

Assessment of the Bacterial Biodiversity of Halophilic Bacteria at Akola District

Maryam Jabeen¹, Zodpe S.N²

Department of Microbiology, ShriShivaji College Akola-444001(M.S).India

Abstract: The interest of current research work lies in the fact that, soil is the richest source of microorganisms and many efficient enzyme producing microorganism can be produced by soil. Akola District is reported to have saline belt spread over an covering large region of Purna river tract. Present investigation has been undertaken to study the isolation of halophilic organism from saline belt of Akola District. Total 30 samples from different saline sites were collected from various 6 areas. Total 10 isolate were obtained, out of which 5 isolates were halophilic where as 5 were non halophilic. Identification was done on the basis of Bergey's manual. Different parameters such as temperature and pH, as well as halocline production ability of isolate by Disc Diffusion Method was also carried out. The results was recorded in terms of zone of inhibition. Isolates S2 and S4 shows very less zone of halocin production i.e 0.8 mm and 0.9 mm. Very excellent and promising result was obtained in case of S5 which shows 2.6 mm zone indicating better halocin ability. Biosurfactant activity of the obtained isolates was studied and surprisingly best result was recorded.

Keywords: Saline soil, Halophilic bacteria, Biosurfactant.

1. Introduction

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. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations with occasionally depending on environmental and nutritional factors for the growth and tolerance (DasSarma, 2001; DasSarma and Arora, 2002; Das *et al* 2006).

Halophilic organisms

Halophiles can be classified as slight, moderate, or extreme. While slight halophiles grow best in an environment containing the range of 1-5% sodium chloride concentration, moderate halophiles thrive in environments containing 5-20% sodium chloride concentration. The moderate halophiles make up the most diverse group of halophiles, including various methanogenic archaea and anaerobic bacteria. Most of these two organisms are Gram positive or Gram negative aerobes or facultative anaerobes (Ventosa 1998). Extreme halophiles require environments containing up to 30% sodium chloride concentration.

Non-Halophilic Organisms:

Non-Halophilic organisms are defined as those requiring less than 1% NaCl, whereas if they can tolerate high salt concentrations are considered as halotolerant microorganisms.

Halocins are proteinaceous antimicrobial compounds produced by halophilic archaea and is considered as their universal feature (Torreblanca *et al*, 1994). Halocins vary in their spectrum of activity and known to act across domain barrier (Atanasova *et al.*, 2013). Halocins are bacteriocins produced by halophilic Archaea and a type of archaeocin.

Biosurfactants are natural surface active agents produced by bacteria, yeast, and fungi, having very different chemical structures and properties (Rone and Rosenberg, 2001; Chen *et al.*, 2007). These biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic domains that find application in an extremely wide variety of industrial processes involving emulsification, foaming, detergency, wetting, dispersing or solubilization (Rodrigues *et al* 2006). Many microorganisms such as *E.coli*, *Acinetobacter junni*, *Aeromonas caviae*, *Pseudomonas fluorescens*, *Klebsiella pneumonia*, *Bacillus sp* synthesize an extracellular polymeric substances called as biosurfactant (Cameron *et al.*, 1988).

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 Akola district

2. Materials and Methods

• Collection of soil sample from saline zone area :

Soil sample were collected from surrounding villages of Akola District. Soil sample were collected at specified depths upto 10-12 cm using a soil corer. The corer was cleaned after each sampling with water followed by methanol and sample were placed in zip-lock plastic bags & stored for no longer than 7 days at 6°C (Gary *et al.*, 2006).

Screening of Salt Tolerant Bacteria

Screening of salt tolerant bacteria from soil sample was done by using modified Nutrient agar i.e containing varied concentration of NaCl & halophilic agar medium containing amount of NaCl (5%, 10%, 15% & 20%)

1) Processing of sample

Collected soil samples were dissolved in distilled water i.e. 1gm of soil is dissolved in 10 ml of distilled water to get a soil suspension.

