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Isolation and Characterization of Secondary Metabolites from Halophilic Bacillus Species from Marin drive in Mumbai

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ABSTRACT

The present study was focussed on production of novel antibiotics from Halophilic bacterial species in Marin drive Mumbai. *Bacillus pumilus* were isolated from soil and screened for the production of antibiotics by plate assay and then cultured in shake flask fermentation at 30ºC for further studies. The bioactive secondary metabolites producing bacterial isolates were studied for their ability to tolerate 3% NaCl. Identification of *Bacillus pumilus* strains was done by using biochemical test as well as 16S r-RNA sequencing method. Identification of antibiotics was done by column chromatography as well as thin layer chromatography. Antibiotics was found to be produced by three are bacterial and one are fungal pathogen strains against *E-coli* (ATCC#2939), *Staphylococcus aureus* (ATCC# 96), *Pseudomonas aeruginosa* (ATCC# 2488) and one are fungal pathogen strains against *Candida albicans* (ATCC# 227) proved to be resistant to antibiotics produced by *Bacillus pumilus.* The maximum production of antibiotics from *Bacillus pumilus* against *E-coli*, *Staphylococcus aureus* and *Candida albicans.* Maximum zones of inhibition were observed after 48 hours of incubation at 30ºC against *E-coli*, *Staphylococcus aureus* and *Candida albicans.* After that structural elucidation was done by using IR, MS and NMR spectroscopy respectively.

INTRODUCTION

Bacillus species are gram-positive aerobic or facultative anaerobic, sporulating rod shaped bacteria that are widely spread in nature (Ali Janabi *et al.,* 2006; Graumann, 2007). The Halophiles are extremophile organisms that thrive in environments with very high concentrations of salt. Halophiles are categorized slight, moderate or extreme, by the extent of their halotolerance. Halophiles can be found anywhere with a concentration of salt five times greater than the salt concentration of the ocean. During early research on the microbiology of hypersaline environments, the halophilic bacteria were often neglected, even though they inhabit a wide range of habitats such as saline lakes, saltern ponds, desert and hypersaline soils, and salted foods (Kushner *et al.,* 1978; Ventosa *et al.,* 1998), a range much less restricted than the habitats in which the halophilic archaea thriven. Moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt (Kushner *et al.,* 1978). Halophiles are found distributed all over the world in hypersaline environments, many in natural hypersaline brines in arid, coastal, and even deep sea locations, as well as in artificial slatterns' used to mine salts from the sea end for NaCl. Extreme environment including alkalophilic, oligotrophic, piezophilic, xerophilic and halophilic will be a best source for bioactive compound producing microorganisms. The marine environment as a prolific source of bioactive compounds from microorganisms. The halophiles can be loosely classified as slightly, moderately or extremely halophilic depending on their requirement for NaCl (Ghosh *et al.,* 2010). These include mostly microorganisms e.g. photosynthetic *green algae,* Cynobacteria, green and purple bacteria, sulfur oxidizing bacteria, sulfate reducing bacteria, gram negative and gram positive heterotrophic bacteria and methano gens and halophilic *archaea.*

MATERIALS

Water and soil sample were collected from arebic sea marine drive, Mumbai. Casamino acid, yeast extract were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai. Magnesium chloride, sodium chloride, potassium chloride, trisodium citrate, ferrous chloride and other chemicals used were of analytical grade.

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METHODOLOGY

The gram positive moderate halophilic bacteria produce an extensive array of ribosomally synthesized peptide antibiotics and lantibiotics. Several species of Bacillus produce other compounds including polyketides, isoprenoids, pumulin, sporulenes, ocumarines and zwittermicin therefore, in the present study bioactive secondary metabolites from moderate halophilic bacterial species were isolated, characterized and studied for bioactivity against *E.coli, Staph aureus, Pseudomonas aerugenosa* and *Candida albicans.*

Isolation & screening of Halophilic bacteria

Water and soil sample were collected from coastal area of arobic ocean (Mumbai), were used for enrichment of halophilic bacterial species and they in the Dunddas and Sehgal & Gibbons medium broth. The enrichment broths were used for isolation of halophilic bacterial species, then pick-up the distinct colony and streak on the slant. A total 12 distinct halophilic bacterial isolated colony were checked against susceptible test organism i.e. *E-coli, Pseudomonas auregenosa, Staph-aureus* and one fungal pathogen such as *Candida albicans*. After incubation the plates were checked for the appearance of zone of inhibition around the well.

