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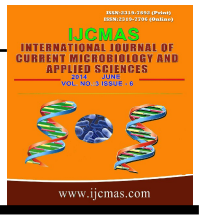

Original Research Article

Antimicrobial Activity of Secondary Metabolites from Halophilic *Bacillus pumilus* sp.

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A B S T R A C T

Halophilic bacteria are potential sources for novel bioactive compounds. Soil samples were collected from coastal area of Arabic ocean (Mumbai). 15 halophilic bacterial isolates were obtained from soil and screened for the production of antibiotics The bioactive secondary metabolites producing bacterial isolates were studied for their ability to tolerate 25% NaCl . Identification of *Bacillus pumilus* strains was done by using Biochemical test and 16S r-RNA Sequencing method. Separation of antibiotics was done by Column chromatography as well as Thin layer chromatography. Antibiotics were produced by two bacterial strains *Bacillus pumilus* (NKCM 8905) and *Bacillus pumilus (*AB211228) and tested against *E-coli* (ATCC# 2939), *Staphylococcus aureus* (ATCC# 96) , *Bacillus subtilis (*ATCC#441*)* and fungal strain *Aspergillus niger*(A1781*) and Aspergillus flavous* (A873) to be sensitive to antibiotics produced by *Bacillus pumilus.* The maximum production of antibiotics from *Bacillus pumilus* against, *Staphylococcus aureus, Bacillus Subtilis* and *Aspergillus niger and Aspergillus flavous*.

Keywords

Halophilic *Bacilli, E.coli, Staphylococcus aureus, Bacillus subtilis. Aspergillus niger and Aspergillus flavous.*

Introduction

The word “antibiotic” is derived from Greek term antibiosis, which literally means “against life”. It can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or for fundamental studies (Robbers *et al*., 1996; De Mondena *et al*., 1993). The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage and compost. Of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be of use in medical practice. A marine isolate of the bacterium *Bacillus pumilus* was obtained from a sediment sample collected in Marine Drive In Mumbai. Preliminary Studies showed that extracts of liquid shake culture of *Bacillus pumilus* exhibited antimicrobial activity against *E-coli, Staphylococcus aureous,Bacillus subtilis, Aspergillus niger and Aspergillus flavous.*

Materials and Methods

Soil sample were collected from Saltern house, Yeast extract, Casine acid hydrolysate were purched from Himedia Laborateries Pvt Ltd Mumbai. Potassium chloride, Magnesium Chloride, sodium chloride, trisodium citrate, ferrous chloride etc. chemicals used were of analytical grade.

Isolation of secondary metabolites producing Microorganism:

Soil sample were collected from Saltern house, and on cheked for the presence of antibiotics producing microorganism by using batch fermentation. Make a soil dilution add in Sehgal and Gibbons broth. Plates were incubated at 30°C for 24 hrs.

Identification of Bacillus Species:

Isolated strains were identified on the basis of their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974). And 16S r-RNA sequencing.

Production of secondary Metabolites by using *Bacillus* species:

Inoculum preparation

Inoculum was prepared in Sehgal and Gibbons broth by inoculating secondary metabolites producing *Bacillus* species separately and incubating at 30°C for 72 hours in an orbital shaker at 120 rpm.

Production media

About 10% inoculum of both the isolates was added in separate flasks, each containing 100 ml Sehgal and Gibbons media for checking the secondary metabolites production. Sehgal and Gibbons media contained (g/L) Sodium chloride 25gm, Magnessium Chloride Pottasium chloride,Ferrus chloride and Casine acid hydrolysate , Yeast extract, pH 7. Flasks were incubated at 30°C in an orbital shaker at 120 rpm. After every 24 hours, samples were taken up to 144 hours, centrifuged to get cell free supernatants.

Agar diffusion assay

Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites (Sen *et al*., 1995). Twenty-four hours fresh cultures of *Staphylococcus aureus, E-coli, Bacillus subtilis* and *Aspergillus niger, Aspergillus flavous* were diluted with presterilized normal saline and the turbidity of the 0.1 µl, cultures was spread on plate with bacterial lawn. Wel were prepared by using Borer and add 0.1ml broth Plate were incubated at 30°C for 24 hrs. Antimicrobial activity was measured in terms of zone of inhibition (mm) Figure1.

