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Research Article

SCREENING OF COMMERCIALLY IMPORTANT HALOPHILIC MICROBIAL COMMUNITY FOR BIOACTIVE COMPOUNDS

Shamim Nezami, Katrin Joseph, Anam Ahsan, LVS Aparna, Shubha R. Sharma* and Santosh K. Jha

Department of Bio-engineering, Birla Institute of Technology,
Mesra, Ranchi-835 215, Jharkhand, India.

ABSTRACT

The importance of antibiotics was well established with the discovery of penicillin in the year 1928. The discovery of many such antibiotics paved way for a better and increased life span. Unfortunately the development of multiple drug resistance in microbes has led to these bio active compounds rendering useless. Thus the search for novel microorganisms producing bio active compounds has become of great importance. Extreme environments are those which were once considered unfavourable for the growth of microbes. But these environments too harbour a number of microbes producing novel bio active compounds. The Sambhar Salt Lake is India's largest salt lake located 96km south west of the city of Jaipur. In this study some halotolerant and halophilic strains of bacteria were isolated from the water and soil samples of the Sambhar Lake. These strains can grow in Nutrient media with different salt (NaCl) concentrations. Microbial assemblages and their species distributions in specific environments are mostly determined by their specific environmental conditions, which may translate into novel chemistry. Morphological, physiological and biochemical characteristics of these strains were studied by optimizing their growth conditions such as pH, salt concentration and temperature. It was found that the six isolated samples grew best in salt concentrations of 11% and at an optimum temperature of 45°C. They were found to be Gram negative, rod shaped and motile bacteria. Upon conducting tests for bio active compound production it was found that they produced bio active compounds against certain bacteria and fungi. Further analysis is needed in future to explore the type of bioactive compounds by the isolated microbes, the knowledge of which can lead to discovery of various products that may of medicinal as well as industrial use.

Keywords: Halophilic, bioactive compounds, biochemical tests, well diffusion, disc diffusion.

INTRODUCTION

The Sambhar Lake is one of the largest inland saline and alkaline lake of India which is situated in state of Rajasthan that experiences desert condition. It is situated in latitude $26^0$58'N and longitude $75^0$5' E on the east of the Aravalli hills. They are home to a number of organisms called halophiles which are salt loving organisms that inhabit hypersaline environments and are well equipped to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Halophiles can be grouped as slightly, moderately and extremely halophilic, on the basis of their requirement for NaCl (Kushner, 1993). Their novel characteristics and capacity for large-scale culturing make halophiles potentially valuable for biotechnology (Das Sarma and Arora, 2001). Halophiles produce a large variety of stable and unique biomolecules including hydrolytic enzymes (Birgul Ozcan *et al*, 2009). Screening of halophiles from various saline habitats of India, resulted in isolation of halophilic bacteria producing industrially significant and useful hydrolases (amylases, lipases and proteases).

High osmolarity in hypersaline conditions is deleterious to most cells since water is lost to the external medium. To prevent loss of cellular water, halophiles generally accumulate high solute concentrations within the cytoplasm

(Roberts, 2005; Yancey, 2005). When an isosmotic balance with the medium is achieved, cell volume is maintained. The compatible solutes or osmolytes which accumulate in halophiles are generally amino acids, sugars and polyols, which do not interfere with intracellular processes and have no net charge at physiological pH. Halotolerant yeasts and green algae accumulate polyols, whereas most halophilic and halotolerant bacteria accumulate zwitterionic species (containing both positive and negative charges), such as glycine, betaine and ectoine. Compatible solute accumulation may occur by biosynthesis, de novo or from storage material, or by direct uptake from the medium. A major exception is found among the haloarchaea and some extremely halophilic bacteria, which accumulate potassium chloride (KCl) equal to the external concentration of NaCl. These organisms produce acidic proteins that can function in high salinity by remaining solvated and reducing aggregation, precipitation and denaturation (Madern et al., 2000).
Antimicrobial substances are widely produced by bacteria. A number of bacteria produces Bacteriocins and bacteriocin like inhibitory substances (BLIS) that are ribosomally synthesized antimicrobial peptides and are often effective against closely related species (Riley and Wertz 2002, Cherif et. al 2003). They have received increasing interest, because of their potential use as food additives and their efficiency for the biological control of spoilage and pathogenic organisms (Delves-Broughton, 1990).

MATERIAL AND METHODS
Materials required
Water and soil sample were collected from Sambhar Lake, Rajasthan. Nutrient agar, yeast extract were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai. Sodium chloride, and other chemicals used were of analytical grade. Starch agar, tributyrin agar, peptone, gelatin, dextrose, phenol red, milk agar, SIM agar, Kovac's Reagent, MR-VP broth, Barritt's reagent, Simmon's Citrate Agar, 3% hydrogen peroxide, oxidase reagent, TSI Agar, urea agar were used for different biochemical tests.

