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Isolation and Characterization of Hydrolytic Halophiles from Sea Water

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Abstract: ***Halophilic bacteria were more abundant in the sea water. These groups of organisms have stability under extreme conditions like high temperature, extreme pH, High concentration of salt. Halophilic bacterial extracellular enzymes are important with respect to their industrial applications mainly in fermented food, textile, pharmaceutical, leather industries, nutrient recycling and biodegradation process. In this novel it shows haloenzymes like Amylase, lipase, protease helpful in industrial applications.***

Keywords: Halophiles, Haloenzymes, Starch agar, Skim milk agar, Tributyrin nutrient agar, *Salinicoccusroseus* strain.

1. Introduction

Halophytes include prokaryotic and eukaryotic salt-loving microorganisms of saline environments which are able to balance the high osmotic pressure. Halophytic bacteria are found in marine, great salt lakes, natural or artificial slatterns, Salina etc. According to salt requirements, halophilic microorganisms are classified as (i) extreme halophiles which require 3.5-5 M NaCl; (ii) moderately halophiles which grow at 0.5-3.5 M NaCl; (iii) weakly halophilies: need 0.3-0.5 M of NaCl and (iv) halo-tolerant which not need salt for growth but their growth is supported at high levels of salt in its medium. Its capacity to grow under high salt concentration is due to adaptation mechanisms such as accumulations of compatible solutes in the cytoplasm. These substances act as biological structures stabilizing and allowing adaption in front of cold, heat, desiccation, etc. Principally, compatible solutes form a water layer generating a cell hydration in presence of high salt in the medium.

Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. These groups of organisms have stability under extreme conditions like high temperature, extreme pH, High concentration of salt etc. and enzymes shown activity at extreme condition is called extremozymes. Halophilic bacterial extracellular enzymes are important with respect to their industrial applications mainly in fermented food, textile, pharmaceutical, leather industries, nutrient recycling and biodegradation process. The major characteristics that they exhibit are because of large proportion of acidic amino acids, principally, aspartic and glutamic acid, allowing a hydration of protein surface because of presence of carboxylic groups of this amino acid. From the several enzymes which include protease, lipase, xylanases, cellulases and amylases have poly extremophilc properties extracellular hydrolytic enzymes such as amylases, proteases, lipases etc have diverse potential usages in industrial and chemical sectors produces extracellular protease. In present investigation, extracellular hydrolytic enzyme producing halophilic bacteria *Salinicoccusroseus* strain.

2. Materials and Methods

The major materials and the standard methods employed in the present study are as follows;

Sample Collection

For conducting the study, Sea water samples were collected from ponnani beach, ponnani, malappuram district. The samples were taken from 30 cm depth from the surface of the water. The samples were collected in sterile polythene covers and containers and stored in refrigerator until the analysis was carried out. The samples were brought to the laboratory and processed for isolation of microorganisms. The examination was always carried out within a period of 5 hours after collection.

Isolation and Screening of Halophiles

Serial dilutions were made with 0.9% saline. 10ml of sea water was transferred to the flask containing 90 ml saline. This is called master dilution. Serial dilution was carried out up to 10^{-6}dilution. From appropriate dilution 0.1 ml of suspension was spreaded on Halophilic Agar medium. The petri plates were incubated at 37ºC for 24-72 hours. Different colonies were picked and sub cultured several times in halophilic agar and halophilic broth to obtain pure cultures. The isolates were characterized for colony characteristics, morphological characteristics and biochemical characteristics and identified with Bergey's manual of determinative bacteriology.

Preparation of pureculture of *Halobacterium* sp

The nutrient agar plate with increased concentration of Nacl was prepared in petri dishes and the isolated colony obtained in the halophilic agar medium were taken carefullyand streaked in a zigzag manner using the inoculation loop. Then the plates were incubated at 37°c for 48 hours.

Determination of respiration type of *Halobacterium* sp

The nutrient agar deep tube was prepared and the pure culture was inoculated using inoculation needle under sterile condition. After inoculation, upper surface of the

medium was covered with the paraffin oil with 0.5 cm thickness and incubated for 48 hours at 37°c.

Screening of Hydrolytic Activities

Hydrolyse producing bacteria among the isolates were screened by plate assay on starch, tributyrin and skim milk agar plates for amylase, lipase and protease respectively.

Amylase Activity

Amylolytic activity of the cultures was screened on starch agar plates with increased concentration of Nacl. The pH was from 7.0 to 10.0. The zone of clearance was determined by flooding the plates with iodine solution. The potential amylase producers were selected based on ratio of zone of clearance diameter to colony diameter.

