International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 12 (2014) pp. 753-760**
http://www.ijcmas.com

Original Research Article

Isolation and biochemical characterization of Halophiles from Sahastradhara region, Dehradun, India

Mohsin Azhar[1], Veena Uniyal[1]*, Neha Chauhan[1] and Devendra Singh Rawat[2]

[1]Department of Microbiology, Baba Farid Institute of Technology, Suddhowala, Dehradun, India
[2]Department of Zoology, Baba Farid Institute of Technology, Suddhowala, Dehradun, India

**Corresponding author*

Keywords

Halophiles, Salt Concentration, Salt loving, enrichment

A B S T R A C T

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. The name comes from the Greek for "salt-loving". While the term is perhaps most often applied to some halophiles classified into the Archaeadomain, there are also bacterial halophiles and some eukaryota, such as the alga*Dunaliellasalina*.Halophiles are categorized slight, moderate or extreme, by the extent of their halotolerance.Isolation of halophiles is done by enrichment culture method and halophilic medium is used as selective media. Biochemical test are done for each isolate. It was found that S8 isolate was an extreme halophile, S5 and S1 were found to be moderate halophile and other isolates were not able to adapt themselves to high salt concentration.

Introduction

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. Halophiles are categorized as slight, moderate or extreme, based on the extent of their halotolerance. Slight halophiles prefer 0.3 to 0.8 M (1.8 to 4.7% - seawater is 0.6 M or 3.5%), moderate halophiles 0.8 to 3.4 M (4.7 to 20%), and extreme halophiles 3.4 to 5.1 M (20 to 30%) NaCl. Halophiles can be found in areas where the concentration of salt is five times greater than salt concentration of the ocean, such as the Great Salt Lake in Utah, Owens Lake in California, the Dead Sea, and in evaporation ponds. (Roohi*et al*.,2012). They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Among halophilic microorganisms are a variety of heterotrophic and methanogenicarchaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes. Halophilic microorganisms have several biotechnological applications like β-carotene production of fermented foods. In recent years, uses of halophilic microorganisms have significantly increased. Many enzymes, stabilizers and valuable compounds from

halophiles may present advantages for the development of biotechnological production processes.

Halophiles may use a variety of energy sources. They can be aerobic or anaerobic. Anaerobic halophiles include phototrophic, fermentative, sulfate-reducing, homoacetogenic and methanogenic species (Oren*et al*., 2002).

The aim of this research is to explore any novel Halophilic, extreme or moderate bacteria, and to examine their morphology, cultural characteristics as well as their biochemical characters. It was also aimed to assess the bacterial biodiversity of Halophilic bacteria at Sahastradhara streams.

Materials and Methods

Sample Collection

Water sample was collected into sterile bottle from the streams of Sahastradhara region, Dehradun and stored at 4ºC in the laboratory until used for isolation of the strains.

Enrichment and isolation of bacteria

Enrichment cultures and techniques to isolate moderately to extremely halophilic microorganism are performed in Halophilic Agar medium and Halobacterium medium. pH was adjusted to 7.2±0.1 before autoclaving. Enrichment cultures were subcultured several times under the same conditions with different NaCl concentration (0%,5%,10%,20%). Aliquots (100 µl) of 10^{-3}-10^{-6} dilutions were plated on to agar medium. After two weeks of incubation at 37 ^{0}C, there were red, orange red, pale pink, yellowish, cream, transparent colonies. Different colonies were picked and streaked several times to obtain pure cultures.

Characterization and Identification of Isolates

These isolates were grown on selective media and were chosen for further characterization. Isolates were examined for colony and cell morphology. Colony morphology were described with special emphasis on pigmentation, colony elevation and opacity. These characteristics were described from cultures growth at optimum temperature, pH, salt concentration. In biochemical tests, Catalase test, Gelatinase, Amylase, Oxidase, Citrate, Urease, Indole test, Tryptophan Deaminase, VogesProskauer, Fermentation/ oxidation (lactose, sucrose, dextrose) were performed.

Different staining and biochemical tests were followed using standard procedure (Aneja, 2003)

Gram Staining

The gram stain is a differential stain which is used to differentiate bacteria into two groups Gram positive bacteria and Gram negative bacteria. The technique is based on the fact that Gram positive cell wall has a stronger attraction for crystal violet when iodine is applied and therefore retain the crystal violet and therefore will remain purple after decolorizing while Gram negative bacteria will be colourless after decolorizing with alcohol, counterstaining with Safranin will make them to appear pink.

