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Research Article

Studies on a marine *Streptomyces fradiae* BW2-7 producing glycopeptide antibiotic Vancomycin effective against skin pathogens

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Abstract: A total of 124 marine actinomycetes were isolated from east and west coastal regions of Indian peninsula and assessed for its antimicrobial activity. A potent actinomycete isolated from west coast of India was selected by its antibacterial and antifungal activities and was identified as *Streptomyces fradiae* BW2-7 using 16S rRNA sequencing which produces antibacterial metabolite extracellularly and antifungal metabolite intracellularly under submerged fermentation conditions. Crude extract of the strain showed board antimicrobial activity spectra against the bacterial skin pathogens *Staphylococcus aureus* (25mm), *Staphylococcus epidermidis* (21mm) and fungal pathogen *Trichophyton rubrum* (15mm). Fermentation conditions for the production of antibiotic were optimized by using statistical method, Response Surface Methodology (RSM). Purified compound was subjected to structural elucidation. After medium optimization the antibiotics activity showed 8% improvement compared with unoptimized condition. The isolated compound MRK1 was found to be Vancomycin and showed effective against skin pathogens. This is the first report, where the compound was isolated from a new source *S. fradiae* and inhibiting the skin pathogens.
Keywords: Marine actinomycetes, Skin pathogens, Response Surface Methodology, NMR, and Vancomycin.

INTRODUCTION

Antibiotics are a group of natural or synthetic compounds that destroy bacteria (bactericidal) or inhibit their growth (bacteriostatic). Antibiotics that are sufficiently nontoxic to the host are used as chemotherapeutic agents in the treatment of infectious diseases of humans, and animals. Nature produces an amazing variety and number of products. About 100,000 secondary metabolites of molecular weight less than 2500 have been characterized, which are mainly produced by microbes and plants [37], Out of which around 50,000 are from microorganisms [12]. Actinomycetes are abundant in terrestrial soils, a source of most isolates shown to produce bioactive compounds [27]. Goodfellow and Haynes [15] reviewed the literature on the isolation of actinomycetes from marine sediments and suggested that this source may be valuable for the isolation of novel actinomycetes with the potential to yield useful new products. Earlier studies considered actinomycetes to be part of an indigenous marine microflora [16, 52]. Others reported them primarily as wash in components that nearly survived in marine and littoral sediments as spores [14]. Jensen *et al.* [20] and Takizawa *et al.* [45] reported a bimodal distribution of actinomycetes in near shore tropical marine environments. The existence of an autochthonous actinomycete population was suggested by the isolation of actinomycetes from marine deep oceanic sediments [34, 45, 52]. These organisms isolating from marine sediments are gaining importance not only for their taxonomic and ecological perspectives, but also for their production of novel bioactive compounds like antibiotics, enzymes, enzyme inhibitors, pigments and for their biotechnological application such as probiotics and single cell protein. Enhanced salt, pH and temperature tolerance of the isolates along with their capacity to secrete commercially valuable primary and secondary metabolites emerges an attractive feature of these organisms [24].

Streptomyces are especially prolific and can produce a great many antibiotics (around 80% of the total antibiotic production) and active secondary metabolites [53]. To make the production of antibiotics feasible, it is necessary to optimize the production conditions. In statistical based approaches, response surface methodology (RSM) has been extensively used in fermentation media optimization [9, 54]. The application of these design techniques in fermentation process results in improved product yield reduced process variability, closer confirmation of the output

response (product yield or productivity) to nominal and target requirements as well as reduced the overall costs [36].

This present work focused on isolating the actinomycetes strain producing effective compound from the marine sediments of various regions of peninsular India (Goa, Kerala, Tamil Nadu and Karnataka) with wide spectrum antibacterial and antifungal activity for skin infections. Identification of the potential antagonistic strain based on morphological, biochemical, physiological and molecular methods. This paper also focused on optimization the cultural conditions and nutritional parameters using RSM for increased antibiotic yield.