Extraction of Polypeptide compound from bacillus Species

Extraction of the Majority of high molecular weight biosurfactants is carried out using ammonium sulphate precipitation, by using dialysis to remove any any small molecules that may be present. Take 100ml of culture broth and remove cells by centrifuging at 10,000 rpm for 25 min. cool the supernatant at 4^0C.and add slowly while steering 23.34g o ammonium sulphate to obtained at 40% saturated solution keep at 4^0C.for overnight. Again centrifuge at 10,000 rpm for 25 min. to obtained the pellate, dissolve pellate in 25 ml distilled water

and extract with equal volume of hexane. Using dialysis tubing with a molecular weight cut of point 5 kDa clamp one end and rinse the tubing with distilled water to check for leaks. Fill the tube with product place in beaker with dialysis buffer of distilled water, place on the steering plate in a cold room at overnight. Remove sample after completion of dialysis.

Characterization of Protein by using Sodium Dodecyl Sulphate Polyacrylamine Gel Electrophoresis (SDS-PAGE)

Prepare 10% resolving gel as well as preparation of stacking gel and running buffer should be prepared. Setup gel electrophoresis as per standard procedure and pour the running gel place 1ml of distilled water saturated with butanol carefully on top of onomer solution and keep for 50 min. to allow the gel to polymerize add the stacking gel buffer with APS and TEMED being added just before pouring insert sample comb at an angle to allow air bubble escape keep set for 20 min. a remove the comb and place gel in the buffers chamber and add running buffer to cover the gel.

Prepare the protein sample to investigated by adding 25µl of proteins to 25µl of sample buffer and 2µl of beta-mercapto-ethanol keep incubation at 60^0C for 15 min. put on lid and connect power supply with 220 volts and run the gel stop the electrophoresis when the dye is less than 1.5 cm from bottom of plate. Remove the gel and immerse in comassie brilliant blue stain and keep at overnight pour off the comassie stain and destain by using 40% methanol in water containing 8% acetic acid cover gel with destain solution and keep at keep for 15 min. then remove. Record image of the gel.

Determination of protein by using Follin Lowery method

We were prepared Reagent A, Reagent B then make a Reagent C by using 50ml of Reagent A and 1. Ml of Reagent B just before use as well as Reagent D (Follin ceociocateaul) commercially available and has to be diluted with distilled water before use. The standard stock solution of the given protein was prepared as 20mg/100ml (200 µg/ml) different dilution were prepared using the above stock ranging from 20 µg in 1 ml to 200 µg/ml. To each 1ml of sample 5 ml of alkaline solution i.e. Reagent C was added and it was allow to stand at room temperature for 15 min. then 0.5ml of diluted Follin cociocateaul was added with shaking. Then makes keep at room temperature for 30 min. then observance was read at 660nm optical density was plotted and the protein concentration in the unknown sample was determined.

Column chromatographic separation of secondary Metabolites

Chromatography was carried out on a DEAE-52 cellulose ion exchange column (2.5 cm×50 cm; Whatman) previously equilibrated with 0.02 mol/L phosphate buffer (pH 6.8). The column was first eluted with 500 ml of the same buffer (pH 6.8) to yield unabsorbed proteins, and subsequently with a linear gradient of 0~1 mol/L NaCl in phosphate buffer0.02 mol/L, pH 6.8) to desorb the adsorbed proteins (fractions I, II, III, IV). Each fraction was analyse by using spectrophotometric study at 280nm.The II fraction shown highest OD and that fractions were used for demonstration of antimicrobial activity against *E.coli, Staphalococus aureus, Bacillus subtilis, Aspergilus niger* and *Aspergilus flavus.*

Antimicrobial activity of secondary Metabolites from Halophilic Bacillus Sp

The II fraction were chek there antimicrobial activity against Bacterial as well as Fungal Pathogen. i.e. *E.coli, Staphalococus aureus, Bacillus subtilis, Aspergilus niger* and *Aspergilus flavus.* The II fractions showing maximum growth inhibitions (figure 2) of three pathogen i.e. *E.coli, Staphalococus aureus* and *Aspergilus niger, Aspergilus flavus.*

Results and Discussion

The present work deals with the isolation, identification and characterization of the bacterial isolates from marine source. and its evaluation for antimicrobial activity against human pathogen.

Antimicrobial Activity of halophilic bacteria

The enriched Dunddas and Sehgal & Gibbons medium broth by water and soil sample marine source showed presence of grams positive rod and cocci and grams negative varied size rod shaped bacteria.
The distinct isolates in terms of cultural and morphological feature showed grams positive nature. The secondary metabolites obtained from these isolates have inhibited growth of human pathogen viz. *E.coli, Staph aureus, Bacillus subtilis, Aspergilus niger* and *Aspergilus flavus.*

The result revealed that the marine isolates have ability to produce secondary metabolites which have bioactivity against human pathogen. The bacterial isolates isolated from coastal region of Mumbai have ability to produce secondary metabolites against *E.coli, Staph aureus.* and *Bacillus subtilis, Aspergilus niger* and *Aspergilus flavus.*

