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PRODUCTION OF ACETAMIDE DERIVATIVE FROM MARINE *Streptomyces* sp. ISOLATED FROM WEST COAST OF INDIA

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ABSTRACT

The purpose of research is to isolate *Streptomyces* species from west coast of India for the production of antibiotic. *Streptomyces* species A3 was isolated from sediments of Alibag west coastal region of India. The species A3 was identified by morphological, cultural, physiological and biochemical characteristics. The antibiotic was produced in maltose yeast extract medium prepared in artificial seawater. Maximum activity was obtained after seven days fermentation at 28^{0}C, at pH 7. By using UV, ^{1}H NMR, ^{13}C NMR and mass spectra, the structure of compound was identified as N- [2-(4-hydroxyphenyl) ethyl] acetamide. The acetamide antibiotic exhibited broad antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi. The minimum inhibitory concentration (MIC) of acetamide antibiotic against different microorganisms ranged from 30 to 105 µg/ml. This is the new report of acetamide antibiotic production from marine *Streptomyces* species.

Key words: Marine *Streptomyces,* acetamide, mass spectra, ^{1}H-NMR, antimicrobial activity.

INTRODUCTION

Antimicrobial compounds of diverse chemical structures have been isolated from microbial flora[1,2]. Most bioactive products of microbial origin have come from terrestrial bacteria. Actinomycetes group of bacteria possesses interesting features. Such bacteria play significant role in cycling of organic matter in the soil[3,4]. Although, this class of bacteria is main source of bioactive compounds, the yield of active metabolites is low. Hence, new sources of bioactive natural products must be investigated[3]. Marine environment has been considered as an exciting source for the isolation of new compounds from actinomycetes[3,5]. Many marine microorganisms contain substances that have antimicrobial, antiviral, anticoagulant and cardioactive properties[5]. Marine environment can produce genetically modified strains capable of producing metabolites with diverse biological activities[6,7].

Few studies have investigated marine actinomycetes as a means to obtain therapeutically useful molecules[8-10]. The *Streptomyces, Micromonospora* and *Nocardia* are commonly observed genera in marine environment[11-13]. In the last decade, a few compounds such as altemicidin, SS-228Y, aplasmomycin, istamycin have been isolated from these microbial species[1,7]. However, only one study has been reported for production antimicrobial metabolites, (2-hydroxyphenyl)

acetamide, from an unidentified marine *Streptomyces* species[14]. The marine actinomycetes from west coast of India have not much evaluated[7,11]. The purpose of this research is to isolate *Streptomyces* species from west coast of India for the production of antibiotic.

MATERIALS AND METHODS

Collection of Marine Sediments

Twenty marine samples were collected from Alibag and Janjira coast of Maharashtra as well as Goa (India). The sediments were collected at the time of low tide in July 1999. The surface layers of sediments were removed and central portions of sediments (approximately 0.5 kg, each separately) were transferred aseptically into sterile plastic bags[12]. Samples were kept in cold box containing ice and transferred to laboratory for evaluation.

Isolation of Actinomycetes from Marine Sediments

Ten gram of sediment was air dried under laminar bench (10-12 hours). The dried sediments, in Petri-plates, were kept at 41^{0}C for 10, 30 and 60 days, respectively[12]. For isolation of marine actinomycetes, each sediment (10g, plain, pre-heated) was suspended in 100 ml of sterile saline water. The flask were mixed on rotary shaker (150 rpm, 30 minutes) and 200µl diluted (1:10, 1:100, 1:1000 and 1:10000) suspension was spread over selective media. The media prepared in artificial seawater include starch casein agar, glucose asparagine agar, glycerol asparagine agar, tyrosine agar, yeast malt extract agar, nutrient agar, maltose yeast extract agar and glycerol glycine medium. The agar plates were incubated at 28^{0}C for 2-3 weeks. Pre-heated samples (10 g) were sprinkled on selective media and incubated for 28 days[4]. Actinomycetes were purified and transferred on slants for storage at 4^{0}C[14,15].