Isolation & screening of Halophilic bacteria
Water and soil sample collected from Sambhar Lake, Rajasthan, were used for enrichment of halophilic bacterial species and were grown in modified nutrient agar and marine broth. The water sample was taken and serially diluted. The serially diluted sample was spread on the modified nutrient agar plates and incubated for 48 hours at different temperatures. The enrichment broths were used for isolation of halophilic bacterial species. Single distinct colonies were picked and slants prepared. A total 6 distinct halophilic bacterial isolated colony were checked against susceptible test organism i.e. *E-coli, Pseudomonas aeruginosa, Bacillus subtilis* and fungal pathogens *Penicillium notetum* and *Aspergillus niger* for bio active compounds production. After incubation the plates were checked for the appearance of zone of inhibition around the well and disc.

Optimization of salinity of growth media
Water samples of the Sambhar Lake were taken and serially diluted. Nutrient agar media with different concentrations of NaCl (i.e, 3%, 4% till 13%) were prepared and autoclaved. The sample was inoculated onto sterile Petri plates containing the modified nutrient agar media. The plates were incubated for 48 hours. Growth was observed and recorded.

Optimization of temperature of growth
The water samples were inoculated onto sterile Petri plates containing sterile modified nutrient agar media. They were then incubated at different temperatures (i.e., 37°C, 45°C and 50°C) for 48 hours. Growth was observed and recorded.

Observation of growth in Marine agar media
Water samples of the Sambhar Lake were taken and serially diluted. Marine agar media solution was prepared and autoclaved. The sample was inoculated onto sterile Petri plates containing the media. They were then incubated at 45°C for 48 hours. Growth was observed and recorded.

Observation of fungal growth
Water samples of the lake were serially diluted. Potato dextrose agar media was prepared and autoclaved. The serially diluted water samples were then inoculated onto sterile Petri plates containing the sterile PDA media. The plates were then incubated at 37°C for 48 hours. Growth was observed and recorded.

Gram staining of the halophiles
A loop full of bacterial culture was selected, spread onto a glass slide and heat fixed. The culture was then gram stained by Crystal violet, Gram's iodine and Saffranin stains. The glass slide was then observed under a microscope under 40X and 100 X magnifications.

Anti-bacterial assay
Anti-bacterial assay was done by two methods:
a) Well diffusion method
b) Disc diffusion method

a) Well diffusion method
Bacterial culture (*B.subtilis*) was prepared and inoculated on to the nutrient agar media plates. Isolated bacterial sample was centrifuged and supernatant was collected. The supernatant was then inoculated into the well of bacterial plates of *B.subtilis*, incubated for 36 hours at 45°C. Zone of inhibition around the well was observed and recorded.

b) Disc diffusion method
Bacterial cultures (*E.coli* and *B.subtilis*) was prepared and inoculated on to the nutrient agar media plates. Isolated bacterial sample was centrifuged and supernatant was collected. Disc made up of blotting paper was dipped into the supernatant and then placed on the bacterial plate of *E.coli* and *B.subtilis*, incubated for 36 hours at 45°C. Zone of inhibition around the disc was observed and recorded.

Anti-fungal assay
Anti-fungal assay was done by well diffusion method. The isolated bacterial sample was cultured. The culture was then centrifuged and the supernatant collected. Different fungal cultures (*A.niger* and *P.notatum*) were inoculated onto sterile Petri plates containing sterile Potato dextrose agar media. The supernatant was then inoculated into the well and tested against the above fungal cultures (*A.niger* and *P.notatum*) through well diffusion method. Zone of inhibition around the well was observed and recorded.

Biochemical tests
Starch hydrolysis
Starch agar was prepared and autoclaved. Petriplates were prepared containing starch agar. Bacterial isolates were inoculated on the starch agar plates and incubate at 45°C for 48 hrs. The plates were flooded with Gram's iodine and the plates were carefully observed for clear zone of hydrolysis around the colonies. Presence of clear zone around the growth indicates a positive result.

Lipid hydrolysis
Tributyrin agar was prepared and autoclaved. Petri plates were prepared containing tributyrin agar. Bacterial isolates were inoculated on tributyrin agar plates and incubate at 45°C for 48 hours. The plates were carefully observed for clear zone of hydrolysis around the colonies. Presence of clear zone around the colonies indicates a positive result.

Gelatin hydrolysis
Nutrient gelatine was prepared and autoclaved. Test tubes were prepared containing nutrient gelatin. Test organism were inoculated in nutrient gelatine and incubated at 45°C for 48 hrs. Gelatin hydrolysis was tested by chilling the tubes with ice or putting it in freezer for 30 minutes. Positive result is indicated by the absence of solidification in the tubes.

