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Isolation and Screening of Moderate Halophiles for Industrially Important Hydrolytic Enzymes

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Abstract:
Halophiles are the salt loving organisms, which are found to survive in wide range of salt concentration. Based on their salt requirement, halophiles are categorized into different categories; 1. Slight, 2. Moderate, 3. Extreme. Moderate halophiles were isolated from Jamnagar Vadinar Bandar area. Total 16 isolates were obtained on N-agar plates containing various salt concentrations. These organisms were exploited for various hydrolytic enzyme activity i.e.; amylase, lipase, caseinase etc. Among 16 isolates, 9 were amylase positive, 12 were lipase positive, 13 were caseinase positive. Identification of these isolates was on the basis of morphology, physiology, biochemical activity. Further research on purification of one of these enzymes is in progress.

Keywords: *Halophiles, hydrolytic enzymes like lipase, catalase, amylase, caseinase*

1. Introduction

Woese and Fox, 1977 suggested a new taxonomy for living systems that uprooted the prokaryote eukaryote view of the world based on base-sequence studies of 16S and 18S ribosomal RNA molecules. This led to the construction of a universal phylogenetic tree comprising three primary groups termed Domains, now referred to as the Bacteria, the Eukarya and the Archaea. (3)
Varieties of organism are found in extreme environment which comes under a common heading extremophiles. The environments which were thought to be sterile have the presence of life. The organism growing in such an environment has quite distinct characters in them. Biomolecules isolated from extremophiles possess extraordinary properties that function under severe conditions, comparable to those prevailing in various industrial processes.
Halophiles are known as "salt loving bacteria" found in the hypersaline environment. There are present in all three domains, i.e. Archaea, Bacteria, Eukarya.

1.1. Hypersaline Environment

Hypersaline environments are found in a wide variety of aquatic and terrestrial ecosystems.(2) Hypersaline environments are found all over the world, in arid, coastal, and deep-sea locations, under- ground salt mines, and artificial salterns.
There are two categories of hypersaline environments:
Thalassohaline and Athalassohaline

1.1.1. Thalassohaline

Hypersaline bodies derived from the evaporation of sea water are called as "thalassohaline" or "thalassic" environments, where Na^+ and Cl^- are predominant ions. Solar salterns, where seawater is evaporated for the production of salt, are typical examples of this kind of environment (7). Precipitation and crystallization of salts like calcite, gypsum, halite, sylvite, and carnallite occurs. The system is designed so that NaCl precipitates during the last stage of salt production. The NaCl concentration of these man-made pools can range from 3% to 35% (saturation). Examples are Great Salt Lake of utha, drainage water, salt mines, etc. (3)

1.1.2. Athalassohaline

This type of environment arose where the salt from non marine proportion gets deposited and major divalent cations are Mg^{++} and Ca^{++}. Athalassohaline waters may be influenced by seawater, but in the main are a reflection of the geology, geography and topography of areas where they develop the brines. Example of such brine is Dead Sea where there are depositions of Mg^{++} ions (3).

Halophilic habitat makes these unique ecosystems fascinating to study. Moderate and extreme halophiles have been isolated not only from hyper saline ecosystems (salt lakes, marine salterns and saline soils) but also from alkaline ecosystems (alkaline lakes). The most widely studied ecosystems are the Great Salt Lake (Utah, USA), the Dead Sea (Israel), the alkaline brines of Wadi Natrun (Egypt), and Lake Magadi (Kenya) (2).

Halophilic Organism are classified on the basis of their salt requirement and growth pattern in saline environment as follow (7): Nanohalophiles (0 - 1.0M NaCl); Slight halophiles (0.2 - 2.0M NaCl); Moderate halophiles (0.4 -3.5); Broderline extreme halophiles (1.4 - 4.0); Extreme halophiles (2.0 - 5.2); Halotolerant (0 ->1.0) and Haloversatile (0 ->3.0).

They have characteristic like sensory rhodopsin, some molecular substances, having distinct purple membrane which is also responsible for the growth and photosynthesis, enzymes which plays an important role in high salt concentration. They also contain gas vesicle for their motility, which provide buoyancy and enabling cells to regulate their position in the water. Halophiles contain cytoskeleton like organelle due to the presence of "fibro crystalline bodies". This appears a bundle of hollow tubes which is present in the cytoplasm. This fibril like structure is sensitive to drug like vincristine. Halophiles include a great diversity of organisms, like moderately halophilic aerobic bacteria, cyanobacteria, sulphur-oxidizing bacteria, heterotrophic bacteria, anaerobic bacteria, archaea, protozoa, fungi, algae and multicellular eukaryotes (1). Halophilic microorganisms include a variety of aerobic, anaerobic, chemoheterotrophs, photoheterotrophs, photoautotrops, photoheterotrophic, methanogenic archaea, photosynthetic, lithotrophic, and heterotrophic bacteria, photosynthetic and heterotrophic eukaryotes (4,5).