MATERIALS AND METHODS

Collection of soil samples

Marine soil samples were collected from different stations of maritime states in India, namely seashore soils covering Murudeshwara [Uttara kannada district, Karnataka, 14.6˚ N and 74.7˚ E], Kannamaly beach [Ernakulam district, Kerala, 10.00˚ N and 76.33˚ E], Colva beach [South Goa district, Goa, 15˚16′33.708˚ N and 73˚55′2.3700′E], Elliot's beach and Pattinapakam [Chennai, Tamil Nadu. 13˚5′2′′N and 80˚16′12′′E], Nallumadai [Ramanathapuram district, Tamil Nadu, 9.28˚N and 79.3˚ E], Kurusadai Island [Mandapam, Tamil Nadu, 9.24690˚N and 79.20945˚E], Shingle Island [Mandapam, Tamil Nadu, 9.24174˚N and 79.23563˚E], Kanyakumari [Kanyakumari district, Tamil Nadu, 8.078˚N and 77.541˚E], Thiruchendur [Thoothukudi district, Tamil Nadu, 8.4833˚N and 78.1167˚E], Karikoil, Kuthamkuli, Kuttapuli, Nadar Ovuary, Ovuary, Perumanal, Anchugramam [Thirunelveli district, Tamil Nadu, 09°04′N 77°30′E] of west and east coasts of India. Samples were collected from 6-10cm depth below soil and transported to the laboratory in sterile polythene bags and stored for further study.

Isolation and Culture Conditions

Starch casein agar medium (Himedia, Mumbai, India) was used for isolation and enumeration of actinomycetes. The medium was supplemented with 10µg/mL amphotericin and 25µg/mL streptomycin (Himedia, Mumbai, India) to inhibit fungal and bacterial contamination respectively [34]. One gram of soil sample was shade dried and mixed in 100mL of sterile distilled water, and mixed thoroughly in a shaker for 30 min at 150 rpm. The suspension was serially diluted up to 10^{-7} dilution. Around of 0.1mL of each dilution was spread plated on starch casein agar plates in triplicates and incubated at room temperature for 7-15 days. After incubation period, the plates were examined for the presence of distinct actinomycetes colonies. These colonies were purified and sub-cultured on SCA plates and were maintained by monthly transfer to agar slant tubes of SCA medium and stored at 4˚C.

Screening of actinomycetes for antidermatophytic activity

The pure cultures of actinomycetes from the collected soil samples were then screened for their antidermatophytic activity by cross streak and well diffusion method. SCA plates were inoculated with pure cultures of actinomycetes, incubated at room temperature for 7- 15 days. *Trichophyton rubrum*, *Staphylococcus aureus* and *Staphylococcus epidermidis* were used as test organisms to screen antidermatophytic activity. After adequate growth of the isolates, *T. rubrum* was inoculated perpendicular to the streak line of the isolates. The plates were then incubated at 30˚C for a period of 7-15 days. In case of well diffusion method after adequate growth at 30˚C in Kuster's agar, 100µl of the actinomycetes broth culture was placed in the wells made with a sterile cork borer on Sabouraud dextrose agar plates seeded with the test fungal culture *T. rubrum* and Muller Hilton agar medium swabbed with bacterial pathogens. The plates were then incubated at 28˚C and observed for antibiosis after 24h [1].

Extraction of antimicrobial compounds

The selected antagonistic actinomycetes isolate was inoculated into production medium (% w/v, Glucose-1.0, Soybean meal-1.0, NaCl-1.0, $CaCO_3$-0.1) and incubated at 28˚C in a shaker (200-250 rpm) for 4-5 days. After the incubation period the cells were separated and the extracellular metabolite was extracted from the cell free supernatant with ethyl acetate as solvent. The ratio of filtrate and solvent (1:1 v/v) was taken in a separating funnel and shaken vigorously. Extraction was continued up to three times with the same solvent. The organic layer was collected and the solvent was evaporated using vacuum rotary evaporator. The cells were then harvested, air dried and the intracellular metabolite was obtained by disintegrating the cells with mortar and pestle using methanol as solvent. The crude extracts of extracellular and intracellular metabolites were studied for their antifungal and antibacterial activity.

Test organisms

The following microorganisms were procured from Institute of Medical Science, PSG Hospitals, Coimbatore, Tamil Nadu, India and used during the investigation as test organisms. *S. aureus, S. epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus vulgaris, Salmonella typhi, Enterococcus faecalis, T. rubrum, Aspergillus niger, Aspergillus fumigatus* and *Candida albicans.*

In vitro antibacterial assay

The antibacterial activity of both of the crude extracts was tested by agar diffusion assay [3]. The plates were incubated at 37˚C for 24h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times

and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (millimeters) produced by the secondary metabolites when compared to the control. Chloramphenicol was used as positive control.