Carbohydrate fermentation
Peptone water broth containing 1% solution of desired sugar (lactose, dextrose and sucrose) was prepared and autoclaved. Phenol red as an indicator was added to the broth. Broth was dispensed in the Durham's tube and the tubes were inserted into the test tubes containing broth. The test tubes were inoculated with bacterial isolates and incubated at 45°C for 48 hrs. Test tubes were observed for gas and acid production. Positive result is indicated by a change in colour from red to yellow and bubble formation in Durham's tube.

Indole production
SIM Agar was prepared and autoclaved. Test tubes were prepared containing SIM agar. Test organism were inoculated into the test tubes and incubated at 45°C for 48 hrs. After incubation Kovac's reagent was added and tubes were observed for the change in colour. Appearance of cherry red colour indicates a positive result.

Methyl Red Test
MR-VP broth was prepared and autoclaved. Test tubes were prepared containing MR-VP broth. Test organism were inoculated into the tubes and incubated at 45°C for 48 hrs. After incubation methyl red indicator was added and test tubes were observed for change in colour. Appearance of red colour in the tubes indicates positive result.

Voges-Proskauer Test
MR-VP broth was prepared and autoclaved. Test tubes were prepared containing MR-VP broth. Test organism were inoculated into the tubes and incubated at 45°C for 48 hrs. After incubation Barritt's reagent was added and test tubes were observed for change in colour. Appearance of rose pink colour indicates a positive result.

Citrate Utilization
Simmon's Citrate Agar was prepared and autoclaved. Slants were prepared in the sterile test tubes. Test organisms were streaked onto the slants and incubated at 37°C for 48 hrs. After

incubation, the change in colour of the slants was observed. Change in colour from green to deep blue indicates a positive result.

Hydrogen Sulphide Test
SIM Agar was prepared and autoclaved. Test tubes were prepared containing SIM agar. Test organisms was inoculated in the test tubes and incubated at 45°C for 48 hrs. After incubation Kovac's reagent was added and the tubes were observed for change in colour. Appearance of black precipitate in the tubes indicates positive result.

Motility Test
SIM Agar was prepared and autoclaved. Test tubes were prepared containing SIM agar. Test organisms was inoculated in the test tubes and incubated at 45°C for 48 hrs. After incubation the test tubes were observed for its growth pattern. If the growth is horizontal, then the organism is motile.

Catalase Test
A drop of 3% hydrogen peroxide was placed on a glass slide. Loop full inoculums was taken and added to the drop of hydrogen peroxide. Catalase production was indicated by prompt effervescence.

Oxidase Test
A drop of freshly prepared oxidase reagent was putted on a piece of filter paper. A test colony was picked with sterile loop and rubbed it on the paper impregnated with oxidase reagent and then change in colour was observed. Positive result is indicated by a colour change from blue to deep purple.

Triple Sugar Iron Test
TSI Agar was prepared and autoclaved. . Slants were prepared in the sterile test tubes. Test organisms were inoculated onto the slants (stab and streak method) and incubated at 45°C for 48 hrs. After incubation the change in colour of the slants was observed.

Probable results

- Alkaline slant (red) and acidic butt (yellow) with or without gas production(breaks in the agar butt).
- Acidic slant (yellow) and acidic butt (yellow) with or without gas production.
- Alkaline slant (red) and alkaline butt (red) or no change (orange-red) butt.

Nitrate Reduction Test
Nitrate broth was prepared and autoclaved. Test tubes were prepared containing nitrite broth. Test organisms were inoculated in the test tubes and incubated at 45°C for 48 hrs. After incubation alpha-naphthylamine and sulphanilic acid was added and the change in colour of tubes was observed. Results were verified by adding zinc powder. Positive result is indicated by the appearance of red colour in the test tubes.

Urease test
Urea broth was prepared and autoclaved. Isolates were inoculated into the broth and tubes were incubated at 45°C for 48 hours. Phenol red (1mg/ml) solution was added drop wise into the tubes. Change in colour was observed. Development of pink colour indicates a positive result.

Carbohydrate fermentation
Peptone water broth containing 1% solution of desired sugar (lactose, sucrose and dextrose) was prepared. Phenol red as indicator was added. Broth was dispensed in the tubes and inverted Durham's tube was inserted in each tube. Isolates were inoculated and incubated at 45°C for 48 hours. Change in colour and gas production was observed. Positive result was indicated by a change in colour and bubble formation in Durham's tube.

Partial extraction of bioactive compounds produced by halophiles
Partial extraction was done to extract bioactive compound from the bacterial culture. Partial extraction was done by two different methods:
a) Ammonium sulphate precipitation method
b) Solvent extraction method

a) Ammonium sulphate precipitation method
The bacterial isolate was inoculated into the modified nutrient broth and incubated at 45°C for 48 hours. After incubation the bacterial sample was centrifuged and the supernatant was collected. For 20ml of supernatant, 20% to 90% of ammonium sulphate was weighed according to the ammonium sulphate precipitation standard chart.
The salt was added to the supernatant slowly. The percentage of the salt was increased gradually and stirred on ice until the ammonium sulphate was completely dissolved. After precipitation was observed between 80 to 90%, the solution was centrifuged and the supernatant and the pellet were separated. The pellet was dissolved in 10ml of methanol.

