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Assessment of the Bacterial Biodiversity of Halophilic Bacteria at Akola District

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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, Hallophilic 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).

Hallophilic 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 biosurfac-tants 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 sampeling with water followed by methanol and sample were placed in zip-lock plastic begs & stored for no longer than 7 days at 6°C (Gary *et al.,* 2006).

Screening of Salt Tolerant Bacteria
Screaming of salt tolerant bacteria from soil sample was done by using modified Nutrient agar i.e containing varied concentration of NaCl & hallophilic 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 hallophilic 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).

Detemination of pH and Tempreture 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 inovulation 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 (25ºC, 37ºC,45º) to find influence of temperature on growth.

- **Screening of hallophiles**

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 (Mullligan *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 hallophilic organisms:

Name of Organism	*pH*					*Temperature*		
	8	*8.5*	*9*	*9.5*	*10*	*25°C*	*37ºC*	*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 dencity 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

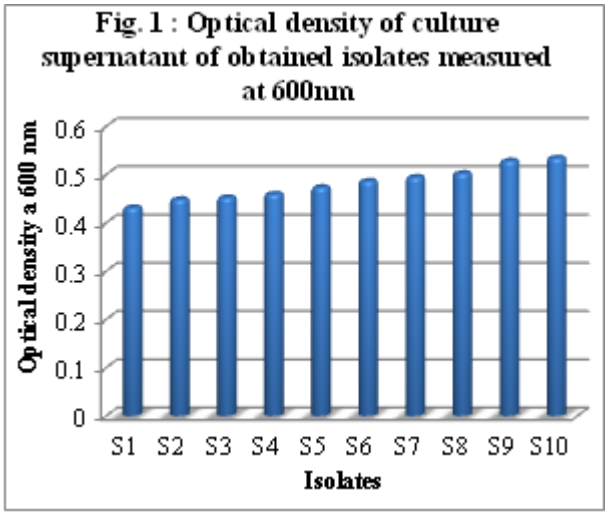

Table 3: Study of Halocin production by Disc Diffusion Method and Antagonestic activity of Hallocin producer organisms

Isolate	Diameter of zone of growth inhibition (mm) by Disc Diffusion Method	Diameter of zone of growth inhibition (mm) by Antagonestic 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 metod and blood homolysis test

Strain	S1	S2	S3	S4	S5
Phenol H_2SO_4 test	+ve	-ve	+ve	-ve	-ve
Blood homolysis test	+ve	-ve	+ve	-ve	-ve

+ Positive test, -ve Negative test

Table 5: Oil sprading test

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

(+) to (++++) crossponding to potential to complete spreeding on the oil surface.

4. Isolation

Hallotolerant organism can grow in either high salinity or in the absence of high concentration of salt. Many hallophiles 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 tolarent 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 Hallophilic agar medium containing NaCl, for further study colonies obtained were transfered 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 tolatrence 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 Tolarent Bacteria from Collected Soil Samples:
Total 10 isolates were obtained from 30 samples, out of which 5 were hallophilic and 5 were non-hallophillic. 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 ºC, 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 coccii. 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 islation and identification of an organisms. These isolates were confirmed on the basis of conventional, cultural and biochemical characterstics. The most frequently encountered bacteria were found to be *S.aureus, E. coli, P. aerugenosa, 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 hallophilic organisms
During the study it was observed that isolate S5 and S10 dosen'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 37ºC- 45°C (Table No 2). Excellent growth was seen at temperature 45ºC, 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 prteinaceous antimicrobial compounds produced by halophilic arechaea and is considered as their universal featmure (Torreblanca *et al.,* 1994) . halocin production 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 is *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 *Pseudomnas aeruginosa, staphylococcus aereus, Escherichia coli, Micrococcus luteus and Proteus vulgaris* by Disc Diffusion Method with Inoculated Media as control.

Like that of halocin production similar pattern results was 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 was 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 confermation of biosurfactant production ability of hallophilic organisms different test were performed to study the production of biosurfactant .The biosurfactant production ability of the organisms was checked by, Phenol sulphuric acid method, Blood homolysis test and Oil spreeding test.

1) Phenol Sulphuric Acid Method:

The isolates S1 and S3 shows phenol sulphuric acid test positive which is indicated by presence and production of orange colour after addition of H_2SO_4 . Whereas rest of other isolates i.e. S2, S4 and S5 shows phenol sulphuric acid test and negative.

2) Blood Haemolysis Test

During the study isolates S1 and S3 shows zone of β Haemolysis on S1 and α Haemolysis on S3. A Haemolysis zone was confirmed by production of greenish coloration zone where as 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 andsurfactant production and they recommended the use of blood agar lysis as a primary method to screen biosurfactant production.

3) Oil Spreading Technique

Oil spreeding technique is one of the important and helpful for studing the emulsification study of organisms. Out of 10 isolates 4 isolates shows 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 techniques for testing biosurfactant production. These result 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.

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