Identification of salinity tolerance Halophilic bacterial isolates

They isolates were identified by performing biochemical test as indicating *Bergey's* Manual of Systematic bacteriology 9th edition (Kuta *et al.*, 2009), and by 16S r-RNA sequencing (Guzmán *et al.,* 2008). The isolates were studied for their ability to tolerate 3% NaCl. The salt tolerancing study was performed by inoculating 12 isolates separately in Sehgal and Gibbons medium containing 3% NaCl. The growth of these isolates was recording terms of turbidity after 48hrs of incubation.

Production and purification of bioactive compound

By using production media it was performed by inoculating 5% active inoculums of *Bacillus pumilus* strains and extracting secondary metabolites from broth by using chloroform. The extracted secondary metabolites of *Bacillus pumilus* strain were analyzed for different components by using TLC (Melo *et al.*, 2009). The solvent systems used were Benzene: Ethyl acetate: Methanol in ratio of 14:4:2. The distinct spot on TLC plate were further purified by column chromatography for obtaining individual compounds. The single eluted component were concentrated and used for demonstration of antimicrobial activity.

Structural analysis of bioactive compound

The single purified fractions were analyzed for structural elucidation by IR, MS and NMR spectroscopy respectively. The data obtained were interpreted for knowing probable structure.

Isolation of secondary metabolites producing microorganisms

Halophilic bacteria were isolated by using Dunddas and Sehgal & Gibbons agar medium. The isolation of halophilic bacteria was carried out by streaking a loopfull of enriched broth on respective media plate and plates were incubated at 30^{o}C for 48-72 hrs. The well isolated colonies were used for cultural and morphological characteristics.

Identification of secondary metabolites producing microorganisms

The bacteria were identified as *Bacillus pumilus* on the basis of their morphological and biochemical characteristics as well as 16S r-RNA sequencing method (Buchanan *et al*., 1974).

Antibiotic production by *Bacillus pumilus*

Samples drawn during batch fermentations were subjected to agar diffusion assay, using *E-coli, Staphylococcus aureus, Pseudomonas aureus* and *Candida albicans* as test organisms. Antimicrobial activity was measured in terms of zone of inhibition (mm) (Figure 1).

Column chromatographic separation of secondary metabolites

The distinct components of secondary metabolites in term of Rf value from three strain *Bacillus pumilus* were separated and separately purified by Column chromatography. The eluted purified fractions were used for demonstration of antimicrobial activity against *E.coli, Staph aureus, Pseudomonas aureoginosa* and C*andida albicans.*

The eluted fractions showing maximum growth inhibitions (figure 2) of three pathogen were analyzed for partial structure determination by using IR, MS and NMR spectroscopy were shown in figures 4, 5, 6, 7 and 8.

RESULTS AND DISCUSSION

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

Halophilic bacteria and 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, Pseudomonas aerugenosa* and *Candida albicans.*

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. Pseudomonas auregenosa* and *Candida albicans*.