In vitro antifungal assay

Antifungal activity of both the crude extracts was determined by using the agar diffusion method. Each fungal inoculums were applied on plate and evenly spread on Sabouraud's Dextrose agar (Himedia, Mumbai, India) using a sterile swab. Agar diffusion assay was followed to evaluate the antimicrobial activity along with amphotericin B as control. The petri plates were incubated at 30˚C for 2 days. At the end of the 48h, inhibition zones formed in the medium were measured in millimeters (mm). All experiments were done as three replicates.

Characterization of antagonistic isolate

Gram staining

A smear of the actinomycetes culture was taken in a clean glass slide and heated gently over a flame. The smear was covered with a thin film of crystal violet for 1 min and washed gently in slow running tap water. Gram's iodine solution was flooded over the smear for 1 min and washed with tap water. 70% of alcohol was used to decolorize the smear until the violet color ceased to flow away. The slide was washed with water and counter stain safranine was flooded over the smear for 2 min, then the slide was washed, drained, air dried, and viewed under microscope. The culture retaining the violet color indicated that it was Gram-positive organism.

Culture Characterization

According to the recommendation of International Streptomyces Project (ISP) [41] the potent antagonistic actinomycetes isolate was further characterized based on morphological, biochemical, cultural and physiological features. Microscopic characterization was carried out by cover slip culture method [21] and formation of aerial and substrate mycelium, and arrangement of spores on mycelium were observed under high power objective of light microscope and Scanning electron microscope. Cultural characteristics (growth, coloration of aerial and substrate mycelia, formation of soluble pigment) were tested in seven different media including, SCN agar, nutrient agar, yeast extract malt extract agar (ISP- 2), oat meal agar (ISP-3), glycerol – asparagines agar (ISP-5) and Tryptone yeast agar with the procedures of ISP. Biochemical tests including IMViC, H_2S production, nitrate reduction, urease, catalase, starch, gelatin and casein hydrolysis, citrate utilization were also performed as recommended by ISP. Physiological characterization such as, the effect of pH (5-9), temperature (25˚-50˚C) and salinity (NaCl concentrations 1-4%) were also studied.

16S rRNA sequencing and phylogenetic analysis

The DNA was isolated by HiPurA bacterial DNA isolation and purification kit (Himedia, Mumbai, India) and its 16S rRNA gene was amplified by PCR using a master mix kit, medoxmix (Medox, India) as per user manual. The primers and the PCR conditions were adapted from Rainey *et al*. [33]. The design of the sequencing primers and the methodology for the sequencing were adapted from previous reports [25, 22, 33]. The 1348 bp fragment of the 16S rRNA gene of the strain was sequenced both the sense and antisense directions by Xcleris Lab (India). The 16S rRNA sequence was analyzed for the similarity and homology with the existing sequences available in the data bank of National Center for Biotechnology Information (NCBI) using BLAST search. The sequences were aligned using ClustalW program and phylogenetic tree was constructed by neighbour joining method using Phylip software version 3.69. Bootstrap analyses of 1000 replicates were carried out.

Optimization of the production medium

Screening of medium components using Plackett-Burman (PB) experimental design

The purpose of the first step in the optimization strategy was to identify the medium components that have significant effect on the antibiotic production. PB statistical experimental design is a fraction of a two level factorial design and allows the investigation of 'n-1' variables in at least 'n' experiments. For the selection of variables, various carbon sources (Glucose, Glycerol, lactose, starch), nitrogen sources (soybean meal, wheat bran, corn flour, sesame oil cake) and cultivation parameters (incubation period, pH, inoculum size) were tested and identified by statistical software package Design expert 8.0 (Stat-Ease Inc., Minneapolis, USA). A total of 11 parameters were included for selection, in which each variable was represented at two levels. The production was set up by inoculating the medium with respective inoculum percentages as suggested by the model and incubated at 27˚C, 200 rpm. For each experiment antibiotic production was calculated in terms of Zone of inhibition (ZOI) measured in diameter.