• Isolation of halophilic bacteria:

0.1 ml of soil suspension was inoculated on Nutrient agar containing excess salt. After inoculation plates were incubated at 37°C and observed after 24 hrs, 48 hrs upto 7-8 or days for occurrence of visible growth colonies.

• Biochemical test

The confirmation of the organisms was done on the basis of conventional cultural and biochemical characteristics of Bergay's manual determine bacteriology (Bergy's, 1986).

Determination of pH and Temperature range for growth

a) pH:- Isolates were transferred into fresh Nutrient broth medium and incubated at 37°C for 48hrs or until growth appeared. After inoculation growth is examined by pH meter and turbidity.

b) Temperature: Isolates were transferred into fresh Nutrient agar medium. All tubes were incubated at various temperatures (25°C, 37°C,45°) to find influence of temperature on growth.

• Screening of halophiles

a) Halocin production :- Halocins are proteinaceous antimicrobial compounds produced by halophilic archaea and is considered as their universal feature (Torreblanca *et al.*, 1994). Halocins vary in their spectrum of activity and known to act across domain barrier (Atanasova *et al.*, 2013).

Halocin production of isolates was detected by disc diffusion method (Shand, 2006). Briefly, supernatant of freshly grown culture of each isolate was obtained by centrifugation and filtered through 0.2 µm syringe filters and tested against other isolates. Seed agar of test isolates was prepared by spreading 100µl of freshly grown culture, adjusted to 0.1 OD at 600nm. Filter paper discs soaked in culture supernatant were placed on seed agar and zone of inhibition was observed. Culture supernatant of Halocin producing strains was also tested against the such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* by disc diffusion method with uninoculated media as control.

b) Biosurfactant Method:-

1) Phenol Sulphuric Acid Method:

In phenol sulphuric acid method 1ml of 5% phenol was added to the supernatant and to this added 5ml of concentrated sulphuric acid drop by drop the colour changes from yellow to orange indicates the positive test for biosurfactant production (Keppeli and Finnerty, 1980).

2) Erythrocyte Homomlysis Method:

Hemolytic assay was performed in 5% sheep blood agar plates (Mulligan *et al* 1984). 50µl of bacterial culture grown in mineral salt medium was spotinoculated on to blood agar plates and incubated for 48 h at 37oC. The plates were visually inspected for clear zone (Haemolysis) around the colony. The diameter of the clear zone is a qualitative method used as an indicator of biosurfactant production (Rodrigues *et al.* 2006).

3) Oil spreading test

Oil spreading experiment was performed using the method described by Morikawa *et al.*, (2000). In brief, 20 ml of distilled water was added to a plastic Petri dish followed by

addition of 20 µl of crude oil to the surface of the water. 10 µl of cell free culture broth was then added to the oil surface. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone and diameter of this clearing zone indicates the surfactant activity, also called oil displacement activity. A negative control was maintained with distilled water (without surfactant), in which no oil displacement or clear zone was observed and Triton X-100 was used as the positive control.

3. Results and Discussion

Table 1: Effect of different pH and temperature on the growth of halophilic organisms:

| Name of Organism | pH | | | | | Temperature | | |
|------------------|-----|-----|-----|-----|-----|-------------|------|------|
| | 8 | 8.5 | 9 | 9.5 | 10 | 25°C | 37°C | 45°C |
| S1 | + | + | - | - | - | - | + | ++ |
| S2 | ++ | ++ | ++ | + | + | - | + | ++ |
| S3 | +++ | +++ | +++ | +++ | +++ | + | + | ++ |
| S4 | + | - | + | + | - | - | - | ++ |
| S5 | - | - | - | - | - | - | - | ++ |
| S6 | + | + | + | + | - | - | + | ++ |
| S7 | ++ | ++ | ++ | + | + | - | + | ++ |
| S8 | +++ | +++ | +++ | +++ | +++ | + | + | ++ |
| S9 | + | - | + | + | - | - | - | ++ |
| S10 | - | - | - | - | - | - | - | ++ |