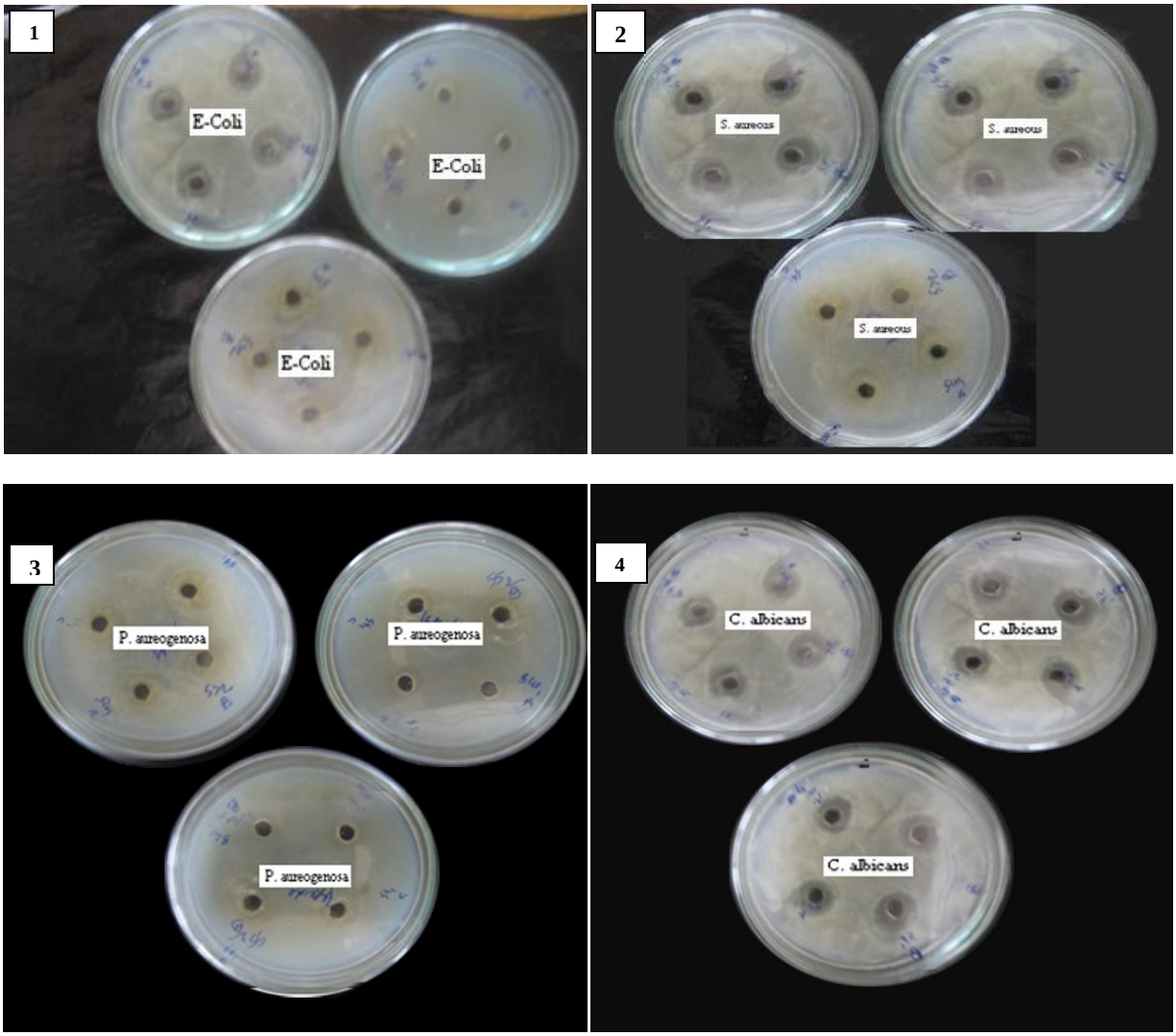

Fig. 1:
Plate. 1: Inhibition of *E. coli* Growth by Secondary metabolites of Halophilic bacterial isolates.
Plate. 2: Inhibition of *S. aureous* Growth by Secondary metabolites of Halophilic bacterial isolates.
Plate. 3: Inhibition of *P. aureogenosa* Growth by Secondary metabolites of Halophilic bacterial isolates.
Plate. 4: Inhibition of *C. albicans* Growth by Secondary metabolites of Halophilic bacterial isolates.