Bacterial culture (*S.typhi*) was inoculated onto sterile petri plates containing sterile nutrient agar media. The pellet and the supernatant were then tested against the above mentioned bacteria (*S.typhi*) through well diffusion method.

Solvent extraction method

The bacterial sample was cultured in modified nutrient broth. This culture was then centrifuged and the supernatant was collected. The supernatant was mixed in three different solvent n-hexane, petroleum ether and iso-butanol separately. The mixture of supernatant and solvent were kept at 4°C for overnight. The organic phase and aqueous phase were separated with the help of pipette. The organic and aqueous phase were tested for antibacterial activity against above bacteria (*Salmonella typhi* and *Pseudomonas aeruginosa*) through well diffusion method.

RESULTS AND DISCUSSION

Optimization of salinity of growth media

The water sample was inoculated on the plates containing modified nutrient agar with different salt concentration (3% to 13%). Optimum growth was observed at 11% NaCl. (Figure1)

Optimization of temperature of growth

The water sample inoculated on the plates containing modified nutrient agar and incubated at different temperature (37°C, 45°C and 50°C). Optimum growth was observed at 45°C. (Figure 2)

Observation of growth in Marine agar media

In marine agar lesser colonies with greater diameter were isolated indicating modified nutrient agar is suitable for isolation of different colonies. (Figure 3)

Observation of fungal growth

Only few fungal colonies were observed on Potato dextrose agar media. (Figure 4)

Gram staining of the halophiles

Different sizes and shapes of colonies were observed after an incubation period of 48 hours. Gram staining revealed that the bacteria present in the sample was gram negative. Pink coloured, rod shaped bacteria was observed. (Figure 5)

Anti-bacterial tests

a) Well diffusion method

Microbial culture was inoculated into the well of bacterial plates of *B. subtilis* and incubated for 36 hours at 45°C, zone of inhibition was observed around the well. (Figure 6)

b) Disc diffusion method

Disc made up of blotting paper was dipped into the microbial sample and the placed on the bacterial plate of *E.coli* and *B.subtilis* and incubated for 24 hours at 37°C. Zone of inhibition observed around well. (Figure 7)

Anti-fungal tests

Well diffusion method

Microbial culture was inoculated into the well of fungal plates of *A.niger* and *P.notetum* incubated for 36 hours at 27°C that result into the formation of zone of inhibition around the well. (Figure 8)

Biochemical tests for 6 different isolates

The biochemical tests for the 6 different isolates are as shown in Table 1.

Identification of bacterial isolates

The isolated bacterial strains were identified on the basis of their morphological characteristics and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology as:

SL1, SL3 and SL6 might belong to genus *Aeromonas*, *Pseudomonas* and *Vibrio.* SL2 and SL5 might belong to *Proteus* and *Yersinia.* SL4 may belong to *shigella* and *yersinia.*

As the bacteria are isolated from hypersaline environment, it thrives best at higher salinity upto 11% NaCl. In Modified Nutrient agar with higher percentage of NaCl, more number of colonies with smaller diameter were isolated while in Marine agar lesser colonies with greater diameter were isolated indicating modified nutrient agar is suitable for isolation of different colonies. Following are the findings:

- The bacteria isolated from the sample are halophiles, Gram (-ve), rod shaped, motile.
- The bacteria produces bioactive compounds which are extracellular in nature.

Partial extraction of bioactive compounds produced by halophiles

a) Ammonium sulphate precipitation method

Ammonium sulphate precipitation method was done and then sample was centrifuged, supernatant and pellet was separated. Antibacterial assay against *Salmonella typhi* was done for both pellet and supernatant. Zone of inhibition was observed only for the supernatant.

This result shows that the antimicrobial components are not a protein as the zone of inhibition was not shown by pellet. (Figure 9)

b) Solvent extraction method
Organic and aqueous phase were separated by using three solvents (petroleum ether, n-hexane and iso-butanol) and antibacterial assay was performed against *Salmonella typhi* and *Pseudomonas aeruginosa* for both phases. Organic and aqueous phases separated by iso-butanol only shows the zone of inhibition.
This indicates that the antimicrobial component may have maximum partition coefficient in iso-butanol. (Figure 10, Figure 11 & Figure 12)

CONCLUSION
After analysing the results it was found that the six isolated samples grew best in salt concentrations of 11% and at an optimum temperature of 45°C. They were found to be Gram negative, rod shaped and motile bacteria. Upon conducting tests for bio active compound production it was found that they produced bio active compounds against certain bacteria and fungi. The bacteria produces bioactive compounds which are extracellular in nature. Further analysis is needed in future to explore the type of bioactive compounds by the isolated microbes, the knowledge of which can lead to discovery of various products that may of medicinal as well as industrial use.