Catalase test

The glass slide was held at an angle and few drops of 3% hydrogen peroxide were allowed to flow slowly over the culture. The emergence of bubbles from the organism was noted. The presence of bubble displayed a positive test indicating the presence of enzyme catalase. If no gas is produced, this is a negative reaction.

Amylase test

In this test, isolate was point inoculated on starch agar plates and incubated at 37^0C for two days. After incubation, iodine solution was poured on the agar and examined for hydrolysis of starch by the production of clear zone around the microbial growth.

Gelatin liquefaction

Gelatin is a protein produced by hydrolysis of collagen. Hydrolysis of gelatin is brought about by microorganism capable of producing a proetolyticexoenzyme known as gelatinase. The degradation of gelatin occurs in the medium by an exoenzyme, it can be detected by observing liquefaction (i.e. flooding the gelatin agar medium with mercuric chloride solution and observe the plates for clearing around the line of growth) because gelatin is also precipitated by chemicals that coagulate proteins while the end products of degradation (i.e. amino acids) are not precipitated by same chemicals.

Urease Test

Urease test is performed by growing the test organisms on urea broth or agar medium containing the pH indicator phenol red (pH 6.8). During incubation, microorganisms possessing urease will produce ammonia that raises the pH of the medium/broth. As the pH becomes higher, the phenol red changes from a yellow colour (pH 6.8) to a red or deep pink colour. Failure of the development of a deep pink colour due to on ammonia production is evidence of a lack of urease production by the microorganisms.

Oxidase Test

The oxidase test is a test used in microbiology to determine if a bacterium produces certain cytochrome c oxidases. It uses disks impregnated with a reagent such as *N, N, N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) or *N, N*-dimethyl-*p*-phenylenediamine (DMPD), which is also a redox indicator. The reagent is a dark-blue to maroon color when oxidized, and colorless when reduced. Oxidase-positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron-containing hemoprotein).[2] These both catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, TMPD dihydrochloride acts as an artificial electron donor for the enzyme oxidase.

The oxidized reagent forms the colored compound indophenol blue. The cytochrome system is usually only present in aerobic organisms that are capable of using oxygen as the final hydrogen receptor. The end-product of this metabolism is either water or hydrogen peroxide (broken down by catalase). Live bacteria cultivated on trypticase soy agar plates may be prepared using sterile technique with a single-line streak inoculation. The inoculated plates are incubated at 37°C for 24–48 hours to establish colonies. Fresh bacterial preparations should be used. After colonies have grown on the medium, 2-3 drops of the reagent DMPD are added to the surface of each organism to be tested. A positive test (OX+) will result in a color change violet to purple, within 10–30 seconds. A negative test (OX-) will result in a light-pink or absence of coloration.

IMViC

Indole Test

Tryptophan is an essential amino acid that can undergo oxidation by the way of enzymatic activities of bacteria and

converted into metabolic products (indole, pyruvic acid and ammonia) is mediated by the enzyme tryptophanase. The presence of indole is detected by adding Kovac's reagent which produces cherry red colour. The colour is produced by the reagent which is composed of p-dimethylaminobenzaldehyde yielding the cherry red colour.. Absence of red coloration demonstrates that the substrate tryptophan was not hydrolysed and indicates an indole negative.

Methyl red test

All enteric microorganisms ferment glucose and produce organic acids. .The methyl red indicator which is used in this test, in the pH range of 4 will turn red, which is the indicative of a positive test. At a pH of 6, still indicating the presence of acid but with a lower hydrogen ion concentration, the indicator turns yellow and is a negative test.

Voges Proskauer test

VogesProskauer test determines the capability of some microorganisms to produce non-acidic or neutral end products, such as acetyl methyl carbinol, from the organic acids that results from glucose metabolism. The reagent used in this test, Baritt reagent consists of mixture of alcoholic alpha- napthol and potassium hydroxide solutions. Detection of acetyl methyl carbinol requires this end product to be oxidize to a diacetyl compound. This reaction will occur in the presence of alpha-napthol catalyst and a guanidine group that is present in the peptone of MR-VP medium. As a result, a pink complex is formed, imparting a rose colour to the medium.

Citrate utilization test

In the absence of fermentable glucose or lactose, some micro-organisms are capable of using citrate as a carbon sources for energy. This ability depends on the presences of citrate permeas that facilitates transport of citrate in the cell. During this reaction the medium becomes alkaline, the carbon dioxide that is generated combines with sodium and water to form sodium carbonate, an alkaline product. The presence of carbonate changes the Bromothymol blue indicator incorporated into the medium from green to Prussian blue. After incubation citrate positive culture are identified by the presence of growth on the surface of slants, which is accompanied by blue coloration. Citrate negative will show no growth and the medium will remain green.