The publication of Bergey's Manual of Systematic Bacteriology (2001), listed 57 validate species, but now it's been undergoing research. Halobacteria are monophyletic group, most distantly related species having a 16S rRNA gene sequence similarity of 83.2%. After that it has been found that this extremophiles belongs to the order *Halobacterials*, which comes under *Halobacteriaceae* family (12).

1.1.3. Osmotic Adaptation of Halophilic Microorganisms

The primary requirement for all halophiles is that the entire intracellular machinery like enzymes and proteins must be adapted to high salt concentration (8, 9). These microorganisms maintain osmolarity in their cytosol by the process of osmoregulation. They do osmoregulation against the turgor pressure, cellular dehydration and desiccation. These mechanisms allow halophiles to proliferate in saturated salt solutions and to survive entrapment in salt rock. As their cell membrane is permeable to water, they have to keep their cytoplasm isoosmotic with the environment. Both the techniques work by increasing the internal osmolarity of cell. Halophiles having two strategies in saline environment, i.e. Compatible solute strategy and salt in cytoplasm strategy which involves accumulation of K^+ and Cl^- ions to maintain osmotic balance (6). Intracellular ionic concentrations are similar to those of surrounding medium. Intracellular ionic composition are different to those of surrounding medium. In the presence of this adaptation three different evolutionary lineages suggest convergent evolution of this strategy.(9) This mechanism is used by a limited number of halophiles. Second strategy involves accumulation of organic compounds in cytoplasm; these osmoprotectants are known as compatible solutes. Intracellular ionic concentrations are different to those of surrounding medium. This are naturally synthesized or they are uptake from surrounding environment, this are basically neutral or zwitterionic and include amino acids, sugars and some derivatives of this.(9). A variety of compounds is used for the purpose, ranging from glycerol and other sugar alcohols, amino acids, and derivatives such as glycine betaine and ectoine (2-methyl -1,4,5,6 - tetrahydropyrimidine -4- carboxylic acid) and its 5-hydroxy derivative, to simple sugars such as sucrose and trehalose .This strategy is widely used in all three domains of life.(6).

1.1.4. Application of Halophiles in Different Fields

Due to their distinct features there is lots of application of the halophiles. By using these microbes we can develop many industries as a part of the pollution free environment. Most common application of the halophiles are in the innovative fermentation process in the field of biotechnology , their enzymes have also different application in different field, process in waste water treatment plant, dye production, biosurfactants production, in the field of medicine, antibiotic production, vaccines production, biodegadation, bioplastics production and bioremediation etc.(11)

1.1.5. Significance

- The present work gains importance as halophiles have unique features that facilitate many industrial procedures.
- As no sterilization procedures due to their NaCl concentration preventing contamination by other organisms.
- Thus owing to simple cultivation requirements.
- In addition, no cell–disrupting devices are required, as cells lyse spontaneously in low salt concentration.
- An extremely simple production system can be developed such as open ponds, saline water and salt panes.
- As they have versatility in the choice of a broad range of substrates and simple carbon sources such as sugars, acetate or succinate that favors the yield of hydrolytic enzymes and growth directly reducing production cost.
- Thus, many industries are being developed with the help of hydrolytic enzymes produce by the halophiles.

2. Materials & Methods

2.1. Collection of Samples

Water samples were collected aseptically during the period of July'2014, in sterile glass bottles from the Vadinar bander saltpan in Jamnagar area, located in Gujarat, India. The samples were brought to the laboratory in an icebox and analyzed within 6h of collection.

2.2. Isolation of Organisms

A loopful of sample was streaked on Nutrient agar plates containing various salt concentrations (0%, 1%, 3%, 5%, 7%, 9%). Plates were incubated at 30°C for 24 hrs. For further studies organisms growing on 5% and 7% NaCl concentrations were selected. Morphologically distinct colonies were picked up, purified by repeated sub-culturing and maintained in their respective media at 4□C.

2.3. Identification

Isolates were identified on the basis of their morphological and biochemical characteristics.

2.3.1. Morphological Characteristics

Cell morphology: Isolates were described according to standard microbiological criteria, with special emphasis on pigmentation, colonial elevation, consistency and opacity.

Motility: was examined by Agar Stab Technique. (14)

Gram's staining: One of the differential staining method which was used to differentiate organism into gram positive and gram negative.

→ Motility Protocol

Sterilize the straight inoculating wire by flaming it in a Bunsen's flame. Dip the sterile wire into the culture of the test organism. Inoculate the culture into the medium by stabbing the medium right into the center of agar. Withdraw the wire back in the same straight line. Incubate the medium at 30°C for 24-48 hrs.