CONFLICT OF INTEREST
The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENT
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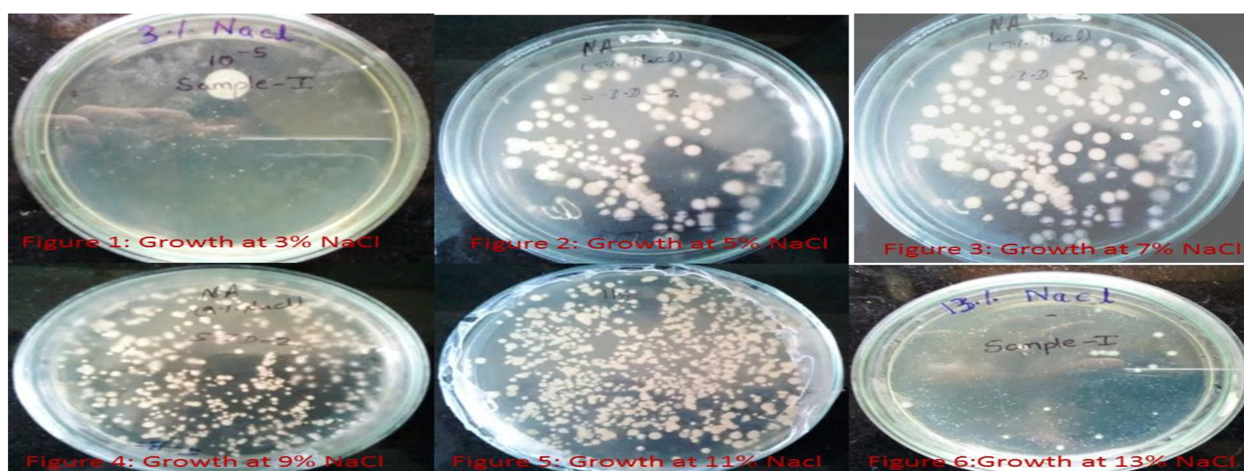

Figure 1: Growth of the isolates at different concentration of NaCl in Nutrient Agar