Morphological and Physiological Characteristics

Morphological and cultural characteristics were studied as described by Shirling and Gottlieb[15]. The growth and growth characteristic of aerial and substrate mycelium of actinomycetes were observed. Spore chain structure (SEM, Cambridge, UK), biochemical tests and carbon utilization (basal medium, 1% (w/v) carbon source) were studied. Starch hydrolysis, proteolytic activity, melanin pigmentation, H_2S production, nitrate reduction and gelatin liquefaction tests were also performed[16,17]. Identification of actinomycetes was performed[18].

Screening of Actinomycetes for Antibiotic Production

A total of eighty actinomycetes were screened for antibiotic production[19]. Among the broad-spectrum antibiotic producing strains, *Streptomyces* species A3 was selected for further studiesd[16,19]. Strains from national collection of industrial microorganisms (NCIM) at National Chemical Laboratory, Pune (India) were used for checking the antimicrobial activity.

Factors Affecting Production of Antibiotic

The effect of media composition for antibiotic production from *Streptomyces* species A3 was screened. The flask containing maltose yeast extract were incubated at 28^{0}C (rotary incubator shaker, 150 rpm) for 7 days. Fermentation broth was assayed for antimicrobial activity. Maltose yeast extract broth (100 mL) in 500 ml flask was inoculated with spore inoculums to get 1 x 10^6 spores /mL of the fermentation medium. The effect of temperature of incubation (4, 15, 28, 44 and 60^{0}C , each separately) for production antimicrobial activity were determined .[20] Similarly, the effect of pH of maltose yeast extract medium (5, 6, 7, 9 and 10, 0.1N HCl or 0.1N NaOH), salt concentration in media (NaCl, 0-5% w/v),[20-22] time of incubation (days) and speed of agitation (50, 100, 150, 200, 250 rpm)[23,24] on production antimicrobial activity were evaluated. The antibiotic producing ability of various maltose (0.4 - 1.4% w/v) and yeast (0.1-0.8% w/v) compositions were also screened.

Production and Purification of Antibiotic

Fermentation broth (7 lit.) consisting of maltose 1% w/v, yeast extract 0.4% w/v and artificial seawater was inoculated by *Streptomyces* species A3 (10%). The flasks were incubated at 28^{0}C for 7 days and the broth was centrifuged for separation of biomass (Remi, RM12C, India, 10,000 rpm, 4^{0}C, 15 minutes). The supernatant was extracted with ethyl acetate (0.5:1, thrice) and crude extract was collected using rotating evaporator (Buchi, Switzerland, 40^{0}C, 50 rpm). The antimicrobial

activity of crude extract against sensitive bacteria and fungi were evaluated.

The number of components in crude mass (1.62 g) was identified by thin layer chromatography (precoated plates, Merck, 60 gel, benzene: methanol: 90:10). The separated components were purified by column chromatography (silica gel column, ethyl acetate: benzene (50:50)[24]. Small quantity of silica was admixed with crude mass prior to purification. The eluents (75 mL) were collected, concentrated and analyzed by TLC. The positive fractions showing identical R_f values were mixed and dried at 37^{0}C[24]. Each fraction was repurified and assayed for antimicrobial activity.

Structural Analysis of Antibiotic

The UV spectrum of purified compound in methanol (50 ìg/mL) was recorded in the range of 200 to 800 nm using Shimadzu UV-170 spectrophotometer[25,26]. The infrared spectrum was scanned (400 cm^{-1} to 4000 cm^{-1}) using Shimadzu FTIR-8400 model[25,27]. The purified compound (3 mg for ^{1}H-NMR and 10 mg for ^{13}C-NMR) was dissolved in 2 mL of $CdCl_3$ and analyzed by NMR (500 MHz, Vavion, USA). The pure compound was subjected to ^{1}H NMR, D_20 exchange, ^{13}C NMR and DEPT and peaks were identified[26,28]. The GCMS spectrum was obtained from Shimadzu instrument (GCMS-QP 5050A)