→ Interpretation

Look for the turbid growth across the line of inoculation indicating motility.

2.3.2. Biochemical Characteristics

Indole production, Carbohydrates fermentation (sugar utilization) test, Methyl red (M-R) test, Voges – Proskauer (V-P) test, Citrate utilization test, Lead acetate paper strip test, Urea hydrolysis, Nitrate reduction, Ammonia production. The results were recorded as incubated 30°C for 24-48 hours. (14)

2.3.2. (a) Carbohydrates Fermentation (Sugar Utilization) Test:-

- Protocol

The Nutrient broth containing NaCl (5% & 7%) was supplemented with different sugar i.e. glucose, fructose, mannose, maltose, sucrose. In each sugar broth, add 2-3 drops andrade's indicator and 1 Durham's tube. These all sugar tubes were autoclaved. Inoculate a loopful of culture into sugar tube and incubate 30°C for overnight. Observe the tube for acid and gas production.

- Interpretation

Acid production changes the color of the medium to pink and gas produced is collected in Durham's tube as a small bubble.

2.3.2. (b) Methyl red (M-R) test

- Protocol

Glucose phosphate broth containing NaCl (5% & 7%) was autoclaved and inoculated with the test culture at 30°C for 48 – 72 hrs. After the incubation, add about 5 - 6 drops of methyl red indicator to the medium. Observe for the development of the red color.

- Interpretation:-

Development of stable red color in the broth indicates the methyl red test positive.

2.3.2. (c) Voges – Proskauer (V-P) test

- Protocol

Glucose phosphate broth containing NaCl (5% & 7%) was autoclaved and inoculated with the test culture and incubated at 30°C for 48 – 72 hrs. After the incubation, add 0.6 ml of α-naphthol and 0.2 ml of KOH solution per ml of culture broth. Shake well after addition of each reagent and slope the tube to increase the aeration. Read results after 15 – 60 minutes.

- Interpretation

The development of the red color within 15 minutes or more, after addition of reagents indicates the presence of diacetyl. The test should not be read after standing for over 1 hour.

2.3.2. (d) Citrate Utilization Test

- Protocol

Simmons's citrate agar slant containing NaCl (5% & 7%) was autoclaved and streak heavily on the surface of the agar slant and incubated the slant at 30°C for 48 – 72 hrs. Record the color change of the slant after incubation.

- Interpretation

Positive test is represented by the development of a deep blue color within 24-48 hours indicating the utilization of the citrate contained in the medium. A positive test may also be read without a blue color if there is visible colonial growth along the inoculation streak line.

2.3.2. (e) Indole Production test

- Protocol

Tryptone broth containing NaCl (5% & 7%) was autoclaved. Inoculate a loopful of test culture and incubated at 30°C for 24 hrs. After incubation add 3-4 drops of xylene in the medium and shake it vigorously. Allow the two layers to separate. Add slowly, 1 ml of Ehrlich's reagent so as to form the layer on the surface of xylene. Observe the formation of pink colored ring at the lower surface of the xylene layer. If Kovac's reagent is to be used (do not add xylene), add 1 ml of reagent on top of the broth and observe for pink ring.

- Interpretation

Development of bright fuchsia red color at the interface of the reagent and the broth within seconds after adding the reagent is indicative of the presence of indole and is a positive test.

2.3.2. (f) Lead Acetate Paper Strip Test

- Protocol

2% peptone broth containing NaCl (5% & 7%) was autoclaved. Inoculate a loopful of test culture in broth. Soak a white filter paper strip in a saturated solution of lead acetate. Place the lead acetate filter paper strip in neck of the tube in such a position that ¼ to ½ of the strip projects below the cotton plug. Incubate the medium at 30°C for 24-48 hrs. After incubation observe the blackening of the filter strip.

- Interpretation

Blackening of filter strip is due to the formation of lead sulfide, which indicates H_2S production by organisms.

2.3.2. (g) Urea Hydrolysis

- Protocol

Urea broth containing NaCl (5% & 7%) was autoclaved and inoculated with a loopful of the test culture. Incubate at 30°C for 48-72 hrs. Observe for the change in color of the broth after incubation.

- Interpretation:

Purple red color throughout the medium indicates alkalinization and urea hydrolysis.

2.3.2. (h) Nitrate Reduction

- Protocol

Peptone nitrate broth containing NaCl (5% & 7%) was autoclaved and inoculated with the loopful of the test culture. The medium was incubated at 30°C for 24-48 hrs. After incubation add 0.5 ml of reagent A and 0.5ml of reagent B to each of the test tube. Observe for the development of red within 30 sec. If no color develops, add a pinch of zinc dust, mix them well and observe the development of red color.