Response Surface Methodology (RSM)

Based on the PB design the effect of 3 factors viz., glucose, soybean meal and incubation period on antibiotic production was studied using one of the RSM methodologies, the Box-Behnken statistical design [5]. The other components of the medium were (% w/v) $CaCO_3$ – 0.1, NaCl-1.0. Each factor in the design was studied at 3 different levels low (-1), middle (0) and high (+1). Once the antibiotic activity (ZOI) was measured, a second- order polynomial model was fitted to the response data obtained from the design.

Fermentation

After optimization of the fermentation parameters for the better production of both antibacterial and antifungal metabolite, scale up studies were carried out

at laboratory fermentor scale. The inoculum was grown in 100mL production medium in 500mL Erlenmeyer flask at 28˚C for 96h on a rotary shaker (200-250 rpm). Of this 5% v/v inoculum was transferred to the 2 L fermentor (Lark Innovative Fine Teknowledge). Fermentation was carried for 120h at 28˚C with aeration (1 v of air/ volume of medium/ min) and agitation (200 rev / min).

Purification

Fermented culture was centrifuged at 8000 rpm for 20 min to separate the cells. The pH of the culture filtrate was lowered to 4.5 and the filtrate was subsequently extracted with butyl acetate (3:1). After separation and subsequent evaporation of the organic phase, the residue was dissolved in methanol and stored at 0-4˚C. The crude extract was purified by silica column chromatography using chloroform: methanol (v:v, 100:0 to 0:100) step gradient. The active fractions were pooled, dried under vacuum to yield a dull white powder. The antimicrobial activities of all fractions were examined against *S. aureus* and *T. rubrum* using well diffusion method. Active fraction was pooled and stored at 0-4˚C and was checked for its purity using Thin Layer Chromatography (TLC).

Structure elucidation

The UV spectrum of the antibiotic was recorded on a Jasco spectrometer. ^{1}H NMR, ^{13}C NMR, and distortionless enhancement by polarization transfer spectra were recorded on a Bruker AVANCE III 500 MHz NMR spectrometer using DMSO-d6 as solvents. IR spectrum was recorded on a Jasco model. The electron mass spectrometry (ESI) were recorded on Thermo Finnigan LCQ Advantage max ion trap mass spectrometer. The 10µl of samples were introduced into the ESI source through Finnigan surveyor autosampler. The mobile phase 90:10 MeOH: H_2O flowed at the rate of 250µl/min by MS pump. Ion spray voltage was set at 5.3 KV and capillary voltage 34V.The MS scan was run up to2.5 min and the spectra's print out are averaged of over 10 scan at peak top in TIC.

RESULTS

Based on the colony morphology and stability in sub culturing, 115 actinomycetes were isolated from the east and west coastal regions of India. All the isolates were named according to their sampling area and examined in Clinical Biotechnology Laboratory, Department of Microbial Biotechnology, Bharathiar University (India). The sampling locations were given in Fig.1. Out of 115 actinomycetes strains, 52 isolates (45.2%) had antimicrobial activity, of which 26 isolates (22.6%) showed antibacterial activity (against *S. aureus*), 17 isolates (14.7%) showed antifungal activity (against *T. rubrum*), 9 isolates (7.8%) showed both antibacterial and antifungal activity (Fig.2).

Morphological, biochemical and physiological characterization

BW2-7 is a gram positive filamentous bacterium. The aerial mycelium is branched, peach in color and the substrate mycelium is branched, pale yellow in color. The mature spores are 0.6-1.0 µm in diameter and length is between 0.8 and 1.0 µm. extensively fragmented substrate mycelium was observed under scanning electron microscopy. The details of morphological, physiological and biochemical characteristics were listed in Table 1.

BW2-7 is an aerobic, non-motile and catalase positive. The utilization of starch and casein showed that the isolate produced the extracellular enzymes amylase, and caseinase respectively to metabolize the polymeric components of the nutrient mixture to monomeric forms for its growth[19]. Apart from these enzymes it also produces esterase and urease. Positive reaction for the catalase enzyme revealed that the isolate could withstand the stress conditions generated by reactive oxygen species. The test on triple sugar iron agar revealed that this organism would not produce gas and acid when incubated in carbon sources such as glucose, sucrose and lactose except for few species variables. BW2-7 was MR positive, Indole and VP negative. It reduced nitrate to nitrite and produce hydrogen sulphide gas [32]. Analysis of the whole cell hydrolysate of strain BW2-7 showed the presence of chemotype I cell wall characterized by LL-diaminopimelic acid and galactose. Chemotaxonomic investigations revealed that BW2-7 strain has cell wall type I which is characteristic of the genus *Streptomyces*. Thus analysis of the morphological and some cultural characteristics of the isolated organism allowed us to determinate its probable taxonomic classification to the genus *Streptomyces*, based on the directions given by Williams [53]. This clearly indicates that BW2-7 belong to the genus *Streptomyces*. The results were found to be similar to that reported by Oskay [29].