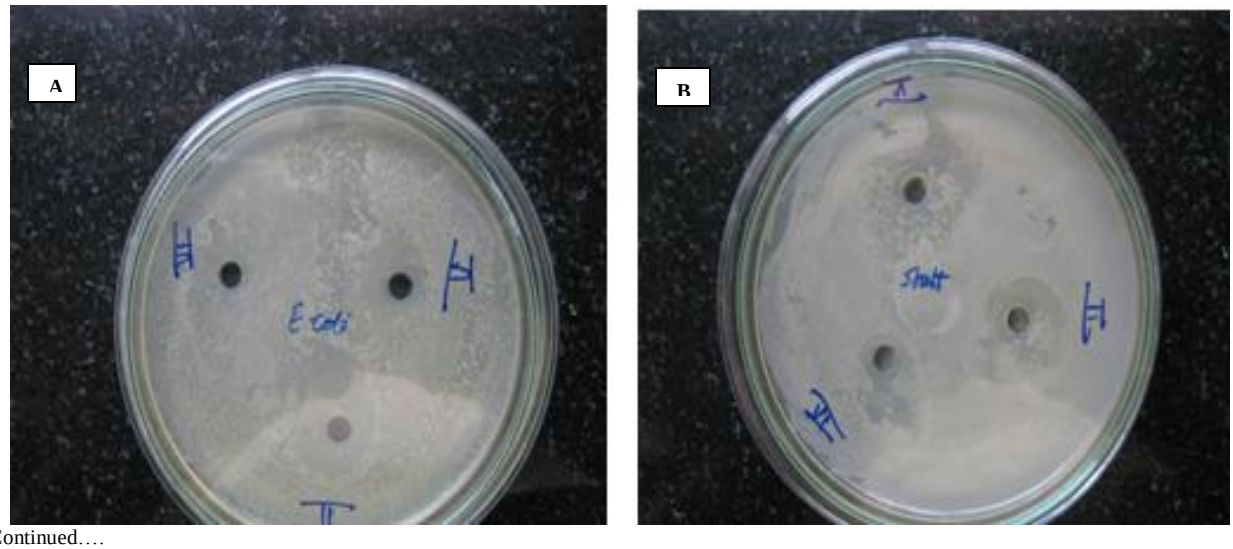

Fig. 2: Continued….

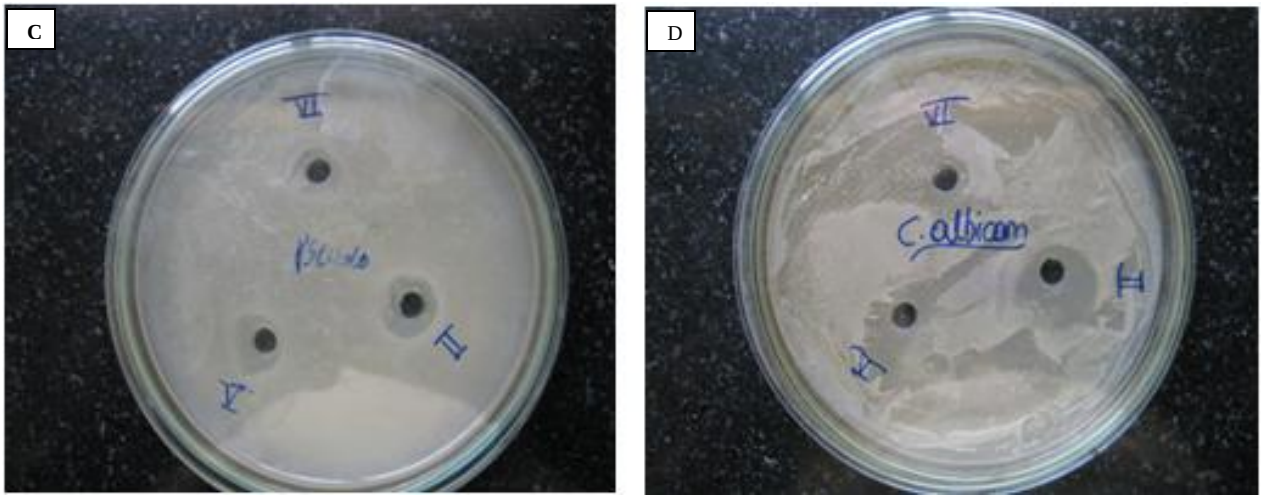

Fig 2. Antimicrobial activity of extracted fraction against Human Pathogen A= *E.Coli;* B= *S. aureous; C= P. aureogenosa; D= C. albicans.*