- Interpretation

The development of red color within 30 sec after adding the test reagent indicates the presence of nitrates and is positive nitrate reduction test. If no color develops after adding test reagent this may indicate that,

a) Nitrate have been not reduced (true negative reaction)
b) They have been reduced to products other than nitrates, such as ammonia, molecular nitrogen (denitrification), nitric oxide (NO), or nitrous oxide (NO_2) and hydroxylamine.
   Since the test reagents detect only nitrates the letter process would lead to false negative result. Thus it is necessary to add a small quantity of Zn dust to all negative reactions. Zn ions reduce nitrates to nitrites and development of red color after adding zinc dust indicates the presence of residual nitrates and confirms a true negative reactions.

2.3.2. (i) Ammonia Production

- Protocol

Peptone nitrate broth containing NaCl (5% & 7%) was autoclaved. Inoculate the loopful of test to PNB and place the red litmus paper strip in mouth of the culture tube in such a way that ¼ to ½ of the strip projects below the cotton plug. Incubate the medium at 30°C for 24-48 hrs. Observe the change of red litmus to blue.

- Interpretation

The change in red litmus to blue or purple indicates the ammonia production and can be read as positive test.

2.4. Screening of Isolates for Enzyme Production

Nutrient agar containing NaCl are used in the following experiments. The NaCl concentrations in the media used is 5% and 7% (13,14,15,16).

2.4.1. Amylolytic Activity

In order to test the amylolytic activity, starch (2 g/L) was added to Nutrient agar containing NaCl (5% & 7%) and was autoclaved. The selected strains were incubated at 30°C for 48-72 hours, and plates were flooded with I2-KI solution (0, 1% I2 – 0, 2% KI). The presence of a clear zone around the colony indicates the starch hydrolysis and strains was recorded positive for amylase production.

2.4.2. Gelatin Hydrolyzing Activity

Gelatin (150 g/L) was supplemented to Nutrient agar containing NaCl (5% and 7%), and 5-6 milliliters were transferred to small testing tubes and autoclaved that were inoculated with the tested strains and incubated at 30°C. For the control, after incubation, the cultures were maintained for 10 minutes at 40°C.The liquefaction of gelatin that indicates production of enzyme gelatinase was recorded.

2.4.3. Lipolytic Activity

Nutrient agar containing NaCl (5% & 7%) was autoclaved and supplemented with 1% vegetable oil (w/v) at 60°C and inoculated with selected strains then incubated at 30°C for 48h. The colonies surrounded by a precipitate were considered positive.

2.4.4. Casein Hydrolyzing Activity

Nutrient agar containing NaCl (5% &7%) was supplemented with 1% casein and was autoclaved. After inoculation the tested strains were incubated at 30°C for 48 - 72 hours. Positive strains were detected based on the presence of a clear zone that indicates the casein hydrolysis by enzyme caseinase.

2.4.5. Catalase Enzymes

Add 5 ml hydrogen peroxide over the growth in a used slant. Observe for the effervescence of oxygen. Rapid appearance and sustained production of gas bubbles or effervescence constitute a positive test.

2.4.6. Dnase Enzymes

Nutrient agar containing NaCl (5% &7%) was supplemented with DNA powder (5 mg/ ml) and was autoclaved. After inoculation the tested strains were incubated at 30°C for 7days. Plates were flooded with 1 N HCL. Positive strains were detected based on the presence of clear zones around the colonies that indicates the DNAse activity.

3. Result and Discussion

A total of 16 isolates had been obtained from the water sample collected from Vadinar bander saltpan, Jamnagar area. Out of which optimum growth of 11 isolates was in 5% NaCl and 5 isolates was in 7% NaCl. From all 16, 4 showed varieties of pigmentation like yellow (3 isolates) and orange (1 isolates).