Antimicrobial Spectrum of Antibiotic

The activity of purified compound was assayed (dimethyl sulfoxide, 0.05-100 mg/mL) against test cultures. Suspensions of test cultures were prepared in saline water. The 100 mL suspension of 0.1 OD (A_{600}) was spread on nutrient agar (bacteria) and sabouraud agar (fungi), each separately. The solution of compound (0.1ml) was added to cups prepared in media. The bacterial cultured plates were incubated at 37^{0}C for 24 hours, and the fungal specimens were incubated at 25^{0}C for 1-4 days[28]. The zones of inhibition corresponding to different concentrations were measured. The activity assays were performed in triplicate.

RESULTS AND DISCUSSION

Identification of Bioactive Actinomycetes from Sediments

The marine flora from west coast of India was screened for potential actinomycetes. The pre-heat treatment considerably reduced contaminating bacteria and fungi. The heat treatment of 41^{0}C for 60 days was efficient in isolating marine actinomycetes. More number of actinomycetes was observed in treated sediments. Researchers have reported diverse approaches for isolation of antibiotic producing strains. Nonomura and Ohara[29] screened new genera by employing pretreatment. The use of dry heating, specialized growth media and longer incubation period were suitable for new species of *Microbispora, Streptosporagium, Thermomonospora* and *Thermoactinomyces.* The results obtained in the present investigation demonstrate suitability of heat treatment techniques. Cross[30] and Williams[31] have successfully applied similar treatment to marine

Table - 1: Cultural characteristics of *Streptomyces* species A3 on different media*

Medium	Growth	Aerial mycelium	Substrate mycelium	Pigment
Starch casein agar	Good	Grey	Grey	None
Glycerol asparagine agar	Good	White	Yellow	None
Glucose asparagine agar	Good	Yellow	Yellow	None
Yeast malt extract agar	Good	White	Green	None
Tyrosine agar	Good	White	Green	Green
Nutrient agar	Scanty	White	Yellow	None
Maltose yeast extract agar	Good	Grey	Yellow	None
Medium glycerol glycine	Moderate	White	Green	Green

* Media prepared in artificial seawater (3% w/v NaCl)

sediments containing actinomycetes. Starch casein and glucose asparagine agar prepared in artificial seawater showed desired number and growth actinomycetes. Starch-glucose (carbon source) and asparagine -casein (nitrogen source) were optimized for improved growth of marine actinomycetes. Kuster *et al.*,[32] had reported similar results for isolation of *Streptomyces* from soil.

The majority of isolated marine actinomycetes were grey or white without pigmentation. The pigmentation pattern of 53 actinomycetes includes brown (5), grey (30), orange (2), black (8), yellow (4), red (2) greenish (2) and 27 showed white colors. Grey and white colour series were found to be predominant. Biochemical and morphological characteristics and slide cultures showed that most of isolates from Streptomyces group. Streptomyces is known to be the most common genus in the marine environment especially in the shallow organic rich coastal area.[33] *Streptomyces, Micromonospora, Nocardia, Streptosporangium, Micropolyspora* and *Streptoverticillium* have been recorded in marine sediments by Ellaiah and Reddy.[34] Out of 20 bioactive actinomycetes, we observed that 16 from *Streptomyces* group, one of each from *Streptoverticillum, Catellatospora, Nocardia* and *Actinopolyspora*. Morphological and cultural characteristics of *Streptomyces* species A3 are shown in Table -1 and 2. The eighty actinomycetes were analyzed. A total of thirty-five showed antimicrobial activity including twenty has strong activity against test organisms. The antimicrobial spectrum of isolates was predominantly evident against Gram-positive than Gram-negative bacteria or fungi. Okami et al.,[3] reported that 27% of 136 actinomycetes strains were antagonistic against microorganisms. Chandramohan et al.,[33] found 60% of 69 strains of *Streptomyces* species, from sediments of Andaman and Nicobar Islands, showed antagonistic property against test cultures. However, in our study 43.75% of 80 actinomycetes exhibited antimicrobial activity. The west cost of India is suitable for isolation, screening for new actinomycetes and bioactive compounds. A *Streptomyces* species A3 showed broad-spectrum ability on agar medium as well as in fermentation broth. This activity was observed against different types of microorganisms.