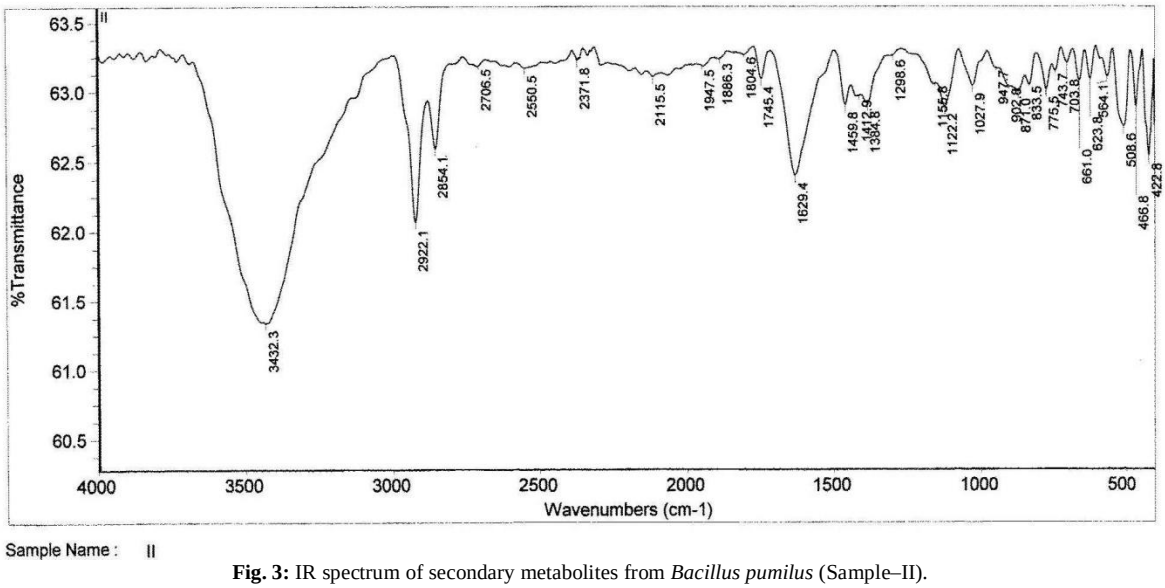

Fig. 3: IR spectrum of secondary metabolites from *Bacillus pumilus* (Sample–II).