Note : -No growth,+ Poor,++ Good,+++Excellent

Table 2: Optical Density of culture supernatant of obtained isolates measured at 600nm

| Isolates | Optical density at 600nm |
|----------|--------------------------|
| S1 | 0.431 |
| S2 | 0.448 |
| S3 | 0.452 |
| S4 | 0.459 |
| S5 | 0.473 |
| S6 | 0.486 |
| S7 | 0.494 |
| S8 | 0.502 |
| S9 | 0.528 |
| S10 | 0.534 |

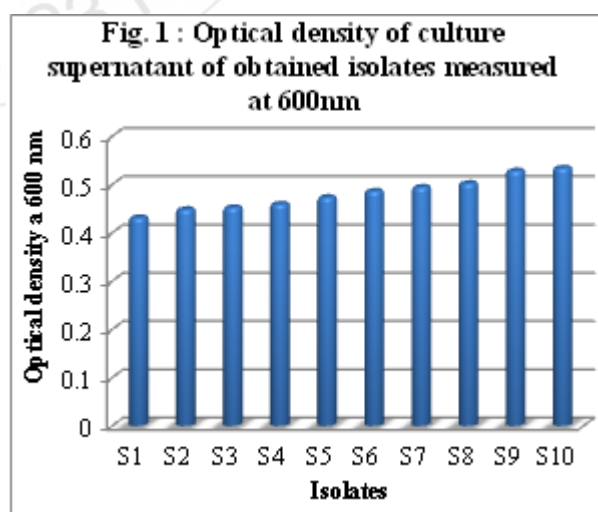


Table 3: Study of Halocin production by Disc Diffusion Method and Antagonistic activity of Halocin producer organisms

| Isolate | Diameter of zone of growth inhibition (mm) by Disc Diffusion Method | Diameter of zone of growth inhibition (mm) by Antagonistic activity |
|---------|---|---|
| S1 | 1 mm | 1.2mm |
| S2 | 0.8 mm | 0.8 mm |
| S3 | 1 mm | 1 mm |
| S4 | 0.9 mm | 0.7 mm |
| S5 | 2.6 mm | 1.8 mm |

Table 4: Phenol sulphuric acid method and blood homolysis test

| Strain | S1 | S2 | S3 | S4 | S5 |
|--|-----|-----|-----|-----|-----|
| Phenol H ₂ SO ₄ test | +ve | -ve | +ve | -ve | -ve |
| Blood homolysis test | +ve | -ve | +ve | -ve | -ve |

+ Positive test, -ve Negative test

Table 5: Oil spreading test

| Strain | S1 | S2 | S3 | S4 | S5 |
|-----------|-----|-----|----|----|------|
| Motor oil | + | +++ | + | + | ++ |
| Corn oil | +++ | + | ++ | ++ | + |
| Olive oil | + | ++ | + | ++ | ++++ |

(+) to (+++++) corresponding to potential to complete spreading on the oil surface.

4. Isolation

Halotolerant organism can grow in either high salinity or in the absence of high concentration of salt. Many halophiles and halotolerant microorganisms can grow over a wide range of salt concentrations, with requirement or tolerance for salts sometimes depend on environmental and nutritional factors.

Total 30 samples from different saline sites were collected from various 6 villages of Akola district (Umri, Morna, Nimvadi, Akola and shirla). For isolation of soil tolerant bacteria from soil samples, two medium containing higher salt concentration was used. Throughout this investigation, salt used is NaCl. Every collected sample was diluted with distilled water in 1:1 proportion. This was inoculated on Halophilic agar medium containing NaCl, for further study colonies obtained were transferred to media with same composition. All these separate isolated colonies were selected on the basis of their ability to grow on medium containing NaCl. These were subjected to screening of salt tolerance by inoculating them on salt medium. Percentage of different isolates obtained. In an average 20% of all the isolates were obtained and is shown in Fig no 1. Meseguer *et al.*, (2004), reported the use of different NaCl concentrations allowed isolation of tolerant, moderate and weak halophilic bacteria. Similar findings were obtained from Madigan *et al.*, (2004), about the isolated strain were predominantly Gram positive and only a few Gram negative.