Table - 2: Characteristics of *Streptomyces* species A3

Spore chain morphology	Long, circular (upto 5-10)
Growth in 3.5% (w/v) NaCl:	
Colour of colony	Grey
Soluble pigments	-
NaCl concentration (% w/v) for growth	2- 4
Growth at 20°C	+
Optimum temp. (°C)	26-30
Max. growth temp. (°C)	45
Enzyme production:	
Amylase	+
Gelatinase	-
Nitrate reductase	+
Protease	+
Lipase	+
Cellulase	+
Melanin pigmentation	+
H_2S production	+

+ : positive, - : negative

Effect of Environmental Factors

The effects of factors affecting on production of antimicrobial compound from *Streptomyces* species A3 were studied. Maximum activity was found against *A. niger* at 28^{0}C, indicating optimum temperature. Similar trend was observed against *S. aureus*. Optimum temperature for production of the antibiotic was 28^{0}C. The broth of pH 7 medium showed maximum activity against *A. fumigatus* and *B. subtilis*. Maltose yeast extract medium containing 3% (w/v) NaCl was found to be best for antibiotic activity. The salt requirement or tolerance data suggested that some actinomycetes might be terrestrial forms that have adapted to the salinity of seawater and sediments. Time course of antibiotic production was observed throughout the growth phase of the species. Maximum antibiotic was produced in stationary phase at 7th day of fermentation. Fig. - 1 clearly shows that the antibiotic production started from 3rd day (log phase) and increased in stationary phase up to 7th day of fermentation. Maximum activity against *Candida albicans* and *B. subtilis* was observed at 150 rpm. However, the antimicrobial compound against test

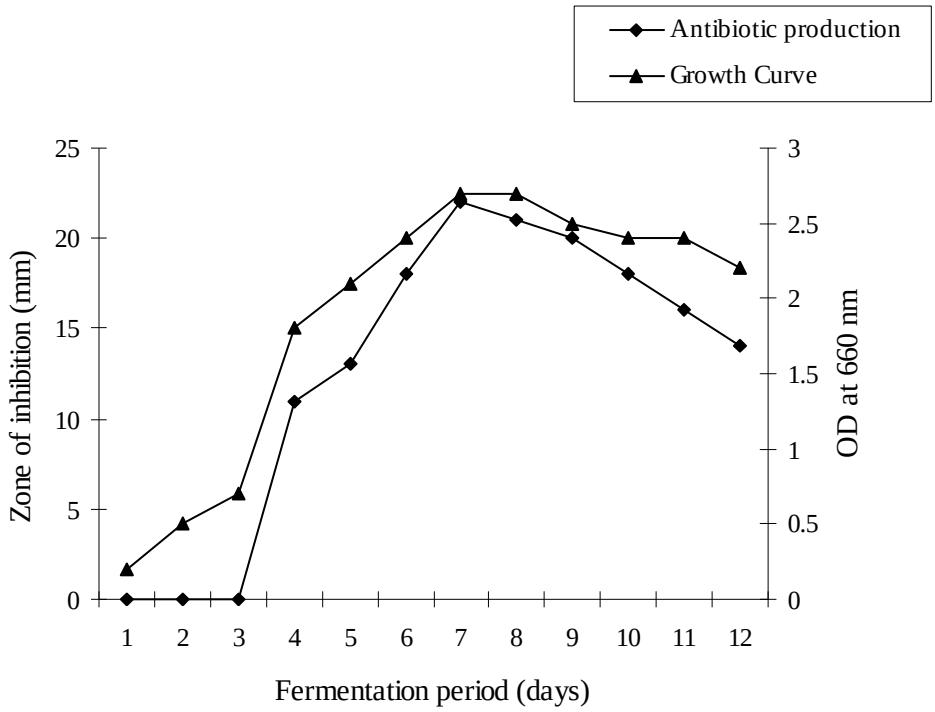

Fig. - 1: Time course for antibiotic production against *Aspergillus niger* and growth phases of *Streptomyces* species A3

microorganisms was found to be produced within wide range of shaking speed ranging from 50 to 250 rpm at 28^{0}C. Antimicrobial activity was found within concentration ranging from 0.4 to 1.4% (w/v) of maltose. However, maximum antimicrobial activity was observed with 1% (w/v) maltose concentration. Maximum antimicrobial activity was observed with 0.4% (w/v) yeast extract.