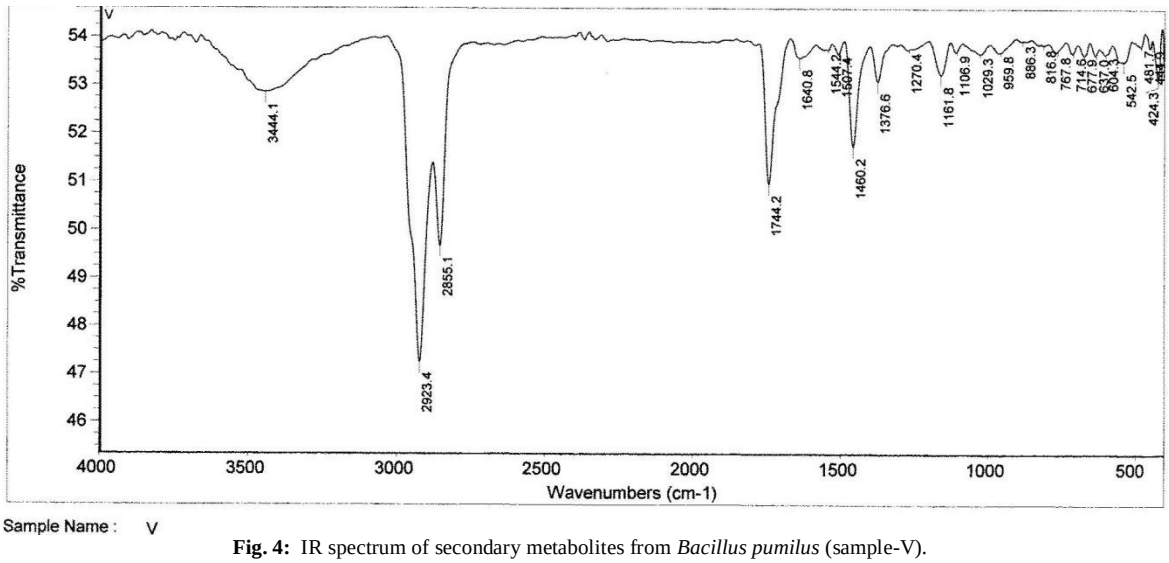

Fig. 4: IR spectrum of secondary metabolites from *Bacillus pumilus* (sample-V).

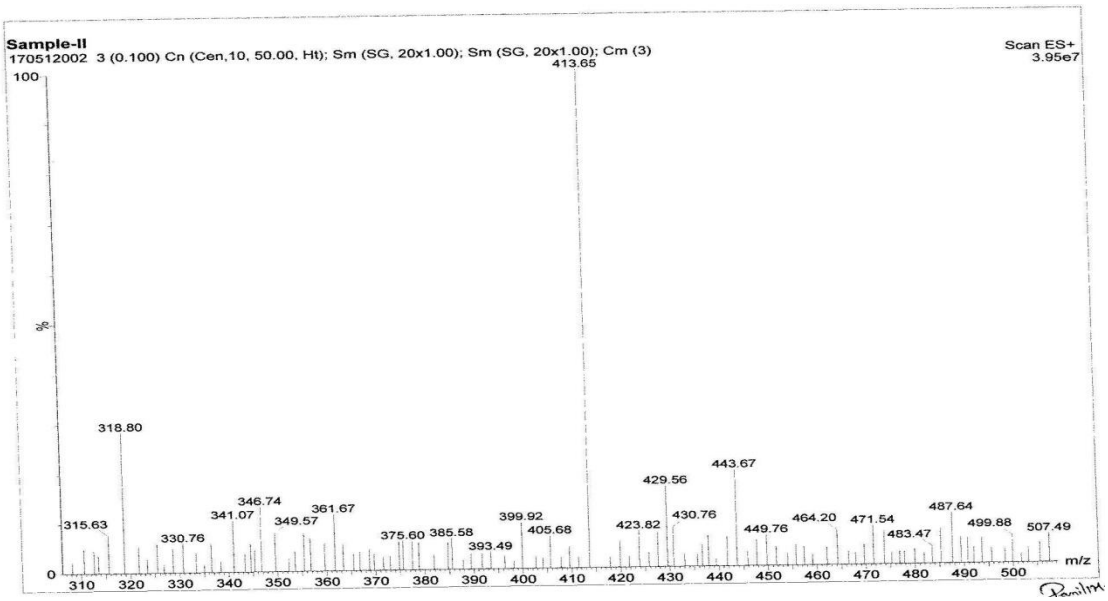

Fig. 5: MS spectrum of secondary metabolites from *Bacillus pumilus* (sample-II).