Screening of Salt Tolerant Bacteria from Collected Soil Samples:

Total 10 isolates were obtained from 30 samples, out of which 5 were halophilic and 5 were non-halophilic. As a results reported from Meseguer, (2004) the selected bacterial strains of their study can survive at salt concentrations as high as 14% of NaCl. Which indicated that

these strains are moderately halophilic bacteria. Mudryk *et al.*, (1991), reported that the bacterial strain isolated from saline environmental shows growth of these strains at 14% NaCl concentration suggests that sodium chloride has an influence on their metabolic activities.

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 pH 7, suggesting that these isolates should be considered as halophilic according to the definition of Ventosa *et al.*, 1998. Out of 10 isolated, 4 were found to be Gram Positive and 6 were found to be Gram negative. Strains S2, S3, S5, S7, S8 and S10 were found to be Gram negative rods, whereas, S1, S4, S6 and S9 were found to be Gram positive cocci. Similar result were obtained from Kanekar *et al.*, (2008) reporting Gram's positive bacterial strains from Lonar lake. Similarly Vidyasagar *et al.*, (2007), isolated Gram negative, rods shaped halophilic strains from solar evaporation pond.

Biochemical Identification

The sample were further subjected for the isolation and identification of an organisms. These isolates were confirmed on the basis of conventional, cultural and biochemical characteristics. The most frequently encountered bacteria were found to be *S.aureus*, *E. coli*, *P. aeruginosa*, *M. luteus* and *P. vulgaris* (Table No 1).

5. Determination of pH and Temperature range for growth

Influence of pH and Temperature on growth of halophilic organisms

During the study it was observed that isolate S5 and S10 doesn't show growth at any pH where as S3 and S4 shows excellent growth at all pH range. However S2 and S7 shows good growth at pH range of 8-10. Indicating that there is variation in pH range of all obtained isolates (Table No.2). Similar result were obtained from Kanekar *et al.* (2008), about the pH range for the organisms isolated from Lonar lake and the optimum pH is 9.8 and optimum temperature is 23°C.

After this it was inoculated in medium containing pH 8.5 and incubated at various temperatures. The range of different temperature in which isolates could grow on temperature ranges between 37°C- 45°C (Table No 2). Excellent growth was seen at temperature 45°C, and even a good growth was seen at temperature 37°C, but there is no growth or a very less growth is observed at this temperature of 25°C.

Yumoto *et al.*, (1989), reported that most species (60%) have temperature optima of 40°C. 25% of these have temperature optima of 38-40°C, and approximately 50% have temperature optima of 32-37°C. The remaining 25% have temperature optima between 30 and 32°C.

Halocin production

Halocin are proteinaceous antimicrobial compounds produced by halophilic archaea and is considered as their universal feature (Torreblanca *et al.*, 1994). Halocin production by isolates was detected by disc diffusion method (Shand, 2006).

Halocin production by the obtained isolates S2 and S4 shows very less zone of halocin production i.e. 0.8 mm and 0.9 mm respectively, followed by these two isolates S1 and S3 shows 1 mm zone. Very excellent and promising result was obtained in case of S5 which shows zone of 2.6 mm indicating better halocin ability. Previous reports have mentioned that pigment production in *S. aureus* has been associated with enhanced bacterial survival in harsh environment (Samuel Katzlf, *et al.*, 2005).

Similarly culture supernatant of halocin producing strains was also tested against the human pathogenic bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Micrococcus luteus* and *Proteus vulgaris* by Disc Diffusion Method with Inoculated Media as control.