Carbohydrate Fermentation

Fermentation of carbohydrates (glucose, sucrose, lactose etc.) are carried out by microorganism, under anaerobic condition in which a Durham tube is placed in inverted position to trap the gas bubble formed due to production of gas. The fermentation broth contains ingredients of nutrient broth, a specific carbohydrate and a pH indicator (phenol red), which is red at neutral pH (7) and turns yellow at or below a pH of 6.8 due the production of an organic acid.

Result and Discussion

After several dilutions and subculturing in the liquid as well as solid medium, colonies were isolated in the enrichment medium containing 20% NaCl. A total of 8 Halophilic strains were isolated under aerobic conditions from the sample. These strains were examined and characterized morphologically as well as biochemically.

Colony and Cell Morphology

Most of the isolated colonies were circular, smooth, convex. Rarely the colonies were

found to be transparent and translucent. Most of colony pigmentation ranged from blood red, orange, yellow and pink. Optimum growth occurred at 10% and 20% NaCl (w/v) at 37°C, and pH7, suggesting that these isolates should be considered as halophilicaccording to the definition of Ventosa *et al*., 1998. Out of 8 strains isolated, 6 were found to be gram positive and two were found to be gram negative. Strains S1, S3, S4, S5, S6, S7 were found to be gram positive, out of which S1,S5 and S6 were found to be gram positive rods and S3, S4, S7 were gram positive coccii. S2 and S8 were found to be gram negative rods (Table1.)

Optimization of growth conditions:

The growth conditions of all the isolated strains were optimized for pH and salt tolerance. The purpose of optimization of the strains was to find their optimum growth in different pH. From the results, it was concluded that the halophilic bacteria species grow best at 7-8 pH. Similarly NaCl tolerance was checked from these species, most strains, grow best in the range of 10-20% at temperature 37^0C. (Table2).

Biochemical Tests

Isolated strains were tested for Biochemical characteristics (Table 3).

Catalase Test: Isolates S1, S2 and S5 were found to be catalase positive as they showed bubble formation on addition of H_2O_2. S3, S4, S6, S7, S8 were found to be catalase negative as no bubble were produced.

Amylase test: S1, S3, S5, S6 were found to be Amylase positive as they showed clear zones around the colonies by addition of iodine solution. Other isolates were found to be amylase negative.

Gelatinase Test: No isolates showed gelatinase test positive.

Urease Test: All isolates except S4 and S6 were found to be urease positive as the media color changes from yellow to pink.

Oxidase Test: All isolates except S1 and S3 were found to be oxidase positive and other isolates were oxidase negative as no color change was observed.

IMVIC Test

Indole Production: No isolates showed indole production test.

Methyl Red : NO isolates showed methyl red positive test

VogesProskauer Test: Two strains S7 and S8 showed VP test positive as pink color was observed in to Carbohydrates.

Citrate utilization: Only 1 strain S5 were found to be citrate utilization test positive as the color of media turned blue.

Fermentation of carbohydrates

Lactose: Only 2 strains S4 and S6 showed fermentation of lactose with production of lactic acid and gas.

Sucrose: S4 and S7 showed gas and acid production.

Dextrose : S1 showed acid production

Bacteria isolated from the sample collected from Sahastradhra region were found to be highly diverse group of halotolerant and halophilic bacteria with different phenotypic characters (Table1). Several studies have been conducted on the ecology, taxonomy and phylogeny of halophilic bacteria as well

as their biotechnological applications (Lichfield and gillevet, 2002).

Halophilic bacteria were categorized on the basis to tolerance of different NaCl concentration into slightly, moderately and extremely halophilic bacteria. According to our study, Table No.2 shows growth of isolated colonies at different salt concentration at 37^0C. In this study, extreme halophilic isolates was found to S8 as this survived and tolerated such a high concentration of NaCl. Moderately halophilic isolates were found to be S5 and S1, other were not able to adopt to high concentration of salt.

Our study showed that slightly and moderate halophilic bacteria were more abundant then the extremely halophilic bacteria. 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.