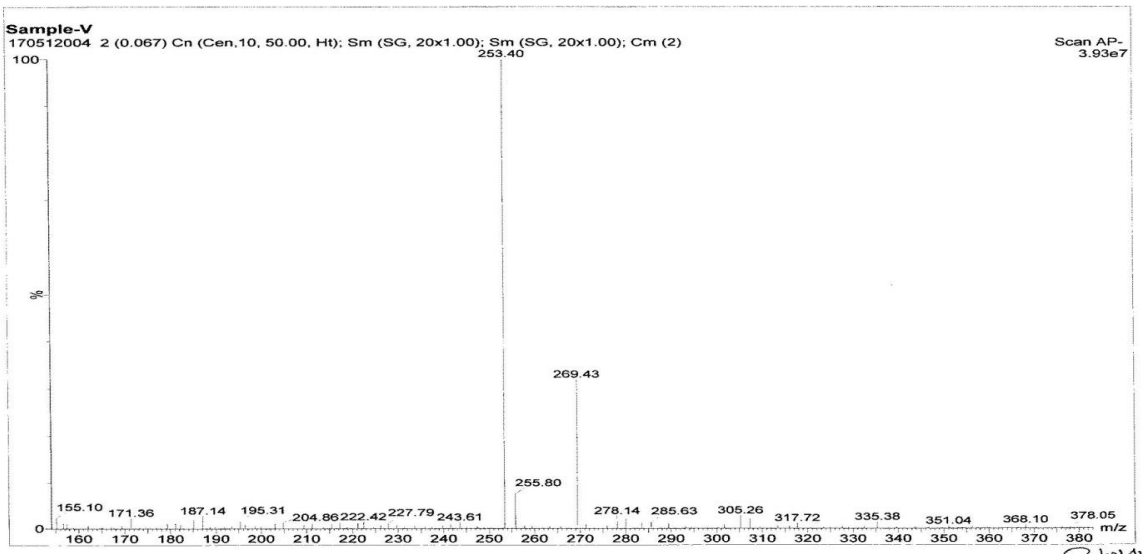

Fig. 6: MS spectrum of secondary metabolites from *Bacillus pumilus* (sample-V).

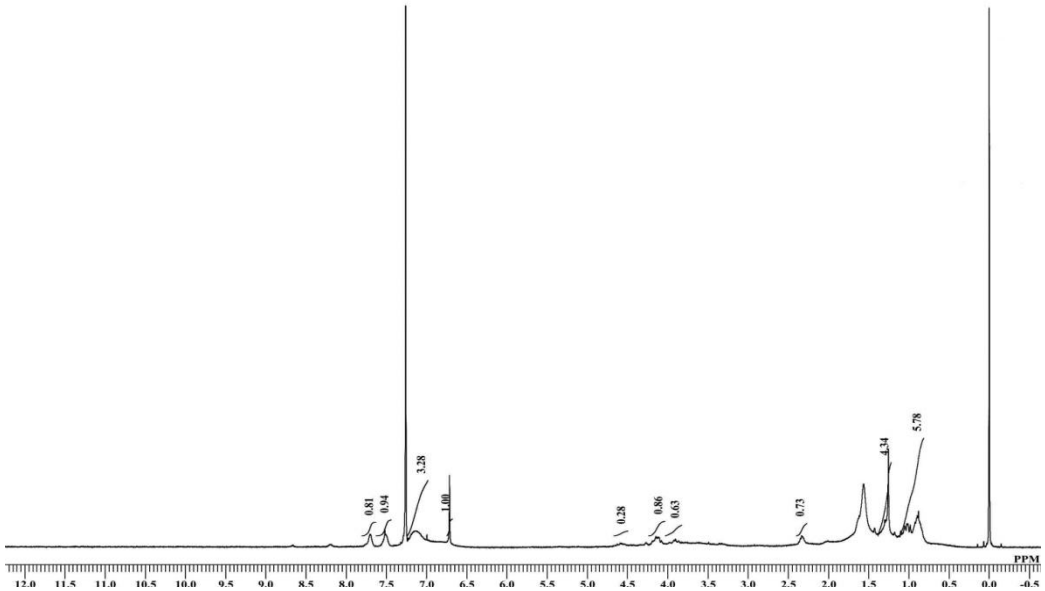

Fig. 7: NMR spectrum of secondary metabolites from *Bacillus pumilus* (sample-II).

