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Isolation and Screening of Antibiotic producing Halophiles from Ratnagri coastal area, State of Maharahstra

Sandip Todkar*, Rohit Todkar, Leena Kowale**, Kirti Karmarkar**, Arvind Kulkarni*****

*Department of Biotechnology, **Department of Microbiology, ***Department of Zoology,
Gogate - Jogalekar College, University of Mumbai,
Ratnagiri- 415612, Maharashtra, India

***Abstract*-** The prime purpose of this study is to isolate and screen out antibiotic producing halophilc bacteria and to determine their activity against different pathogenic strains by agar cup method viz., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and fungi like *Aspergillus niger*, *Penicillium* sp. *Candida albicans* etc. The isolates were obtained from marine environments by crowded plate technique and were subjected to primary screening.

Enrichment of these organisms were carried out in Balanced Salt Medium broth and finally dialyzed so as to purify the proteins then it was characterized by SDS-PAGE electrophoresis. The purified crude extract was prepared. This is the first time we report at our coastal area, the presence of halophiles having antibacterial activity against various pathogenic microorganisms.

***Index Terms*-** halophiles, marine environment, antibacterial activity, zone of inhibition, SDS-PAGE, protein purification.

I. INTRODUCTION

Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt and inhabit hypersaline environments all over the world. There are currently 15 recognized genera in the family (Gutierrez, 2002) Halophilic microorganisms are found in all three domains like *Archare*, *Bacteria* and *Eukaryotes*. Moderate halophiles are those organisms growing optimally between 0.5-2.5 M salt (Kushner, 1927) Halophiles are categorized slight, moderate or extreme, by the extent of their halo tolerance. Halophiles can be found anywhere with a concentration of salt five times greater than the salt concentration. The metabolism of halophiles includes oxygenic and anoxygenic prototrophs, aerobic hetrotrophs, fermenters, denitrifiers and sulphate reducers. Halobacterium glycoprotein requires high NaCl concentration for structural stability. When suspended in low salt concentration, the wall protein denatures and this leads to lysis and cell death (Soo-Hoo, 1967) The emergence of antibiotic resistance is an evolutionary process that is based on selection for organisms that have enhanced ability to survive doses of antibiotics that would have previously been lethal (Cowen, 2008). Different antibiotics likes penicillin, erythromycin and gentamycin which used to be one of the important cures are now less effective because bacteria have become more resistant (Pearson, 2008). The requirement of high salt concentration for the structural stability of the protein be attributed to the low content of hydrophobic residues and accordingly weak hydrophobic interactions within the protein molecules. This advantage of halophiles has showed many medical applications (Lowe, 1993). Analysis of most of the halophiles strain E-367 harbors three different plasmids (pVC1, pVC2 & pVC3) as well as megaplasmids. Other plasmids that have been isolated and detected are pH11 from *Chromobater israelensis* (48 kb), pHS1 from *Halomonas subglaciescola*, about 70kb (Vergas C, 1995). *Halomonas elongota* containing a plasmid named pMH1, shown to have resistance against kanamycin , tetracyclin and neomycin (Fernandez Crastillo R, 1992). A comparatively wide range of taxa have been isolated from saltern crystalliser ponds, including members of the following genera: *Haloferax, Halogeometricum, Halococcus, Haloterrigena, Halorubrum, Haloarcula* and *Halobacterium* families (Oren, 2002). Thus the presence of plasmids might be responsible for the antibacterial activity.

II. MATERIALS AND METHODS

The study was carried out at Department of Microbiology and Department of Biotechnology, at Gogate-Jogalekar College, Ratnagiri during November 2011 to Febuary 2012.

A. Isolation of Halophiles from Marine environments

The organisms (halophiles) used in this study were isolated from the Ratnagiri coastal area, Maharashtra, India. The water sample was collected from the Mandvi beach (Arabic ocean) during December 2011 and was brought to the Microbiology laboratory in highly aseptic conditions. Halophiles from the sample had been isolated by pour plate, streak plate technique on Balanced Salt Medium agar and incubated at room temperature at 48 hours. For enrichment the broth inoculated with 1ml of sample was kept on rotary shaker at room temperature at 150rpm for 48 hours.

B. Screening of Halophiles for Antimicrobial activity

The primary and secondary screening was done. In primary screening the antimicrobial activity of crude culture filtrate were used to determine the effect of isolate by agar well diffusion method on Muller-Hilton agar per National Committee for Clinaical Laboratory Standards (NCCLS, 1999). The medium is modified with addition of certain essential amino acids except tryptophan so as to enhance growth of halophiles. Secondary screening was carried out with purified protein extract by ammonium sulphate precipitation and dialysis. The pathogenic strains used were *Staphylococcus aureus*, *Klebsiella pneumoniae*,

Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and fungi like *Aspergillus niger, Penicillium* spp. *Candida albicans* etc. obtained from NCIM Department National Chemical Laboratory, Pune.

C. Characterization of Halophiles from Secondary screening

The active isolates obtained from secondary screening were characterized by morphological method described by Nakazawa *et al, (*2006). Morphological method consists of Microscopic methods. The microscopic characterization was done with Trinocular Research Microscope (Carl Zeiss) Germany. Gram staining and motility was done so as to determine their Gram nature.

D. Enrichment

Enrichment step was carried out in 500ml of flask containing 100ml of BSM broth inoculated with 1 ml of sample and kept on rotary shaker for 48hours at 150rpm.