Characterization of Protein by using Sodium Dodecyl Sulphate Polyacrylamine Gel Electrophoresis (SDS-PAGE)

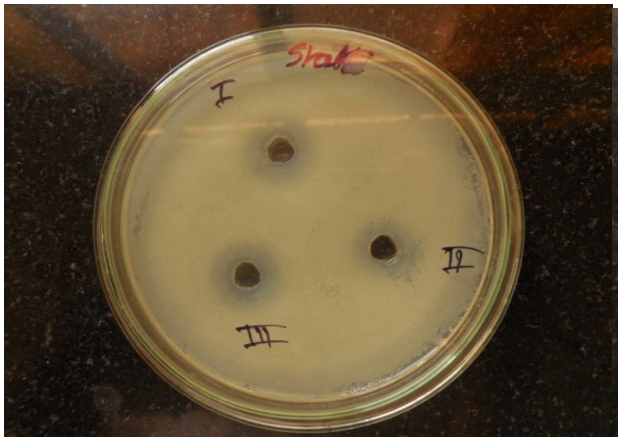
SDS-PAGE is commonly used for proteomic experiments and extremely useful for separation of protein mixtures. In present study to separate the Protein by using Sodium Dodecyl Sulphate Polyacrylamine Gel Electrophoresis Method. We were used three sample and got a various type of band were present in that method.

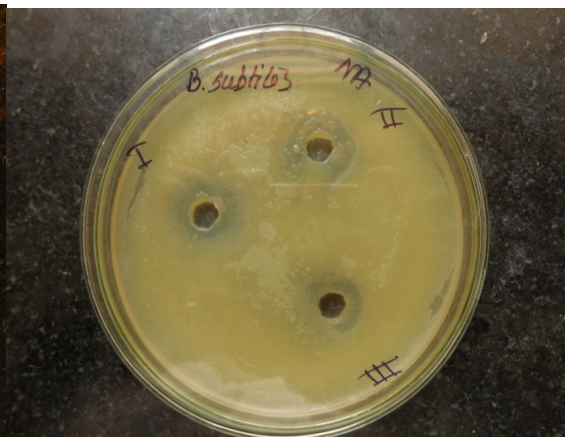
Determination of protein by using Follin Lowary method

Usally Follin Lowry method using for determination of protein after the dialysis method to determine the percentage of protein in crude extract. Taking A,B,C and D reagent performing the assay after that observance was read at660nm optical density was plotted and the protein concentration in the unknown sample was determined.

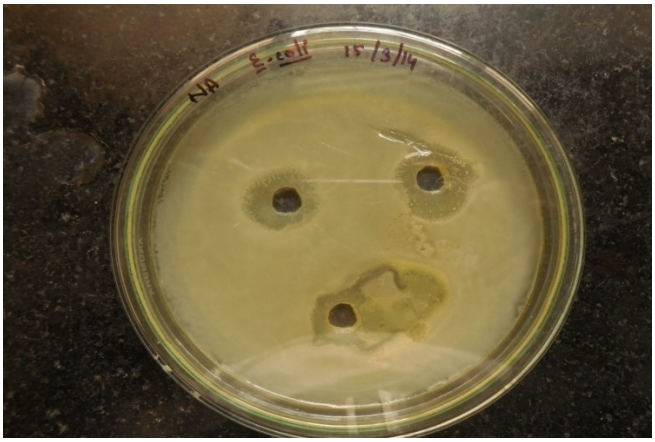
Column chromatographic separation of secondary Metabolites

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. Once the packing is complete, the sample can be loaded directly to the top of the column. Normally, a minimum amount of a polar solvent, 5-10 drops, is used to dissolve the mixture. The solution is then carefully added to the top of the column using a pipet without disrupting the flat top surface of the column.





Antibacterial activity Against *Staphylococcus* aureus. Antibacterial activity Against Bacillus subtilis.



Antibacterial activity Against *E. coli*

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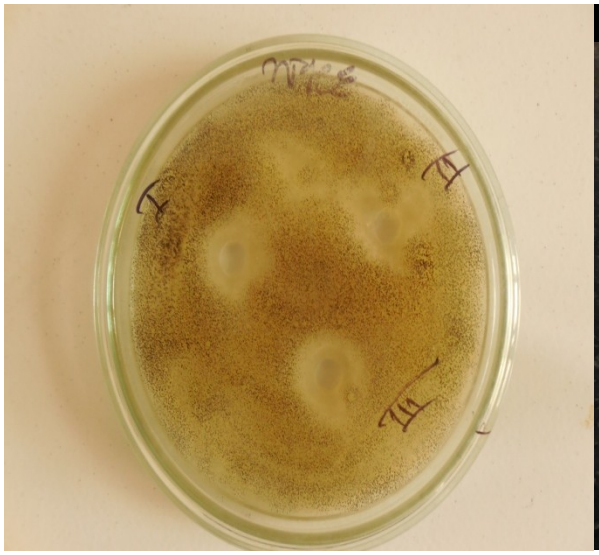