Production of antibiotic was influenced by media components and cultural conditions. The effect of fermentation conditions on antibiotic production differs from organism to organism[35]. *Streptomyces* usually produce antibiotic near 28^{0}C, whereas *Thermoactinomycetes* species produce antibiotic above 40^{0}C. Productivity of *Streptomyces griseus* strain SS-254 changed with the cultural temperature. Culturing the SS-254 strain at 15^{0}C produced holomycin and amicetin C. But culturing same strain at 27^{0}C produced amicetin C and plicacetin[36]. The optimum pH for antibiotic production was 7.0. The pH affects antibiotic production in all species[35]. In large-scale fermentation of antibiotics, aeration and agitation conditions are selected depending on the optimal concentration of dissolved oxygen. The relation between production of antibiotics and the oxygen concentration is reviewed[35]. Maltose yeast extract medium is widely used for production of antibiotics by *Streptomyces.* This medium was supplemented with 1% (w/v) maltose and 0.4% (w/v) yeast extract, which enhanced antibiotic production. Farooq Biabani, *et al*[37] isolated antranilamides from *Streptomyces* species by using yeast extract as a nitrogen source in fermentation medium. Media containing starch and soybean meal as carbon and nitrogen source, respectively, are known to favour the production of aminoglycoside antibiotics. One such medium was successfully used in the aminoglycoside directed screening programme[38]. However, certain media favor the production of specific groups of antibiotics. In the present study, artificial seawater and natural seawater were found most suitable for production of antibiotic. Usually 0.5% (w/v) NaCl is added in the medium for antibiotic production to terrestrial organisms. Okami *et al*[39]

found that aplasmomycin antibiotic was produced best in the presence of NaCl as high as 1 to 3% (w/v).

Purification and Structural Analysis of Antibiotic

A TLC of antibiotic residue (ethyl acetate) in benzene: methanol (90:10) system showed six bands of different R_f values. The bands were separated by column chromatography. The separated bands showed identical R_f values. Bands having R_f (ethyl acetate: benzene, 50:50) values 0.79 and 0.34 showed zone of inhibition against different test organisms. These two fractions were re-purified using column chromatography. The two fractions having R_f values 0.79 and 0.34 were named as SR-I (0.28 mg/lit) and SR-II (0.57 mg/lit) respectively. SR-I antibiotic showed small impurities in TLC. It also showed less activity. SR-II antibiotic was found to be in pure form by TLC and was found to be active against different test microorganisms. The SR-II was subjected to structural analysis.

UV spectrum of SR-II antibiotic showed l_{max} at 266 and 270 nm, indicating the presence of benzoid ring structure. The infrared spectrum of SR-II depicted -NH stretching vibration in the form of broad band at 3357 cm^{-1}. The stretching at 3199.7 cm^{-1} was assigned for phenolic -OH group. A vibration at 3090 cm^{-1} indicated a sharp -CH stretching while signals at 1641 cm^{-1} depicted to -C=0 stretching vibration of -CO-NH group. A signal at 1371 cm^{-1} was assigned as $-CH_3$ bending vibration. All the vibrations in the area of 912 to 732 cm^{-1} depict the out of plane -C-H bending.