The strain could grow up to 4% NaCl concentration with optimal concentration of 1% and it showed optimum growth when cultivated at 30˚C and pH 8.0. The cultural characterization of the isolate was determined using various media which are listed in Table 2. It produces pink diffusible pigment in most of the media. Based on these results the strain BW2-7 was concluded that it belongs to the genus *Streptomyces* sp.

Molecular characterization

The genomic DNA of *Streptomyces* sp BW2-7 was isolated and subjected to 16S rRNA gene amplification for the identification of the potent antagonistic producer. PCR product of the length 1,348 bp was purified and sequenced in Xcleris Lab (India). The 16S rRNA sequences of the isolate BW2-7 was subjected to BLAST using megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/). Among the 19 closest neighboring strains of NCBI-BLAST used in the phylogenetic analysis, the 16S rRNA partial gene sequence of the isolate showed high percentage

nucleotide similarity with *Streptomyces fradiae* NBRC 3439 (99%)(Fig 4). The phylogenetic tree was constructed based on neighbour joining method and the percentage difference in the genetic relationships between the allied strains of *Streptomyces*. The physiological and biochemical characteristics and the 16S rRNA sequence analysis confirmed that the strain *Streptomyces sp* BW2-7 was identical to *S. fradiae*. The 16S rRNA gene sequences of the strain *Streptomyces sp* BW2-7 has been deposited in the GenBank (NCBI, USA) under the accession number JF340438.

Antimicrobial assay

An extracellular brown colored crude active metabolite recovered by solvent extraction method using ethyl acetate showed higher antimicrobial activity against gram positive bacteria than gram negative bacteria (Fig.3). Bacterial pathogen *S. aureus* had more susceptible. The intracellular metabolite was also studied for its antimicrobial activity. Ethyl acetate extract of *S. fradiae* BW2-7 showed potent antimicrobial activity against *S. aureus* (25 mm) followed by *S. epidermidis* (21 mm), *S. typhi* (18 mm) *P. aeruginosa* (11 mm) and *T. rubrum* (15 mm). Methanolic extract of *S. fradiae* BW2-7 showed antifungal activity against *T. rubrum* (14 mm) followed by *A. niger* (12 mm).

Optimization of the production medium

Statistical optimization: Placket-Burman design (PB)

Since process optimization by single-dimensional search is laborious and time consuming, an attempt was made also to use a more practical and simple, fractional factorial method. The potential effects of 11 variables on antibiotic production were evaluated against *S. aureus* using the PB design. The results of antibiotic production by a PB design are presented in Table 3. The model F-value is 129.00, which implies the model is significant. Values of 'Prob >F' less than 0.0500 indicates model terms are significant. In this case the variables A, C, D, F, G, J, K, L are significant model terms (Fig.5). The low probability value of soybean meal, glucose, incubation period shows that they were significant for the enhancement of antibiotic production and hence they were selected for further optimization using RSM. Sodium chloride, Calcium carbonate, inoculum size and temperature were maintained at a constant level.

Response surface analysis

Response surface designs are commonly used to explore nonlinear relationships between independent (medium components) and the dependent (antimicrobial activity) variables [38]. The Box-Behnken design was conducted in the optimum vicinity to locate the optimum concentration of Soybean meal, Glucose and Incubation period for maximum antibiotic production. The range and the levels of the three variables were selected in RSM (data not shown). The results were analyzed by using ANOVA, i.e., analysis of variance suitable for the experiment design used. The ANOVA of the quadratic model indicates that the model is significant (Table 4). The Model F-value of 46.69 and value of $P < 0.0001$ indicate the model terms to be significant. When the values of "Prob > F" is less than 0.0500 that also indicate model terms are significant, while values greater than 0.1000 indicate the model terms are not significant.