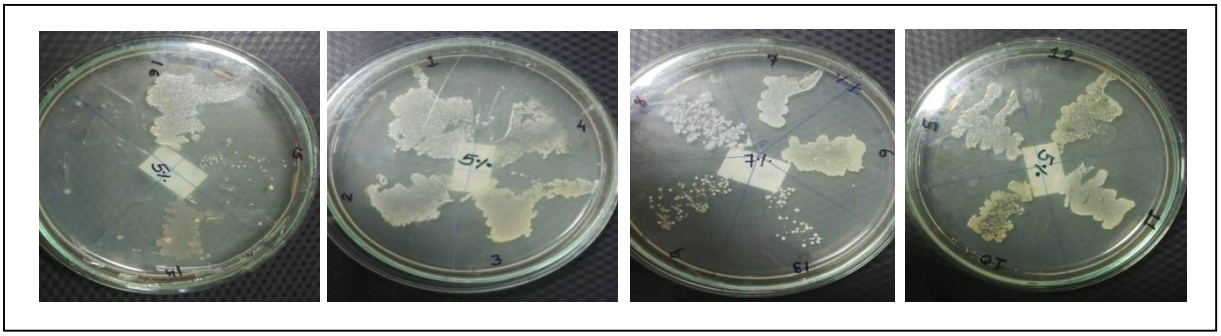

Figure 1: Halophilic Isolates

The Gram's staining result showed both Grams positive (11 isolates) and Gram negative (5 isolates) bacteria. All Grams positive and negative showed various shapes like rods (4 gram positive and 4 negative) and cocci (7 gram positive and 1gram negative).

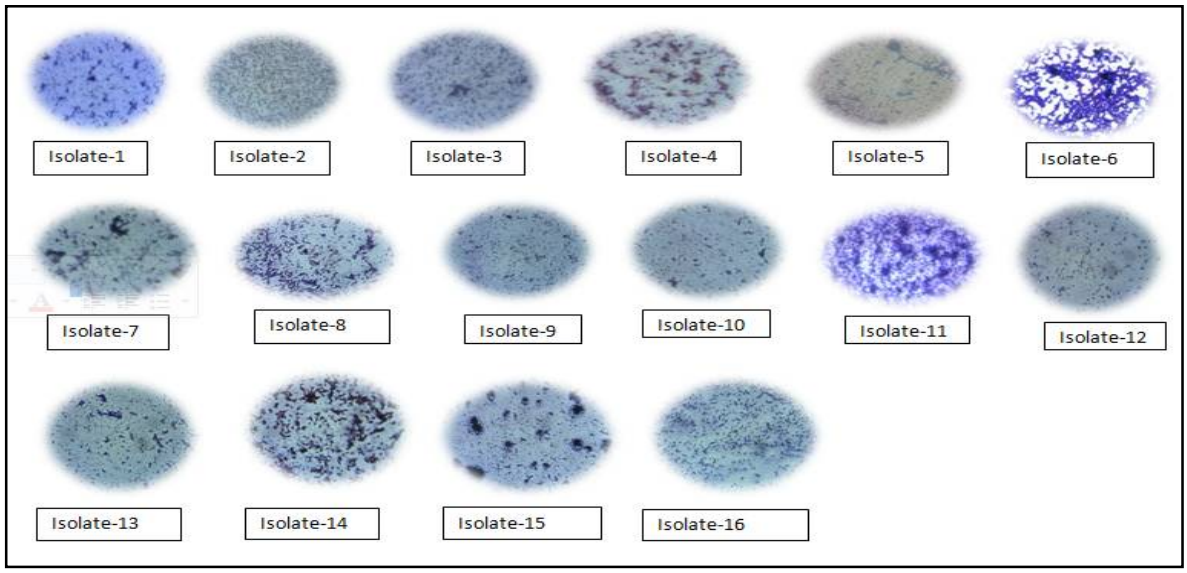

Table 1: Results of Gram's staining

ISOLATES	COLONY CHARACTERISTICS							
	SIZE	SHAPE	ELEVATION	CONSISTENCY	OPACITY	PIGMENTATION	MARGIN	TEXTURE
1	Small	Round	Flat	Moist	Opaque	Off- White	Entire	Smooth
2	Pinpoint	Round	Flat	Moist	Opaque	Off-White	Entire	Smooth
3	Small	Round	Raised	Moist	Opaque	Off-White	Entire	Smooth
4	Pinpoint	Round	Flat	Moist	Opaque	Off-White	Entire	Smooth
5	Pinpoint	Round	Flat	Moist	Opaque	Off-White	Entire	Smooth
6	Medium	Round	Raised	Moist	Opaque	Yellow	Entire	Smooth
7	Pinpoint	Round	Raised	Moist	Opaque	Cream	Entire	Smooth
8	Big	Round	Raised	Moist	Opaque	Off-White	Entire	Smooth
9	Medium	Round	Raised	Moist	Opaque	Off-White	Entire	Smooth
10	Small	Round	Raised	Moist	Opaque	Yellow	Entire	Smooth
11	Pinpoint	Round	Flat	Moist	Opaque	Off-White	Entire	Smooth
12	Pinpoint	Round	Flat	Moist	Opaque	Yellow	Entire	Rough
13	Medium	Round	Raised	Moist	Opaque	Off-White	Entire	Smooth
14	Pinpoint	Round	Flat	Dry	Opaque	Orange	Entire	Rough
15	Small	Round	Raised	Moist	Opaque	Off-White	Entire	Smooth
16	Pinpoint	Round	Flat	Moist	Opaque	Off-White	Entire	Smooth