The ^1H NMR spectra of SR-II (Fig. - 2) depicted the proton signals for the benzoid ring structure with side chain. Benzoid ring represented the aromatic signals at 6.78 ppm (C_3H and C_5H). Proton signals at 7.03 were assigned for C_2H and C_6H. The nature of the side chain present on the aromatic ring was found to be two-carbon chain containing peptide group. A strong singlet at 1.94 ppm showed the presence of $-CO-CH_3$ methyl proton. C_7 methylene protons appeared at 1.25 ppm while C_8 methylene protons nearer to -NH group was assigned for the signal appearing at 2.73 ppm while the -NH was assigned for a signal appearing at 3.47 ppm. The above spectral study indicated the presence of 4-disubstituted aromatic compound and presence of -OH group at 4-position.

The ^{13}C spectra of SR-II reveled the characteristic signals of 1,4 substituted benzoid ring

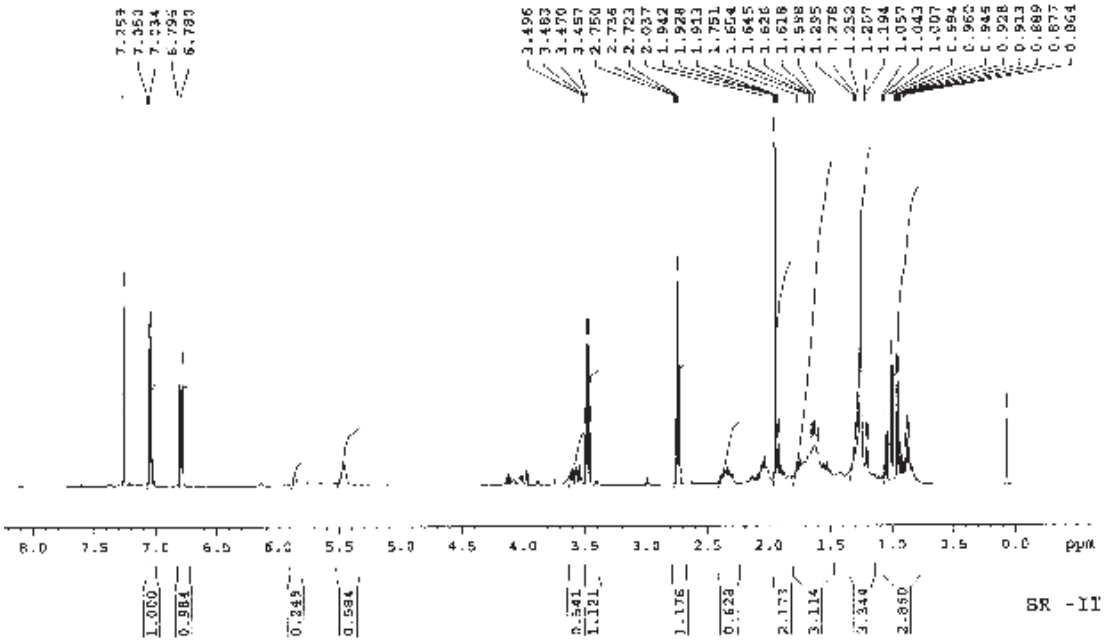

Fig. - 2: ^1H NMR spectrum of acetamide derivative

Table - 3: Antimicrobial activities of fermented broth and MIC of acetamide derivative isolated from *Streptomyces* species

Microorganisms	Test Culture	Diameter of Zone of Inhibition of fermented broth (mm)	MIC of pure antibiotic (µg/ml)
Bacteria	*Bacillus subtilis*	18	50
	Staphylococcus aureus	20	30
	Staphylococcus epidermidis	19	33
	Escherichia coli	12	>100
	Serratia marcescens	14	100
	Enterobacter aerogenes	12	>100
Fungi	*Aspergillus niger*	22	20
	Aspergillus fumigatus	16	45
	Aspergillus flavus	19	40
	Fusarium oxysporum	16	55
	Trichoderma species	13	>100
	Penicillium species	11	>100
	Cryptococcus species	14	>100
	Candida albicans	15	100

structure (Fig. - 3). The signals of aromatic carbons C_3 and C_5 appeared at 115.64 ppm while C_2 and C_6 carbons were signaled at 129.71 ppm. Carbons C_1 and C_4 arrived at 129.91 and 155.13 ppm, respectively. Two methylene carbons C_7 and C_8 were signaled at 34.62 and 40.98 ppm. A methyl carbon present on carbonyl group appeared at 232.2 ppm. ^{13}C NMR signal appeared at 170.73 ppm was attributable to carbonyl carbon, which is associated with -NH group. The DEPT edited ^{13}C NMR spectra had shown all the $-CH_3$, $-CH_2$ and -CH as per expectation.