The coefficient estimates and the corresponding *P* values suggested that, among the test variables used in the study, A, B, C, AC, A^2, B^2, C^2 are significant model terms (where A= Glucose, B= Soybean meal, C= Incubation period.). The "Predicted R-Squared" of 0.8753 is in reasonable agreement with the "Adjusted R-Squared" of 0.9625. "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 17.267 indicates an adequate signal. The following regression equation was developed by the application of the respose surface methodology, yielding an empirical relationship between the logarithimic values of the antibiotic activity and the coded units of the test variables (medium components).

$$ZOI = +27.60 + (3.25 \times A) + (1.63 \times B) + (2.63 \times C) + (0.000 \times A \times B) + (2.00 \times A \times C) + (1.25 \times B \times C) - (5.18 \times A^2) - (4.93 \times B^2) - (4.92 \times C^2)$$

Where, ZOI is the Zone of inhibition of antibiotic activity and A, B and C are coded values of the glucose, soybean meal and incubation period, respectively. The three dimensional response surface plots and contour plots are obtained with the help of the statistical software. Response surface plots (Fig.6) as a function of two variables at a time maintaining the third variable at a fixed level were helpful in understanding both the main and the interaction effects of these variables. These values and the analysis of variance (ANOVA) suggested that the concentration of glucose and incubation period have a direct effect on antibiotic activity and hence yield. All of the above consideration indicated an excellent adequacy of the regression model. RSM analysis by Box-Behnken indicated that there is an increase in ZOI for antibiotic production against *S. aureus* (27mm), *S.epidermidis* (24mm) and *T. rubrum* (21mm) (average of three replicates) in diameter. The optimum medium compositions and culture conditions for the maximum antibiotic production were glucose 1.5%, soybean meal 1.5% and incubation time for 5 days.

Production and purification of antibiotic compound

After medium optimization by statistical method the strain *S. fradiae* BW2-7 was cultured in 500mL Erlenmeyer flask at 28˚C for 96h on a rotary shaker (200-250 rpm). Of this 5% v/v inoculum was

transferred to the 2 L fermentor (Lark Innovative Fine Teknowledge) for 120h at 28˚C. The brown colored crude active compound was extracted using ethyl acetate as solvent [50]. The ethyl acetate phase that contains antibiotic substances was separated from the aqueous phase. The crude extract was evaporated to dryness in water bath and the residue obtained was weighed. The crude extract was subjected to flash chromatography on silica gel column with Chloroform: Methanol (v:v). All the fractions were checked for its antimicrobial activity against *S. aureus* and *T. rubrum.* Antimicrobial activity was found in the 30% and 40% methanol eluates. The fractions containing the inhibitory compound MRK1 was further chromatographed on a silica gel column. The fractions eluted with Chloroform- Methanol (v:v, 7:3) showed antibacterial and antifungal activity against *S. aureus* and *T. rubrum* respectively. The active fractions were pooled and concentrated in vacuum. It was moderately soluble in chloroform and acetone but soluble in water and methanol. The UV spectral pattern of the compound has absorption maxima at 282nm in water, corresponds to the characteristic of glycopeptide antibiotics.

Structure elucidation of antibiotic MRK1

The molecular formula of the compound MRK1 was determined as $C_{66}H_{75}Cl_2N_9O_{24}$ on the basis of the ESI-MS, which gave a ([M^+], 100) ion at m/z 1450.1. The IR and NMR (Table5) spectral information of the MRK1 was searched in different databases and the results were very similar to the report by Pearce and Williams [31], hence the compound MRK1 was confirmed as Vancomycin (Fig.7). This indicated the IUPAC name of Vancomycin as (1*S*,2*R*,18*R*,19*R*,22*S*,25*R*,28*R*,40*S*)- 48- {[(2*S*,3*R*,4*S*,5*S*,6*R*)- 3- {[(2*S*,4*S*,5*S*,6*S*)- 4- amino- 5- hydroxy- 4,6- dimethyloxan- 2- yl] oxy}- 4,5- dihydroxy- 6- (hydroxymethyl) oxan- 2- yl] oxy}- 22- (carbamoylmethyl)- 5,15- dichloro- 2, 18, 32, 35, 37- pentahydroxy- 19- [(2*R*)- 4- methyl- 2- (methylamino) pentanamido]- 20, 23, 26, 42, 44- pentaoxo -7,13 - dioxa-21, 24, 27, 41, 43- pentaazaoctacyclo [26.14.2.2^{3,6}.2^{14,17}.1^{8,12}.1^{29,33}.0^{10,25}.0^{34,39}] pentaconta 5, 8(48), 9, 11, 14, 16, 29(45), 30, 32, 34,36,38,46,49- pentadecaene- 40- carboxylic acid.