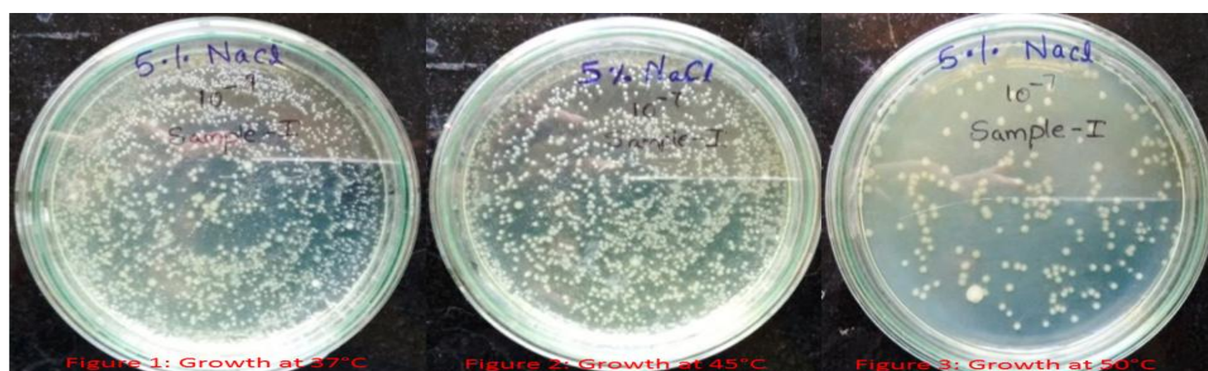

Figure 2 : Growth of isolates at different temperature conditions

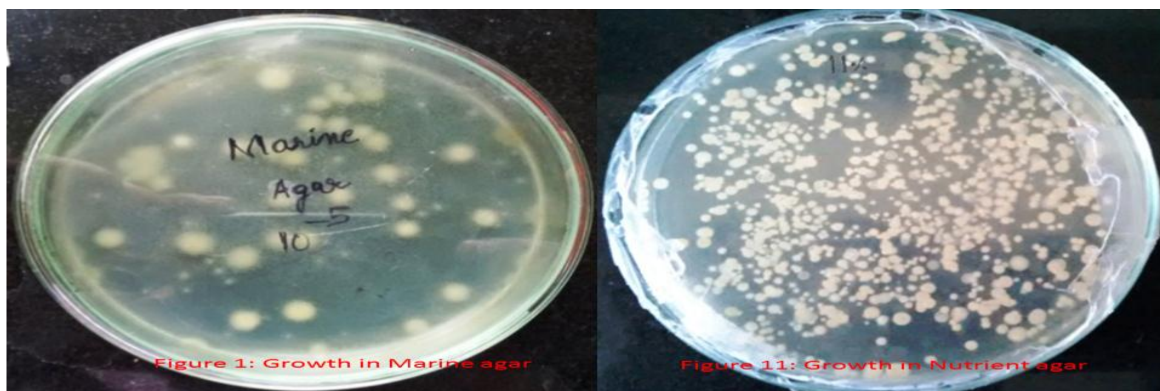

Figure 3 : Growth in different bacterial growth media

Figure 4 : Fungal growth

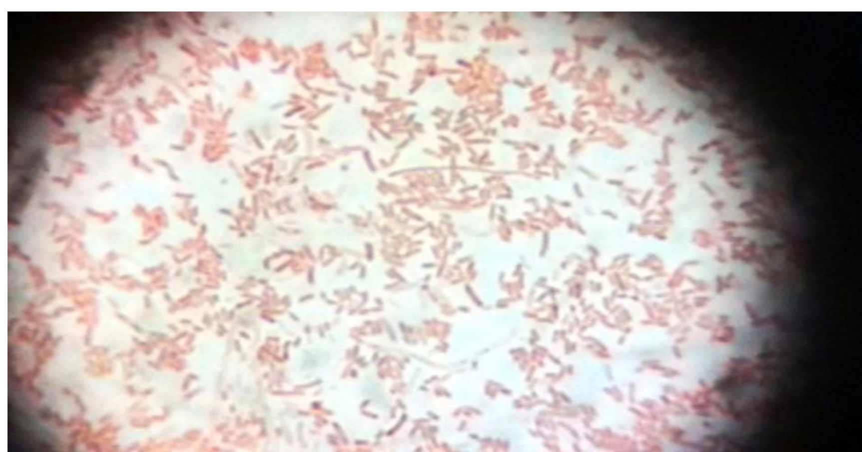
Figure 5 : Gram staining

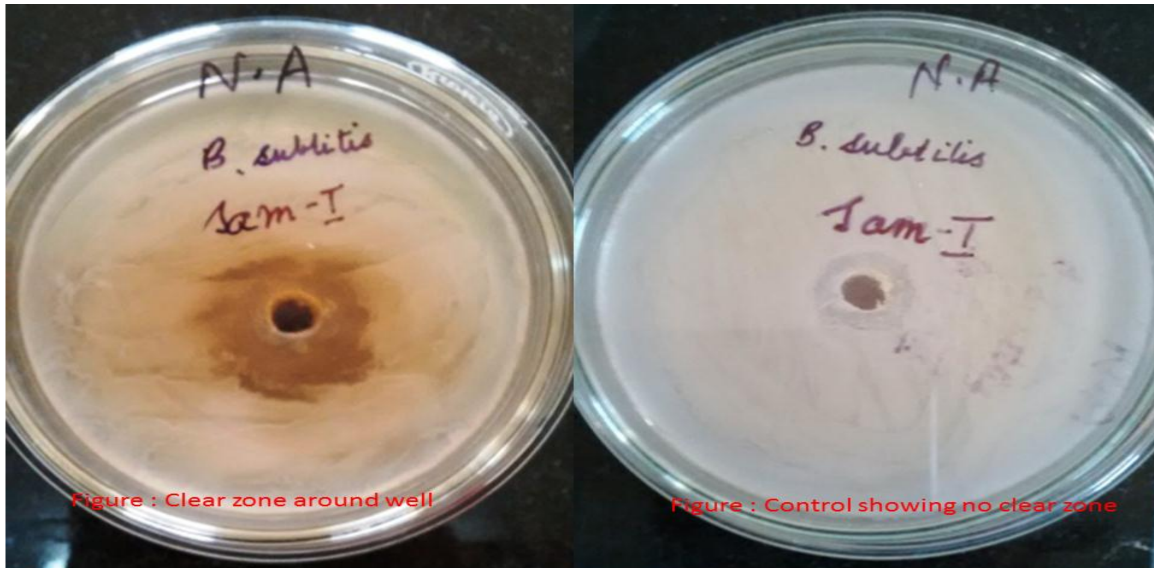

Figure : Clear zone around well

Figure : Control showing no clear zone

Figure 6 : Antimicrobial effect shown by compound produced by bacterial isolates

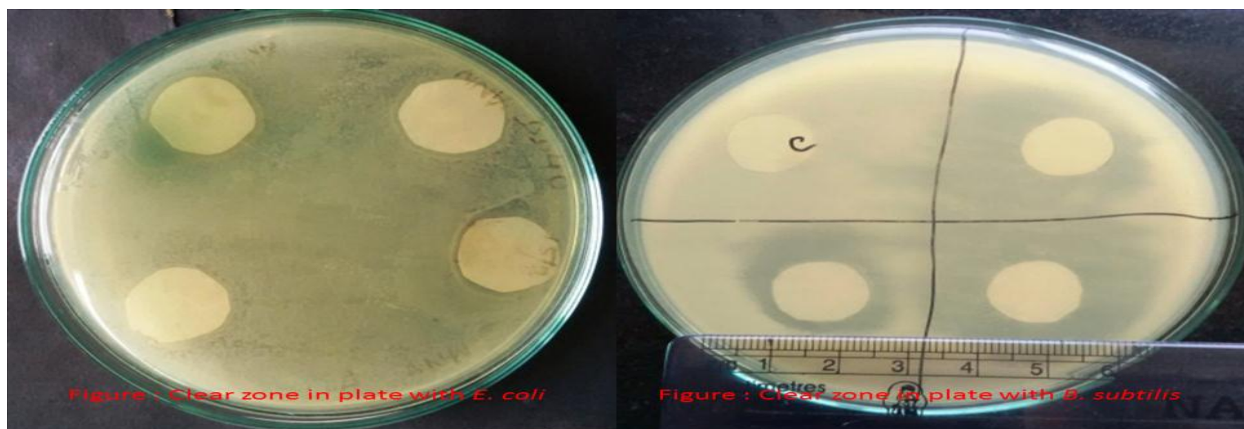

Figure : Clear zone in plate with *E. coli*

Figure : Clear zone in plate with *B. subtilis*

Figure 7 : Clear zone in plate with *B. subtilis* by disc diffusion method

Figure : Clear zone surrounding well in plate with *P.notatum*

Figure : Clear zone surrounding well in plate with *A.niger*

Figure 8 : Antifungal effects shown by the compounds produced by bacteria

Figure 9 :For isolate SL1 and SL2 Fig: For isolate SL3 and SL4 Fig: For isolate SL5 and SL6

Antimicrobial components are not a protein as the zone of inhibition was not shown by pellet.