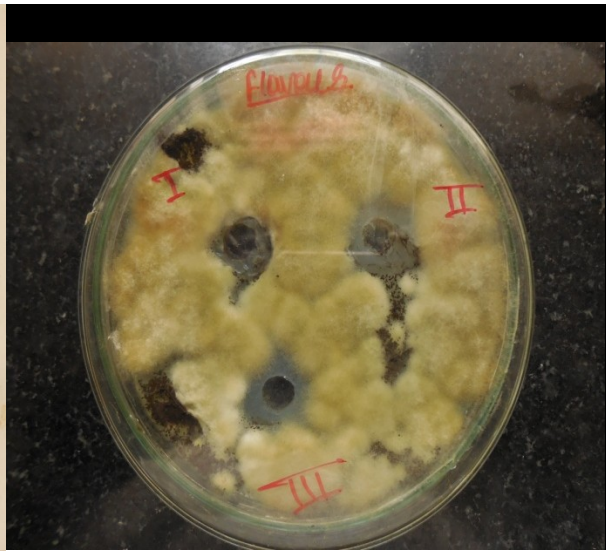

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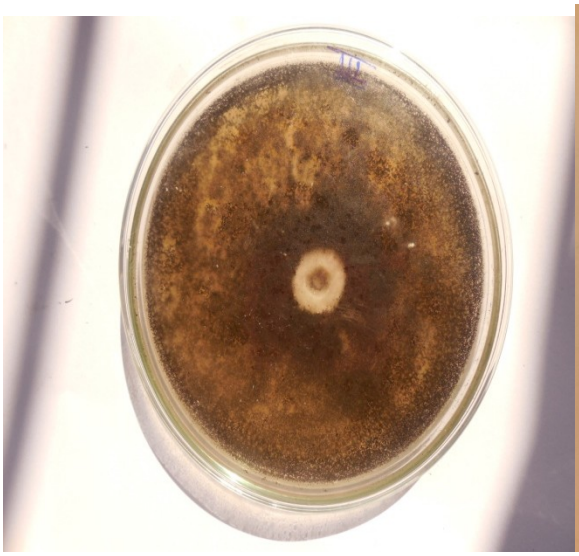
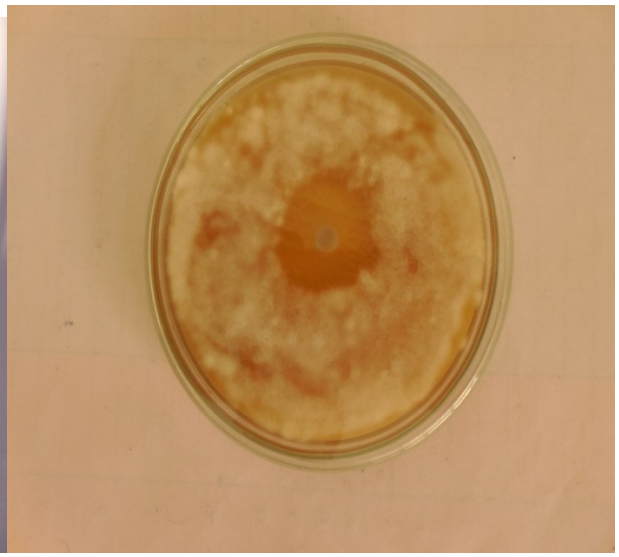
Antifungal Activity Against *Aspergillus niger* Antifungal Activity Against *Aspergillus flavus*



Antifungal Activity Against *Aspergillus niger*



Antifungal Activity Against *Aspergillus flavus*

A thin horizontal band of sample is best for an optimal separation continuously add the solvent eluent while collecting small fractions at the bottom of the column.

Production of Antibiotics from Halophilic *Bacillus Sp*

The collected fraction was taken so as to determine the antibacterial as wel as antifungal activity by agar wel method (Zamanian et al, 2005) 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. The fraction No.2 is given good activity against antibacterial as wel as antifungal.

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. 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 protein crude purified extract showed greater activity than the crude culture filtrate It has been studied that, arobic coastal area of Mumbai shows tremendous diversity of various microorganisms (Todkar et al, 2011). In this study the maximum activity was observed against *Staphylococcus aurous and Aspergillus niger.*

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