Table 2: Colony characteristics of halophile

Test	ISOLATES															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Methyl red	+	-	+	-	+	-	-	-	+	+	+	-	-	-	+	+
Voges-Proskauer	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S production	+	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Ammonia production	+	+	+	+	+	-	-	+	-	+	-	+	-	-	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 3: Biochemical test

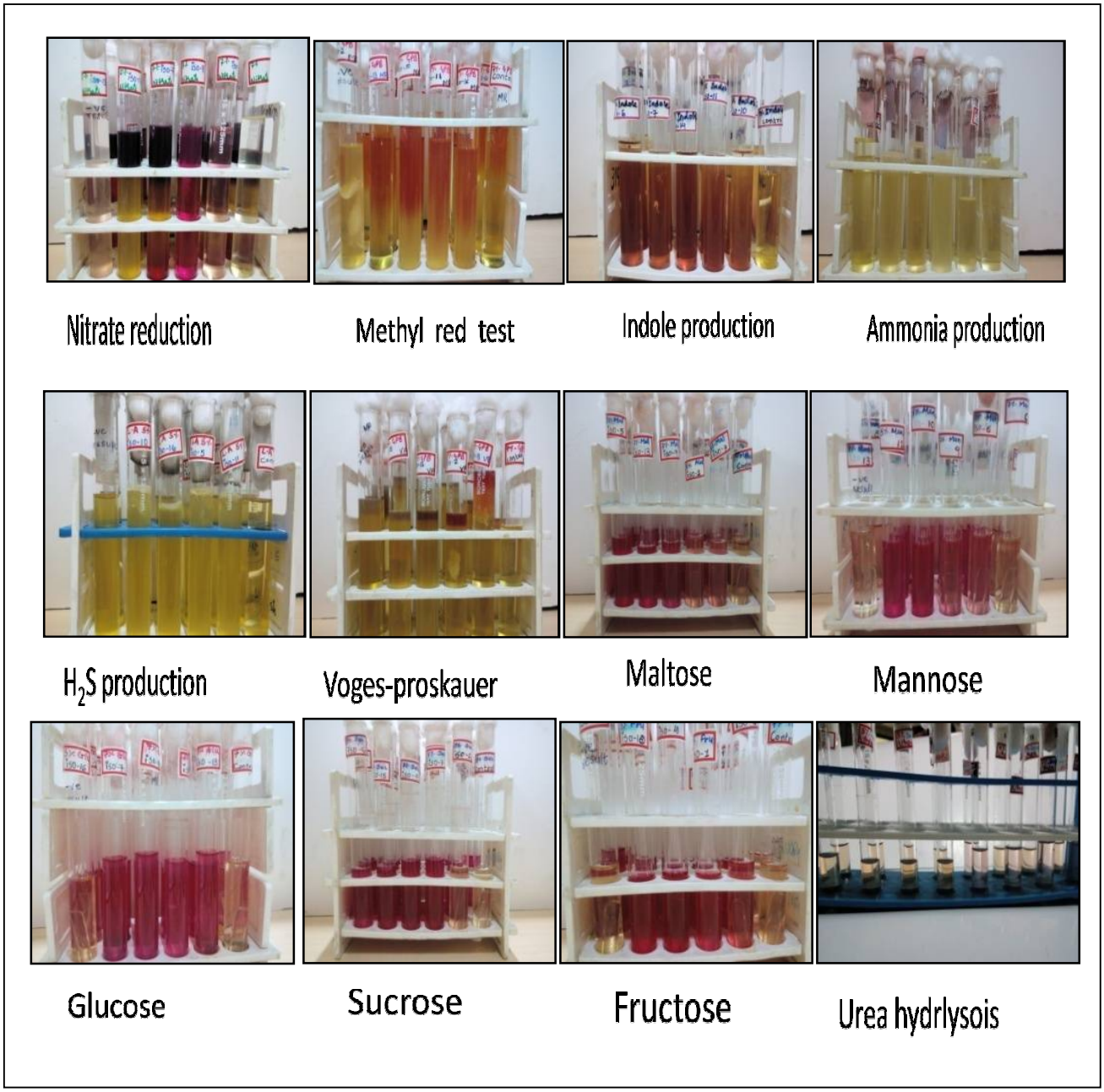

Figure 2: Tubes showing results of biochemical test

Test	Isolates															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Gelatinase	-	-	+	-	-	-	-	-	-	+	-	+	-	-	+	-
Catalase	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+
Casienase	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+
Lipase	+	+	+	+	+	n	+	-	-	+	+	+	+	n	+	+
Amylase	+	+	+	+	+	n	-	N	n	+	+	+	-	n	n	+
Dnase	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-