The mass spectra of SR-II showed a weak molecular ion peak at m/e 179 (Fig. - 4). The base peak with 100% relative abundance was observed at m/e 120, which seemed to be due to the loss of $-NH-CO-CH_3$ (m/e 58). The above structural studies and physicochemical characteristics of SR-II antibiotic indicates the presence of 4-disubstituted aromatic compound. The presence of -OH group at 4 position and a side chain ($-CH_2-CH_2-NH-CO-CH_3$) as evident for IR, ^1H-NMR, ^{13}C-NMR data and mass fragmentation pattern. Thus on the basis of structural elucidation, SR-II antibiotic was found to be N-[2-(4-hydroxyphenyl) ethyl] acetamide.

Antimicrobial Spectrum of SR-II Antibiotic

The SR-II showed good antibacterial as well as antifungal activity. The spectrum of antibiotic produced by *Streptomyces* species is given in Table - 3. The spectrum of antibiotic was very broad. It includes activity against Gram-positive bacteria like *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtilis*, Gram-negative bacteria like *Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens* and *Enterobacter aerogenes* as well as weak activity was reported against fungi like *Aspergillus niger, Aspergillus flavus, Aspergillus fumigatus, Candida albicans, Penicillium* species*, Cryptococcus* species and *Fusarium oxysporum*. Minimum effective concentrations were found between 20 µg/mL to 100 µg/mL and more against different test microorganisms.

A microbial metabolites N- (2 hydroxyphenyl) acetamide was isolated by Pusecker et al[14] from an unidentified marine *Streptomyces* species. This compound was isolated after fermentation of species in yeast malt extract medium at 28^{0}C for 72 hours. The yield was 1.4 mg/lit. Our studies isolated metabolites, N- [2-(4-