E. Protein Purification

The crude culture filtrate was mixed with saturated ammonium sulphate and kept overnight at 10^0C and then centrifuged. The precipitate was dialyzed in phosphate buffer having pH 7.5 so as to purify the protein. The sample is allowed to run on SDS-PAGE electrophoresis.

F. Isolation of Antibacterial components

The components needed for antibacterial activity was obtained from the crude filtrate by solvent extraction method. Ethyl acetate was added to the filtrate in the ratio of 1:1(v/v) and shaken vigorously for 1hour for complete extraction. The Ethyl phase that contains antibiotic was separated from aqueous phase, then it was evaporated in water bath at 60^0C and the residue obtained was used for further analysis.

G. Determination of Antibacterial activity

The same residue was taken so as to determine the antibacterial activity by agar cup method (Zamanian *et al,* 2005). The partially purified extract obtained by the evaporation of the ethyl acetate extract was dissolved in 1Ml 0.2M phosphate buffer (pH 7). Then 0.1ml of sample was added into well bored against test organisms and plates were kept for incubation for 37^0C for 24hours and observed. The diameter of zone of inhibition was measured with the help of Kirby-Bauer's chart.

III. RESULTS

Thus, the halophiles were isolated from marine environments having antibacterial activity and they found to be gram negative non-motile organisms. The halophiles which inhibits the growth of test organisms were *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and fungi like *Aspergillus niger, Penicillium* sp. *Candida albicans* and shows significant zone of inhibition against them.

Table 1 shows the antibacterial activity of halophiles (protein crude extract) against test organisms. The inhibition was found to be maximum against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* followed by *Klebsiella pneumonia*.

Table 2 shows the antifungal activity of halophiles (protein crude extract) against test fungus and inhibition was found to be maximum against *Candida albicans* followed by Aspergillus niger*, Penicillium spp.*

Table 1: In vitro antibacterial activity of halophiles (protein crude extract)

Organisms	Zone of Inhibition in (mm) Isolate I
Escherichia coli,	8.4
Pseudomonas aeruginosa,	5.4
Bacillus subtilis	5.2
Klebsiella pneumonia	4.2

Values are mean of three replicates

Table 2: In vitro antifungal activity of halophiles (protein crude extract)

Organisms	Zone of Inhibition in (mm) Isolate I
Candida albicans	8.4
Aspergillus niger	6.2
Penicillium sp.	6.4

Values are mean of three replicates

The result of SDS-PAGE shows the presence of potent proteins in the crude residues, responsible for the antibacterial and antifungal activity. The molecular weight of the isolate I was found to be 66 kDa.

IV. DISCUSSION

The antibacterial and antifungal assays of halophiles (protein crude extract) have shown that, the marine environments represent a potential source of new antimicrobial and antifungal agents. The marine environments and mangrove rhizosphere has enormous diversity of all aerobic as well as facultative anaerobic bacteria (Todkar *et al,* 2011). The protein crude purified extract showed greater activity than the crude culture filtrate. The antibacterial and antifungal profile of this (Isolates I) halophiles gives the findings that these strains may contain multiple plasmids as seen in other plasmid containing strains (Ghosh *et al,* 2009). *Halobacterium* is a group of Archaea that have a high tolerance for elevated levels of salinity. Some species of halobacteria have acidic proteins that resist activity of most of other organisms. It has been studied that, Ratnagiri coastal area shows tremendous diversity of various microorganisms (Todkar *et al,* 2011). In this study the maximum activity was observed against *Escherichia coli, Pseudomonas aeruginosa, **Bacillus subtilis*** followed by *Klebsiella pneumonia*. Further analysis of these (isolates I) would be possible by 16S-rRNA sequencing method so as reveal the genus and species level.

V. CONCLUSION

This study reveals the importance of halophiles present in marine environments and that may useful in control in diseases caused by bacterial and fungal pathogenic species. This halophile protein purified extract would have an increased importance in medicine and in health care industry again further research on the above aspects may be undertaken. Thus these protein purified extracts obtained from halophiles seems to be a potential source of arresting the growth and metabolite activities of various

pathogenic microorganisms. Thus it supports previous findings based on presence of *Bacillus spp.* in marine environments.

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AUTHORS

First Author – Prof. Sandip S Todkar**,** Gogate -Jogalekar College, University of Mumbai, Maharashtra, India, Ratnagiri - 415612, India. Email id - sandip_todkar7@rediffmail.com

Second Author - Mr. Rohit S Todkar, (student) Shivaji University, Kolahapur India

Third Author - Prof. Leena Kowale Gogate -Jogalekar College, University of Mumbai, Maharashtra, India, Ratnagiri - 415612, India. Email id - leenakowale@rediffmail.com

Fourth Author - Prof. Kirti S Karmarkar Gogate -Jogalekar College, University of Mumbai, Maharashtra, India, Ratnagiri - 415612, India.
Email id - Kirtikarmarkar85@gmail.com

Fifth Author - Prof. Arvind S Kulkarni Gogate -Jogalekar College, University of Mumbai, Maharashtra, India, Ratnagiri - 415612, India. Email id - drarvindkulkarni13@rediffmail.com

Corresponding Author - Prof. Sandip S Todkar**,** Gogate - Jogalekar College, University of Mumbai, Maharashtra, India, Ratnagiri - 415612, India.
Email id - sandip_todkar7@rediffmail.com
Mob:+919960149669.