Like that of halocin production similar pattern results were recorded in case of antagonistic activity of isolates. Isolates S2 and S4 shows less zone of inhibition i.e. 0.8 and 0.7 mm respectively. Moderate activity was shown by S3 1mm and S1 1.2 mm zone. Excellent results were recorded in case of S5 isolate showing zone of 1.8 mm. So from the result of halocin production and antagonistic activity it was confirmed that isolates S5 tentatively confirmed as *P. vulgaris* 5 in having better ability for halocin production and antagonistic activity.

The recently introduced Kirkup and Riley (2004), rock-paper-scissors model of bacterial antagonism (resistant-sensitive-producer) suggests that the production of antimicrobial substances may promote species diversity in an environment instead of restricting it.

6. Biosurfactant Method

For the confirmation of biosurfactant production ability of halophilic organisms different tests were performed to study the production of biosurfactant. The biosurfactant production ability of the organisms was checked by Phenol sulphuric acid method, Blood hemolysis test and Oil spreading test.

1) Phenol Sulphuric Acid Method:

The isolates S1 and S3 show phenol sulphuric acid test positive which is indicated by presence and production of orange colour after addition of H₂SO₄. Whereas rest of other isolates i.e. S2, S4 and S5 show phenol sulphuric acid test negative.

2) Blood Haemolysis Test

During the study isolates S1 and S3 show zone of β Haemolysis on S1 and α Haemolysis on S3. A Haemolysis zone was confirmed by production of greenish coloration zone whereas colorless zone indicates the zone of β Haemolysis. Bernheimer and Avigad (1970), reported that the biosurfactant produced by *B. subtilis*, surfactin, lysed red blood cells. Carrillo *et al.*, (1996) found an association

between hemolytic activity and surfactant production and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production.

3) Oil Spreading Technique

Oil spreading technique is one of the important and helpful for studying the emulsification study of organisms. Out of 10 isolates 4 isolates show clear zone at the center of oil layer indicating positive test for emulsification, whereas, in case of other isolates and no clearance zone indicating negative results.

Our results are shown in accordance with the result of Youssef *et al.*, (2004), they also reported that oil spreading assay method is as reliable technique for testing biosurfactant production. These results suggested that the other method for biosurfactant detection in the supernatant from a culture medium. Morikawa *et al.*, (2000), reported that the area oil displacement in oil spreading assay is directly proportional to the concentration of biosurfactant in the solution.

7. Acknowledgement

The authors gratefully acknowledge the instrumentation facilities for this work provided by college (Shri Shivaji College of Arts, Commerce and Science, Akola) through DST – FIST.

References

- [1] Atanosova, N., Pietilä, M., Oskanen, H. (2013). Diverse antimicrobial interactions of halophilic archaea and bacteria extend over geographical distances and cross the domain barrier. *Microbiol. Open.*, **2**(5): 811-825.
- [2] Bachana R. E, Gibbons N. E, Cowan S T, Holt J G, Liston Murray R G C, Niven C.F, Ravin A W and Stanier R Y (1974). "Bergey's Manual of Determinative Bacteriology" 8 edition The Williams and Wilkins Company, **580**: 217 - 548.
- [3] Bernheimer A. W, Avigad LS (1970). Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *J Gen Microbiol.*, **61**: 361-369.
- [4] Cameron, D. R., Cooper D., Neufeld R. J. (1988). The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. *Appl. Environ Microbiol.*, **54**(6): 1420.
- [5] Carrillo P. G, Mardaraz C, Pitta-Alvarez SJ, Giulietti AM (1996). Isolation and selection of biosurfactant-producing bacteria. *World J Microbiol Biotechnol* **12**: 82- 84.
- [6] Chen S.Y, Wei YH, Chang JS (2007). Reported pH – stat fed-batch fermentation for rhamnolipid production with indigenous *Pseudomonas aeruginosa* S2. *Appl. Microbiol. Biotechnol.*, **76**(1):67-74
- [7] DaSharma S., Arora (2001) Halophiles. *In Encyclopedia of life sciences, Nature Publishing Group, London.* www.els.net.app1-9
- [8] Keppeli O., Finnerty W. R. (1980). Characteristics of hexadecane partition by growth medium of *Actinobacter* spp. *Biotechnol. Bioeng.*, **22**: 495 – 503.