Table 4: Enzymatic activity

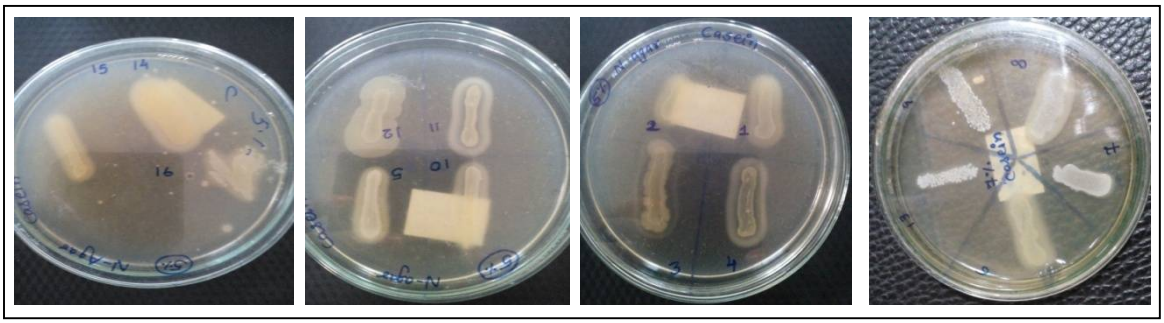

Figure 3: Plates showing Caseinase enzyme activity

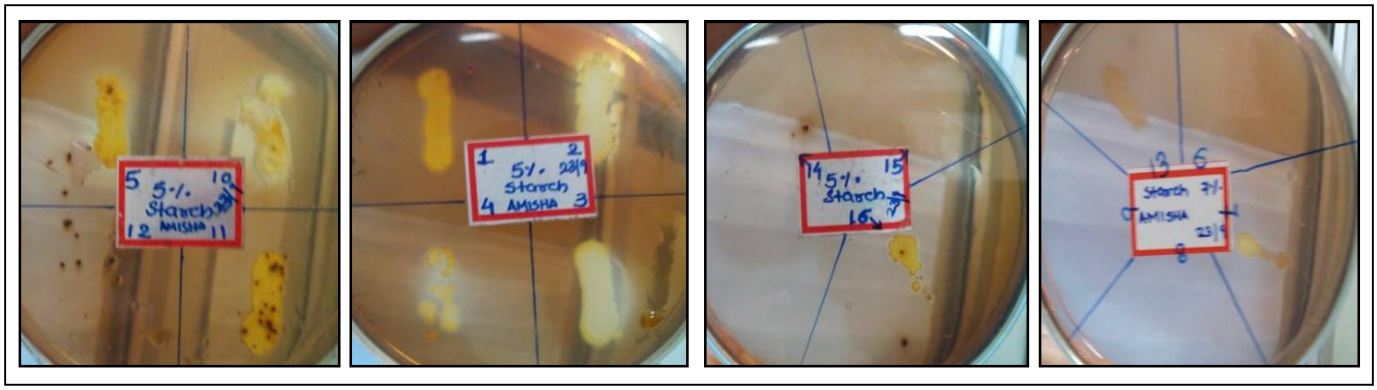

Figure 4: Plates showing Amylase enzyme activity

Figure 5: Plates showing Lipase enzyme activity

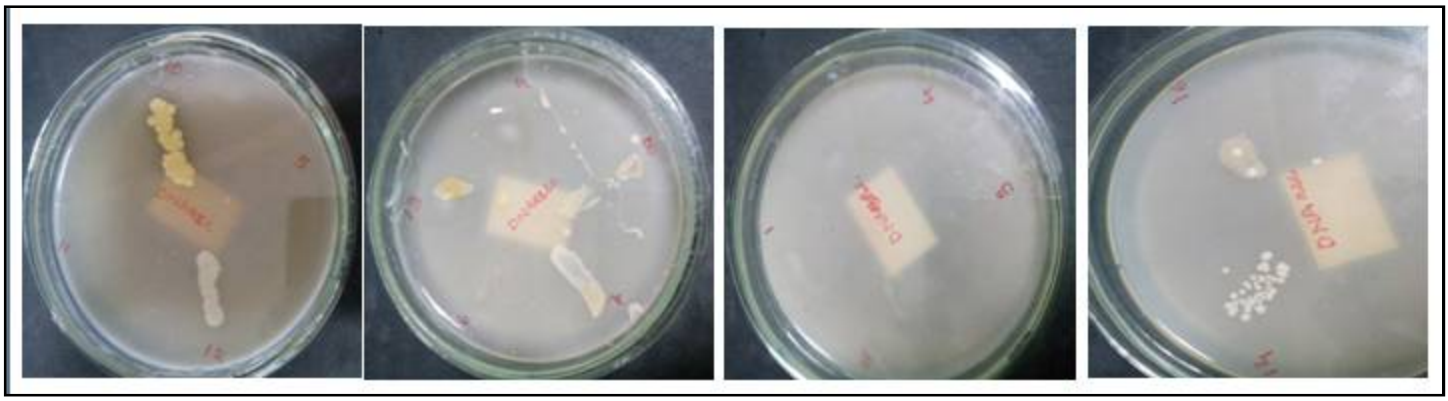

Figure 6: Plates showing DNase enzyme activity

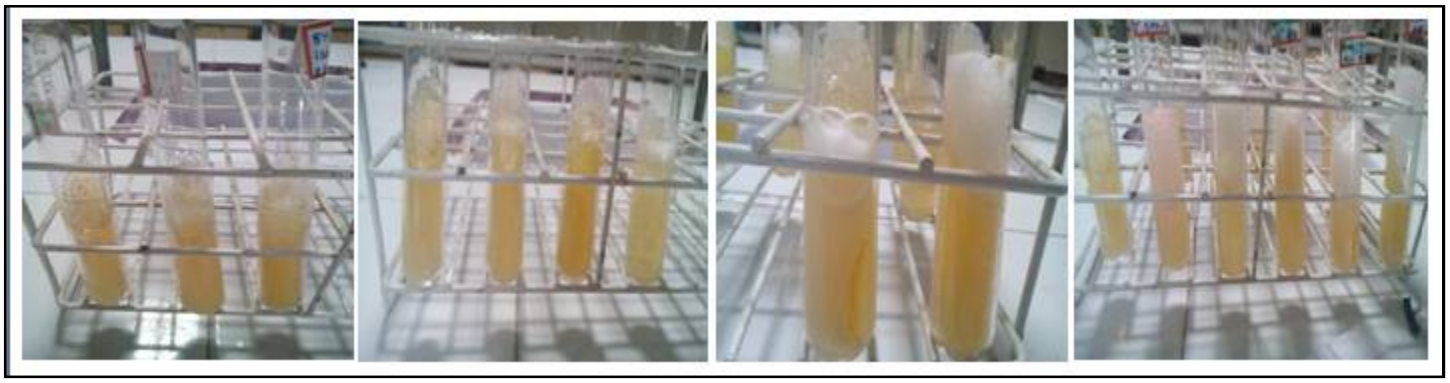

Figure 7: Plates showing Catalase enzyme activity

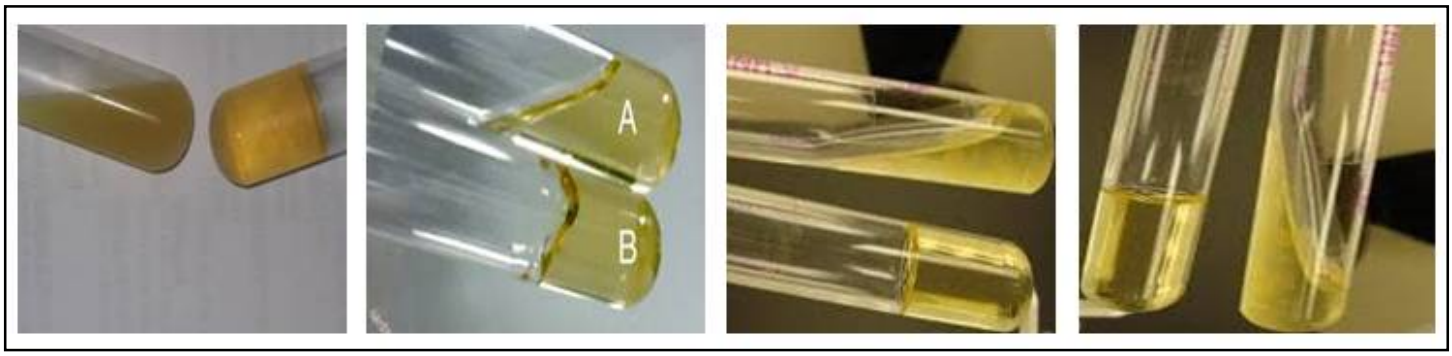

Figure 8: Tubes showing Gelatinase enzyme activity

4. Conclusion

Extremophilic microorganisms have been widely explored industrially and biotechnologically for its valuable products. Halophilic microorganisms can secrete salt loving enzymes viz. protease, lipase, amylase, cellulase, chitinase, etc. These halophilic enzymes can be used to design a wide range of novel biocatalytic processes that are faster, more accurate, specific and environmentally friendly. In order to increase the role of halophilic enzymes in various areas of industries, it is necessary to develop concurrent protein engineering and convergence of bio/nanotechnologies. All these strains obtained through this work could be a good contender for different industrially applicable enzymes under extreme condition.

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