Protease Activity

Proteolytic activity of the isolates was similarly screened on skim milk agar plates with increased concentration of Nacl. The isolates showing zones of clearance were selected and designated as protease producing bacteria.

Lipase Activity

Lipase activity of the cultures was screened on tributyrin nutrient agar plates containing 1% (v/v) of tributyrin. Isolates that showed clear zones of tributyrin hydrolysis were identified as lipase producing bacteria.

Identification and Characterization of Potential Halophilic Isolates

The morphological biochemical characterization was carried out standard methods.

Microscopic Examination

To observing the microscopic morphology the isolate was subjected for Gram staining. Hanging drop method was used to observe the motility character of the pure isolates. In order to check the spore forming ability, Endospore staining were also conducted. Then it is followed by capsular staining.

Biochemical and Physiological Examination

The bacterial isolates were subjected for identification, using the following routine biochemical and physiological tests-Indole Test, Methyl Red Test, Voges-Proskauer Test, Citrate Utilisation Test, Catalase Test, Oxidase Test, Carbohydrate Fermentation Test, Urease Test, Triple Sugar Iron Test, Hydrogen sulphide test.

3. Result and Discussion

Sea water samples were inoculated in halophilic agar medium to obtain bacterial isolates which are halophilic and that were subculture in halophilic broth and agar for producing pure culture. All the strains isolated were found to be gram positive cocci. Only one colony was selected for further study and characterization. Isolated strain was tested for Biochemical characteristics. Isolate was found to be catalase negative as they do not showed bubble formation on addition of H_2O_2. The isolate was oxidase negative as no color change was observed in the oxidase disc. It was found to be urease positive as the media color changes from orange yellow to pink. The isolates doesn't showed indole production test. Methyl red test and voges proskauer test were positive as red and pink colour were observed in those tubes which are marked as MR and VP respectively. The isolate was found to be citrate utilization test positive as the color of media turned to deep prussian blue. It can ferment lactose, glucose and sucrose but not fructose.

The colonies were further screened for hydrolytic activity. The halophilic bacterial isolates were screened for amylase, protease, and lipase activity. For visualising amylase activity, the starch agar plates having bacterial growth were flooded with gram's iodine solution. The appearance of clear zone around the bacterial colonies indicated the utilisation of starch by the halophilic bacteria and thus indicative the presence of amylase activity. The potentiality of halophiles in lipase production was studied by using tributyrin agar medium containing tributyrin as sole carbon source. The hydrolytic activity was confirmed by visual observation of zone around the bacterial species. The lipase production was reasonably good and comparable in the bacterial isolate screened and isolated from sea water. The plates were assessed for the protease activity by monitoring the zone of clearance around the bacterial growth. the zone formation around the colony indicates the hydrolysis of casein present in skim milk due to protease production. . Our study showed that halophilic bacteria were more abundant in the sea water. These results are in agreement with those reported by Roohi, *et al*., 2012, Quesada *et al*., 1982 and Rodriguez Valora (1988) who reported higher frequencies of moderately halotolerant and halophilic bacteria to extremely halophilic bacteria in saline environment. Halophiles known to produce haloenzymes like amylases, proteases, and lipases. From the biochemical and hydrolytic study of halophiles we understood that hydrolytic enzymes (protease, lipase, and amalyse) can play a major role in industrial sector in an effective ways.

Colony and Cell Morphology

Color	Creamy white
Size and shape	Small, Circular
Opacity	Opaque
Elevation	Convex
Margin	Regular
Consistency	Mucoid
Gram staining	Gram positive cocci
Endospore staining	Non-spore forming
Motility	Non motile
Capsule staining	Non-capsulated