- [9] Kirkup B.C, Riley MA. (2004) Antibiotic-mediated antagonism leads to a bacterial game of rock-paper-scissors in vivo. *Nature.*, **428** : 412–414.
- [10] Knekar P. P, Joshi AA, Kelkar SA, Borgave BS, Sarnaik SS (2008). Alkaline Lonar lake, India—a treasure of alkaliphilic and halophilic bacteria. In: The 12th World Lake
- [11] Madigan M.T., Martinko J. M. and Parker J. (2004). *Microbiología de los microorganismos*, Pearson Prentice Hall, S.A. España.
- [12] Meseguer S.I. 2004. Los microorganismos halófilos y su potencial aplicado en biotecnología. *Revista Ciencia e investigación.* **7**(2) : 13 - 17
- [13] Morikawa M, Hirata Y, Imanaka, TA (2000). A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim Biophys Acta*, **1488** : 211 - 218.
- [14] Mudryk Z. and Donderski W. (1991). Effect of sodium chloride on the metabolic activity of halophilic bacteria isolated from the lake Gardno Estuary. *Estuaries.*, **14**(4) : 495-498.
- [15] Mulligan C.N, Cooper DG, Neufeld RJ (1984). Selection of microbes producing biosurfactants in media without hydrocarbons. *J Ferment Technol.*, **62** : 311-314.
- [16] Rodrigues L. Banat.u IM.Teixeria. J Oliveria R (2006). Biosurfactants: Potential applications in medicine. *J. Antimicrob. Chem.*, **57**(2):609-618
- [17] Rone E.Z, Rosenberg E (2001), . review of natural Roles of Biosurfactants. *Environ .Microbiol.*, **3**(4) : 229-236
- [18] Samuel Katzif, Eun-Hee Lee, Anthony B. Law, Yih-Ling Tzeng, William M. Shafer, (2005). CspA regulates pigment production in *Staphalococcus aureus* through a SigB-Dependant mechanisms. *J. Bacteriaol.*, **187** (23) : 8181-8184
- [19] Shand, R. (2006). detection , quantification and purification of halocins peptide antibiotics from haloarchaeal extremeophiles. *Method Microbiol.* **35** : 703-718
- [20] Spring S, Ludwig W, Marquez MC, Ventosa A, Schleifer KH. (1996). *Halobacillus* gen. nov., with description of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of *Sporosarcina halophila* to *Halobacillus halophilus* comb. nov. *Int J Syst Bacteriol.*, **46** : 492–496.
- [21] Torreblanca, M., Meseguer, I., Ventosa, (1994). Production of halocin is practically universal feature of archaeal halophilic rods. *Lett. Appl. Microbiol.*, **99** : 151- 157.
- [22] Ventosa A.(2006). Unusual micro-organisms from unusual habitats: hypersaline environments. In Logan N, Lappin-scott HM, Oyston PCF (eds). S M symposium. 66: Prokaryotic diversity - mechanisms and significance.
- [23] Ventosa, A., Nieto, J. J. and Oren, . (1998). Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.*, **62** : 504–544.
- [24] Ventosa, Antonio, Joaquín J. Nieto, and Haron Oren. (1998). “Biology of Moderately Halophilic erobic Bacteria.” *Microbiology and Molecular Biology Reviews* **62** (2) : 504–44
- [25] Vidyasagar M, Prakash S, Jayalakshmi S K, Sreeramulu K. (2007). Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium *Chromohalobacter* sp. TVSP101. *World J. Microbiol. Biotechnol.*, **23** : 655–662.
- [26] Yumoto I, Hirota K, Sogabe Y, Nodasaka Y, Yokota Y, Hoshino T. (2003). *Psychrobacter okhotskensis* sp. nov., a lipase-producing facultative psychrophile isolated from the coast of the Okhotsk Sea. *Int J Syst Evol Microbiol.*, **53** :1985 – 1989.