Halophilic bacteria grow better at the temperature of 28-37^0C and at pH7.0 - 8.0 on media supplemented with 5 – 20% NaCl concentration. Our study agreed with the study of Roohi*et al*., 2012 and Hongyu*et at.,* 2009 who isolated the halophilic microorganism from ponds of China and Karak region and observed the growth of these microorganism at the temperature of 35 – 40^0C and at pH 7.0 – 8.0 with 20 – 30% (w/v) NaCl.

These properties offer significant advantage to study the activity and metabolism of halophiles at various salt concentration. Such potential halophiles can be used in bioremediationor degradation and transformation of range of organic pollutants in pond, lakes, streams and rivers. Halotolerance of many enzymes derived from halophilic microorganisms can be exploited were as enzymatic transformation are required to function at low water activities, such as in the presence of high salt concentration (Kamekaru, 1986).

In addition, all exoenzymes excreted by halophiles are active in the presence of high salinities found in their medium, even when the organism that produce them are maintain low intercellular ionic concentrations (Oren, 2002).

Finally we conclude from this study that Halophiles have the distinctive advantage to grow in environment having high salt concentration where other potential microorganisms fail to survive. This offers a multitude of potential applications in various fields of biotechnology.

Their compatible solutes are useful as stabilizers of biomolecules and whole cells, salt antagonists, or stress-protective agents. Biopolymers, such as biosurfactants and exopolysaccharides, are of interest for microbially enhanced oil recovery (MEOR).

Enzymes of such organisms such as new isomerases and hydrolases have their own significance due to their potential to remain active and stable in high salt contents. As now the medium composition is optimized, these microorganisms can be used for its various applications in the field of biotechnology.

Table.1 Phenotypical characteristics of the isolates

Characteristics	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8
Colonial morphology	Circular	Circular	Circular	Circular	Circular	Circular	Irregular	irregular
Colony	Convex	Convex	Convex	Translucent	Flat	Convex	Convex	Convex
Colony density	Opaque	Opaque	Opaque	Translucent	Opaque	Opaque	Translucent	translucent
Pigmentation	Cream	White	Cream	Transparent	Cream	Cream	White	White
Cell shape	Rod	Rod	Coccus	Coccus	Rod	Rod	Coccus	Rod
Cell arrangement	Single & paired	Single paired & long chains	Single & paired	Single & paired	Paired & long chains	Single & paired	Single & paired	Paired chians
Gram staining	+ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve

Table.2 pH and Salt concentration for optimum growth at 370C

Isolates	pH	Salt Conc. (%)
S1	7	10
S2	7	10
S3	8	15
S4	8	10
S5	7	15
S6	7	10
S7	7	10
S8	7	20

Table.3 Biochemical characteristics of isolated bacterial species

Biochemical test	S1	S2	S3	S4	S5	S6	S7	S8
Catalase	+ve	+ve	`-ve	-ve	+ve	-ve	-ve	-ve
Amylase	+ve	-ve	`+ve	-ve	+ve	+ve	-ve	-ve
Gelatinase	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Urease	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve
Oxidase	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Methyl red	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Vogesprouskauer	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Fermentation								
Lactose	G	-	AG	AG	AG	G	G	G
Sucrose	A	AG	AG	AG	AG	AG	A	A
Dextrose	A	AG	AG	AG	AG	AG	A	AG

G = gas, AG = acid and gas, A = acid, -ve = negative, +ve = positive

References

Aneja, K. R. 2003. Staining and biochemical techniques.Edition 4th.Experiments in microbiology, Plant Pathology and Biotechnology.

Hongyu, W., L. Yang, L. Shen, B. Hu, L. Zongyun and J. Qijiang. 2009. Isolation and characterization of culturablehalophilic Microorganisms of salt ponds in Lianyungang, China. World Journal of Microbiology and Biotechnology, DOI 10.1007/s11274-009-0068-5.

Kamekura, M. 1986. Production and function of enzymes from eubacterial halophiles.FEMS Microbiological Reviews. 39: 145–150.

Litchfield, C. D. and Gillevet, P. M. 2002. Microbial diversity and complexity in hypersaline environments: a preliminary assessment. Indian Journal of Microbiology and Biotechnology. 28(1): 48-55.

Oren, A., 2002. Diversity of halophilic microorganisms: Environments, phylogeny, physiology and applications. Journal of Industrial Microbiology and Biotechnology. 28: 56-63.

Quesada, E., A. Ventosa, F. Rodriguez-Valera and Ramos Cormenzana, A. 1982. Types and properties of some bacteria isolated from hypersaline soils. Journal of Applied Microbiology. 53: 155-161.