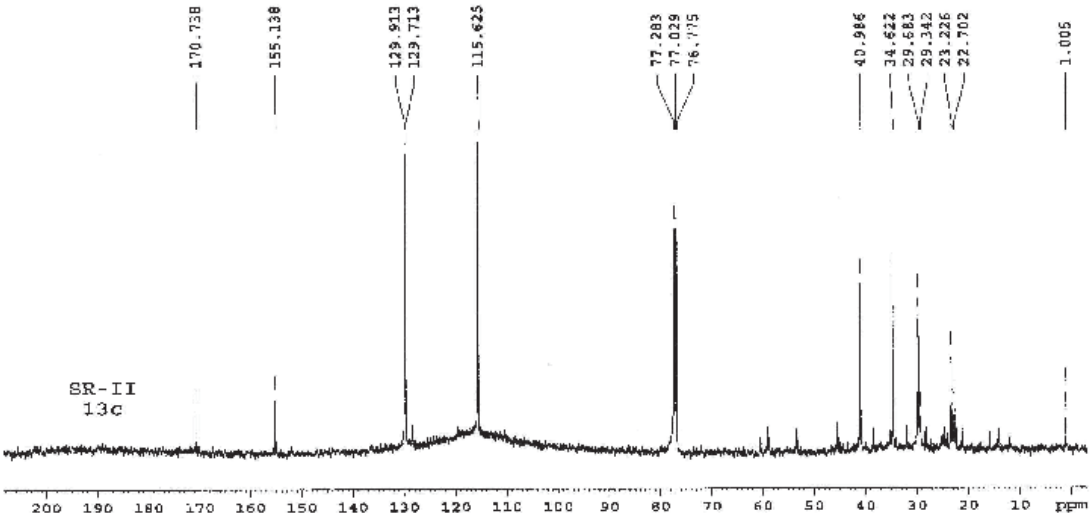

Fig. - 3: ^{13}C NMR spectrum of acetamide derivative

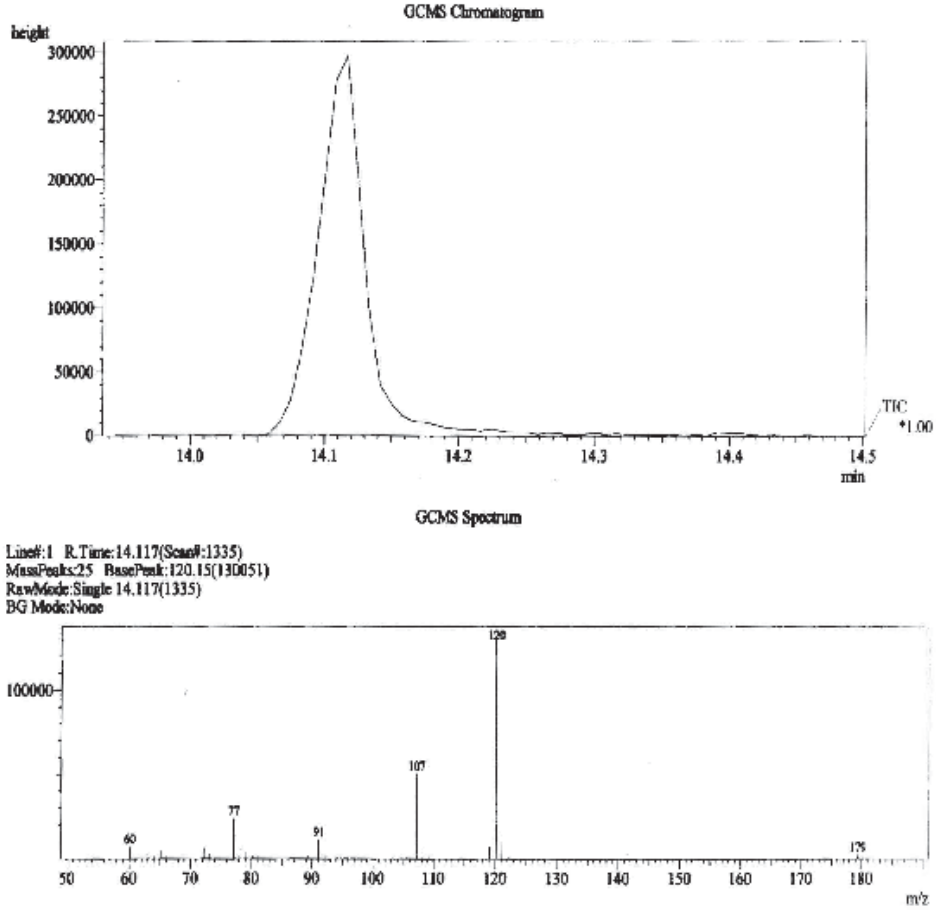

Fig. - 4: Mass spectrum of acetamide derivative

hydroxyphenyl) ethyl] acetamide, from marine *Streptomyces* species A3. The fermentation was performed in maltose yeast extract medium at 28^{0}C for 7 days. The yield was 5.7 mg/lit. This compound showed broad-spectrum activity. There is no report of aromatic substituted ethyl acetamide derivatives from marine *Streptomyces*. These investigations clearly indicate that marine sediments from west coastal regions of India are potent source for the isolation of bioactive actinomycetes and warrants further investigations.

CONCLUSION

The west cost of India has a rich flora of actinomycetes. The microbial analysis reveals that number of actinomycetes species are capable of producing antimicrobial metabolites. *Streptomyces* species A3 has activity against number of test organisms. The fermentation process was optimized to produce a acetamide antibiotic. The fermentation conditions significantly affected its ability to produce a metabolite. The bioactive molecule showed promising antimicrobial activity against Gram-positive, Gram-negative as well fungal species.

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