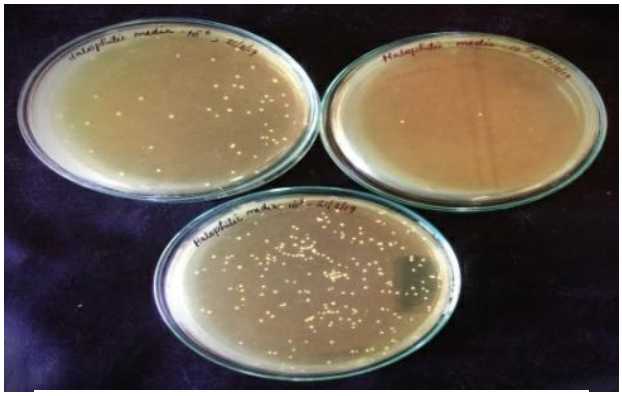
Biochemical Tests

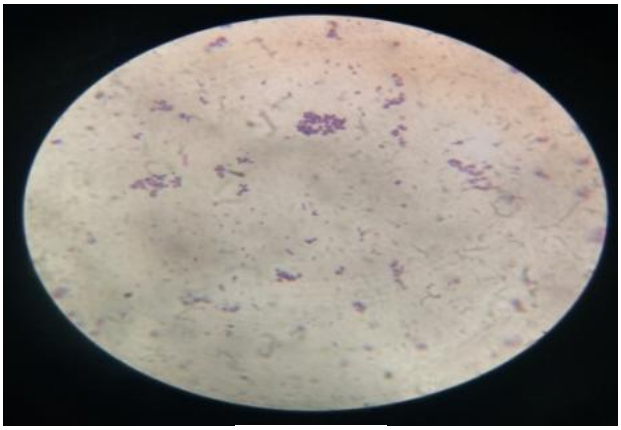
Indole	Negative
Methyl-red	Positive
Voges-proskauer	Positive
Citrate utilisation test	Positive
Oxidase test	Negative
Catalase test	Negative
H_2S Production test	Negative
Urease	Positive
TSI agar test	Acid butt and alkaline slant
CHO fermentation Glucose Lactose Fructose Sucrose	 Positive Positive Negative [acid formation but not gas] Positive

Hydrolytic activity

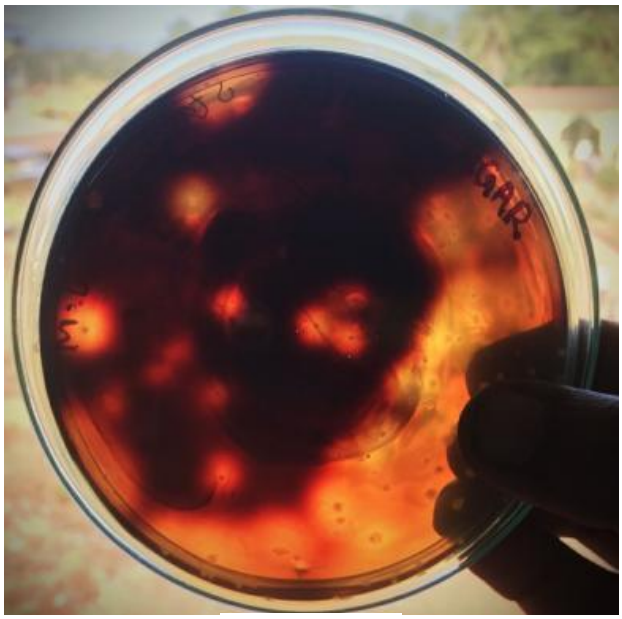
The appearance of clear zone around the bacterial colonies indicated the presence of amylase activity in starch agar, protease activity in skim milk agar, lipase activity in tributyrin agar**.**

Subcultured halophiles

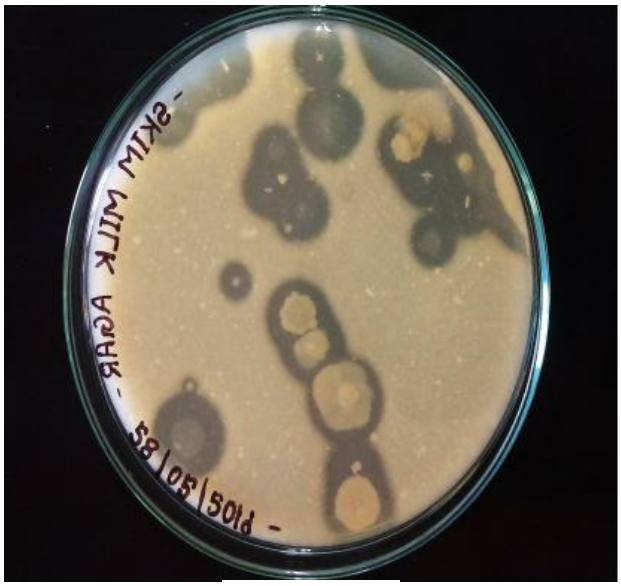

Isolated organism from sea water in Halophilic agar


Gram staining

Biochemical Test



Amylase activity



Protease activity

Lipase activity

4. Conclusion

The present study investigate that there are moderately halotolerant halophilic bacteria to extremely halophilic bacteria in saline environment. Among halophilic microorganisms are a variety of heterotrophic and methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Halophilic bacterial extracellular enzymes are important with respect to their industrial applications mainly in fermented food, textile, pharmaceutical, leather industries, nutrient recycling and biodegradation process. In this novel it shows haloenzymes like Amylase, lipase, protease helpful in industrial applications. From the biochemical and hydrolytic study of halophiles we understood that hydrolytic enzymes (protease, lipase, and amalyse) can play a major role in industrial sector in an effective ways.

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