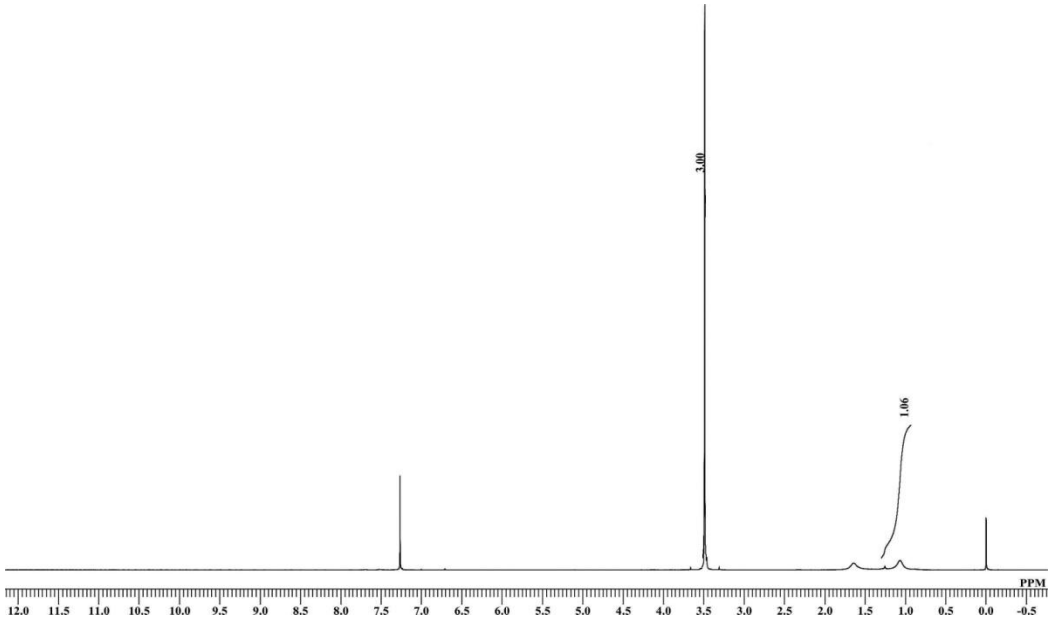

Fig. 8: NMR spectrum of secondary metabolites from *Bacillus pumilus* (sample-V).

Identification of bioactive compound producing bacterial isolates

A three bacterial isolates efficient in bioactivity against selected human pathogen were identified by using *Bacillus pumilus* by using criteria given *Bergey's Manuale of Systematic Bacteriology* for identification of bacitracin producing *Bacillus lichaniformis*. The identified *Bacillus pumilus* showed Citrate, VP, Gelatinase, Starch hydrolysis, Catalase and Nitrate reducing test positive and acid production from D-glucose and D-xylose, *Bacillus pumilus* identified strain also showed these test positive.

Structural elucidation of bacterial bioactive compound

The partial structure elucidated of bioactive compounds of *Bacillus pumilus* strain by IR, MS and NMR, indicated presence of CH3, O=C, C-H, CH2 group, C-H stretching and sub-structured benzene ring.

The presence of such different group and streaching indicates, the bioactive compound produced by *Bacillus pumilus* might have novel structure, which may be responsible for its broad spectrum microbial activity against human pathogen. The structure revealed that the presence of benzene and heterocyclic ring having substitution of OH and ketonic group. The identified compound was pumilin a sesquiterpene lactone antibiotics.

The isolated and purified compound from *Bacillus pumilus* strains in present investigation indicates that compound may have unique structure therefore it shows broad spectrum activity acting against tested human pathogen. The complete structure elucidation by advance instrumentation techniques is essential for explaining its uniqueness. Thus *Bacillus pumilus* moderate halophilic strains finds application in development pharmacological lead compound against multiple antibiotic resistance human pathogens.

Table. 1: Biochemical tests for identification of Bacillus *pumilus*.

Test types	Results
Indole production	-
Citrate utilization	+
Methyl Red	+
Voges-Proskauer	+
Oxidase	-
Catalase	+
Starch hydrolysis	-
Nitrate reduction	-
Casein hydrolysis	+
Gas production from glucose	-

CONCLUSION

A twelve isolated halophilic bacterial isolates were used for production of secondary metabolites these bacterial isolates were screened for bioactivity against human pathogen viz. *E.coli, Staph aures, Pseudomonas aerugenosa* and *Candida albicans.* The partial structures of bioactive compound isolated from halophilic Bacillus pumilus strain were elucidated by using IR, MS and NMR. The presence of CH3 ,O=C,C-H,CH2, group C-N stretching and substituted benzene ring indicated, The detail further identification is of bioactive compound obtained from *Bacillus pumilus* strain.

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