Halophilic Isolate:

Molecular identification of the halophilic bacteria was carried out by 16 S rDNA sequencing. The sequence obtained was submitted to NCBI under the accession number **HQ837638.1**. The sequence was then subjected to BLAST analysis**.** The BALST result showed maximum identity of 98% towards various genera of bacteria like the *Salicola, Pseudomonas and Halovibrio*. The strain isolated in the study was identified up to the class level as the one coming under the class of *Gamma proteobacteria.* This highlights the novelty of the isolated strain and its significance. This was further supported by the phylogenetic analysis (Fig. 4), where the *Gammaproteobacterium* SH1 identified in the study forms a cluster different from other bacteria. Though extremely halophilic halobacteria belong to the order *Halobacteriales* of the domain *Archea*, there are a few halophilic *Bacteria* that resemble the *Archaea* in their salt requirement and tolerance: *Halorhodospira, Halovibrio, Pseudomonas, Halospina, Salicola (Gammaproteobacteria), Actinopolyspora halophila (Actinobacteria) and Salinibacter (Bacteroidetes), Halanaerobiales,* among others (Anton *et al.,* 2002; Oren, 2002a; Kharroub *et al.,* 2006; Maturrano *et a.l*, 2006; Sorokin and Tindall, 2006). Thus SH1 was identified as an extremely halophilic *Bacteria* belonging to the class *Gamma proteobacteria.*

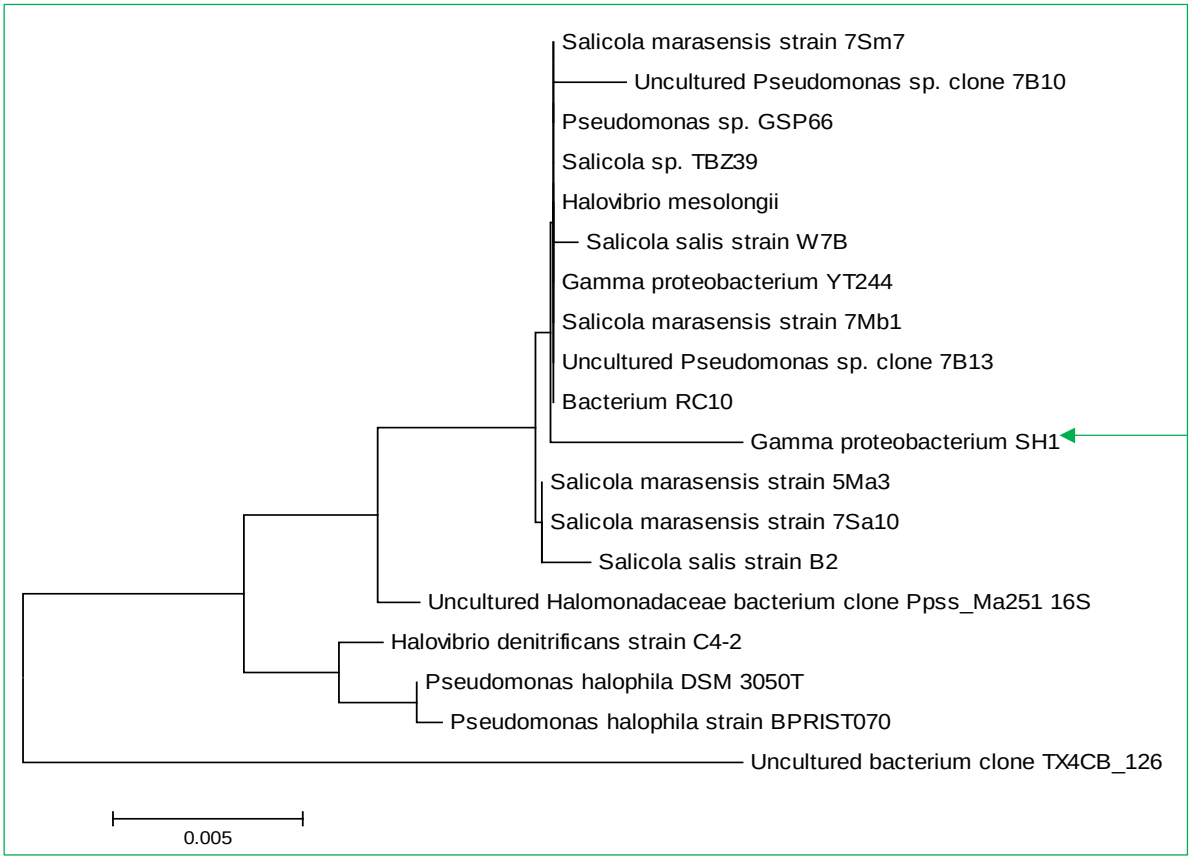

Fig 4. Phylogeneic analysis of *Gammaproteobacterium* SH1 identified in the study along with other sequences from database. The analysis was carried out using neighbour joning method of MEGA.

4.0 Conclusion:

The isolate SH1 was found to be interesting because of its extreme halophilic nature, activity towards different tweens and its growth at wide range of pH. The molecular identification showed the novelty of the isolated strain. Thus the current study identified a novel extremely halophilic bacterium from salted fish. Its lipolytic activity and enzyme productivity at high salt concentration and pH confirms that much industrial potential can be expected from the isolate.

Future scope:

The lipolytic enzyme is expected to be highly stable at extreme conditions and so can be exploited further for applications in industry.

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