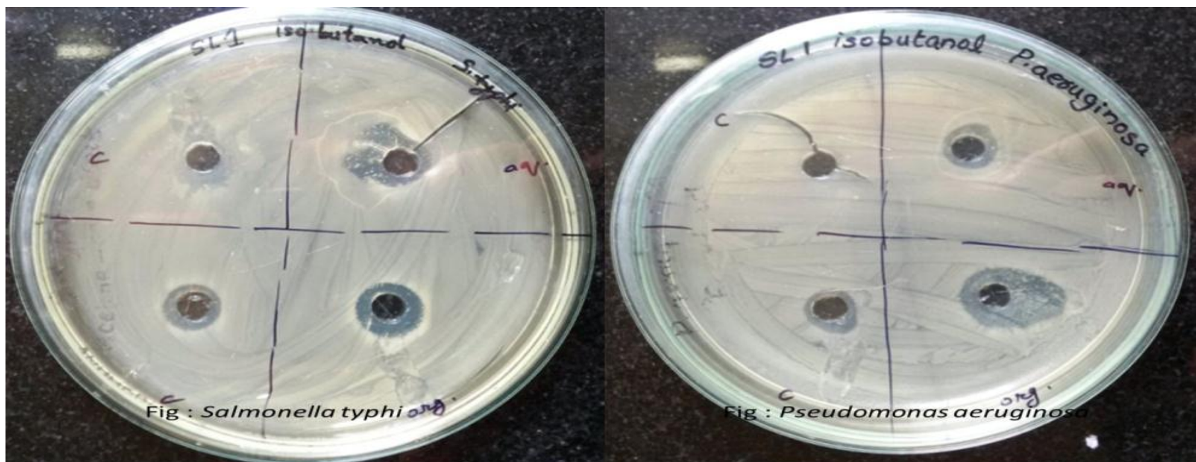

Figure 10: Solvent extraction was done by using iso-butanol

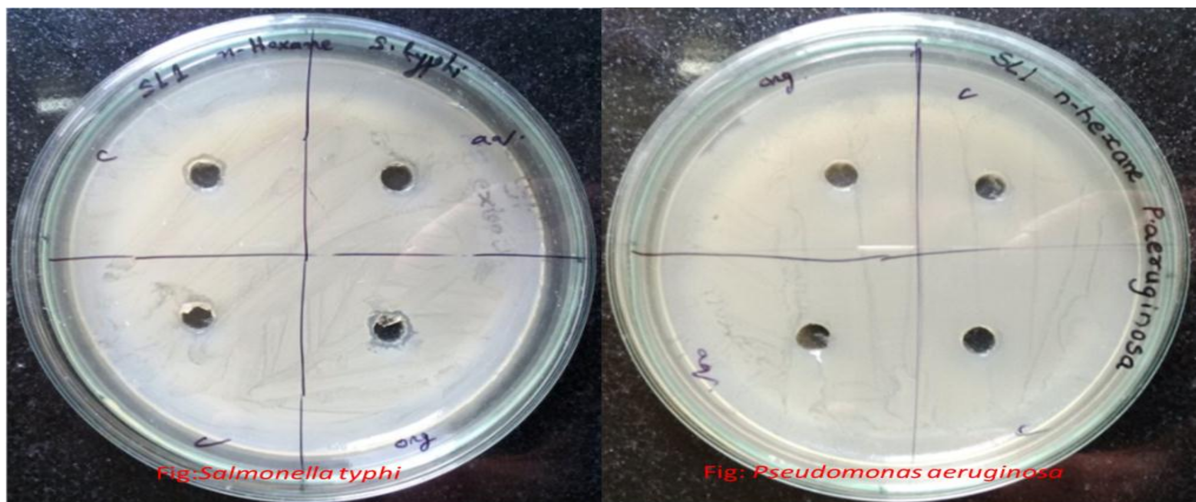

Figure 11 : Solvent extraction was done by using n-hexane

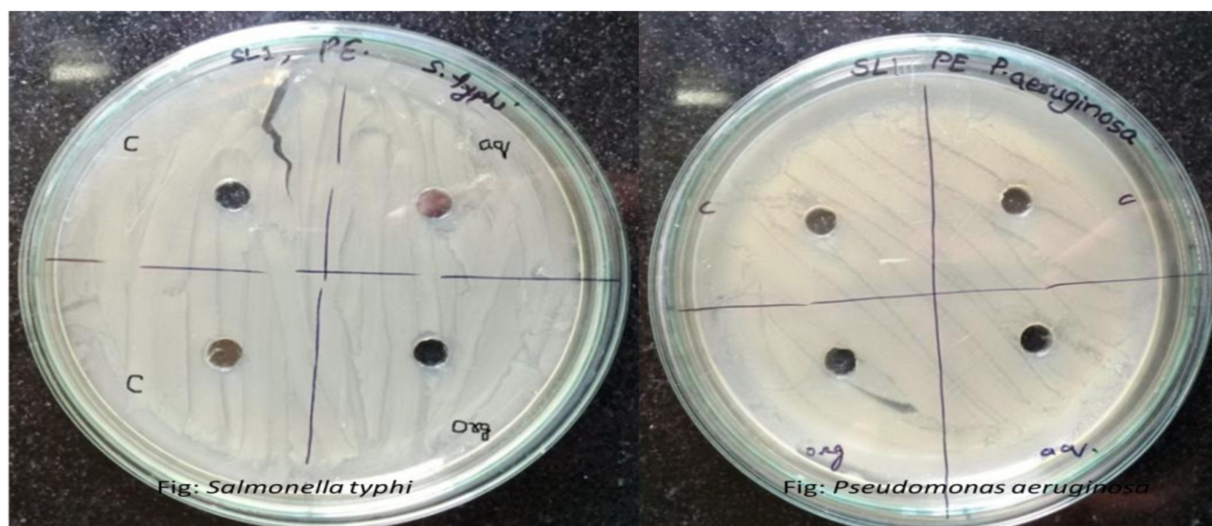

Figure 12 :Solvent extraction was done by using petroleum ether

Biochemical test	SL1	SL2	SL3	SL4	SL5	SL6
Motility test	Non motile	Motile	Non motile	Motile	Motile	Motile
H2S production	-ve	-ve	-ve	-ve	-ve	-ve
Starch hhydrolysis	-ve	+ve	-ve	+ve	-ve	-ve
Gelatine hydrolysis	-ve	-ve	-ve	-ve	-ve	-ve
Indole production	-ve	-ve	-ve	-ve	-ve	-ve
Methyl red test	-ve	+ve	-ve	+ve	-ve	-ve
Voges Proskauer test	+ve	+ve	+ve	+ve	-ve	-ve
Citrate test	-ve	+ve	-ve	+ve	-ve	-ve
Oxidase test	+ve	-ve	+ve	-ve	-ve	+ve
Catalase test	-ve	-ve	-ve	-ve	-ve	-ve
Urease test	-ve	+ve	-ve	-ve	+ve	-ve
Triple sugar iron agar test	Alkaline slant (red) and Acidic butt (yellow)	Acidic slant (yellow) and Acidic butt (yellow)	Acidic slant (yellow) and Acidic butt (yellow)	Acidic slant (yellow) and Acidic butt (yellow)	Acidic slant (yellow) and Acidic butt (yellow)	Alkaline slant (red) and Acidic butt (yellow)

Table 1: Biochemical tests for 6 different isolates (SL1, SL2, SL3, SL4, SL5 and SL6)

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