^{1}H NMR spectrum of the compound MRK1 displayed a broad singlets at δ 9.46 and 9.15 indicates the presence of aromatic –OH group protons. However, the presence of aliphatic –OH group protons in sugar moieties and at Z_2 and Z_6 positions can be found from the δ values obtained in the range of 5.88-5.98. The –$CONH_2$ group proton present in the molecule can be identified by the signal appeared at δ value of 6.94 respectively at W_2, W_3, W_4, W_5, W_6 and W_7 as pointed in the structure. Further the compound exhibited signals in the δ values at 7.99, 6.67, 8.22, 8.63, 6.69 and 8.49 as broad singlets and doublets can be attributed to the presence of –NH group protons. Further the presence of –CH protons between C=O and N-H functional groups can be observed as multiplet, quintet and doublets in the range of 4.19 to 5.75 ppm. The methyl group (-CH_3) protons found in the sugar moiety and at 1 electron position was appeared in the range between 1.07 and 2.43 ppm. Additionally the signals exhibited in the δ value range of 0.862 to 5.27 shows to the presence of aliphatic –CH protons in the molecule whereas the aromatic –CH protons can be found from the δ values in the range of 6.00 to 8.00 respectively for the 2,4,5,6 and 7 aromatic rings as depicted in the structure.

^{13}C NMR spectrum of the compound MRK1 exhibited signals in the δ value range between 167.00 and 173.10 corresponding to the presence of –C=O group carbon atoms respectively at C_1, C_2, C_3, C_4, C_5, C_6 and C_7 positions as marked in the structure. The presence of V_6, V_7, 1e-CH_3 carbon atoms can be identified from the δ values at 17.3, 22.7 and 33.2 respectively. In addition, the signals appeared for –CH_2OH carbon atoms in G_6, Z_2 and Z_6 positions can be detected from the δ values of 61.1, 71.1 and 72.0 respectively. However, the –C-O group carbon atoms of G_3, G_4 and V_4 in the aliphatic regions can be found from the respective δ values at 77.1, 70.0 and 70.6. The signals appeared in the δ values range between 51.4 and 61.6 can be attributed to the presence of aliphatic – C-H carbon atoms found at X_1 to X_7 positions. Further, the other aliphatic –CH carbon atoms positioned at 1a, 1b, 1d, G_1, G_2, V_1 and V_2 positions can be identified from the δ values in the range of 33.6 to 101.7 respectively. The presence of aromatic –CH carbon atom in the aromatic rings 2, 4, 5, 6 and 7 can be found from the δ values in the range of 100.00 to 160.00 ppm. The spectral information obtained from NMR, IR and ESI Mass spectroscopic methods, structure of the compound MRK1 was identified to be Vancomycin.

Figure 1: Map showing the sampling sites of southern peninsula of India

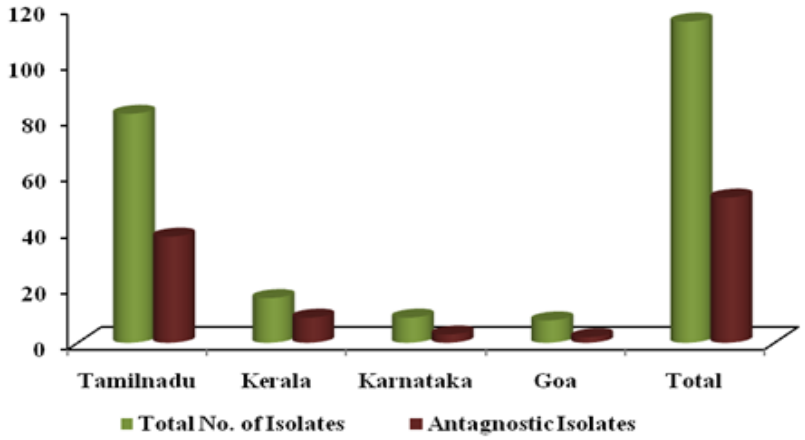


Figure 2: Antagonistic isolates from various regions of Indian Peninsula

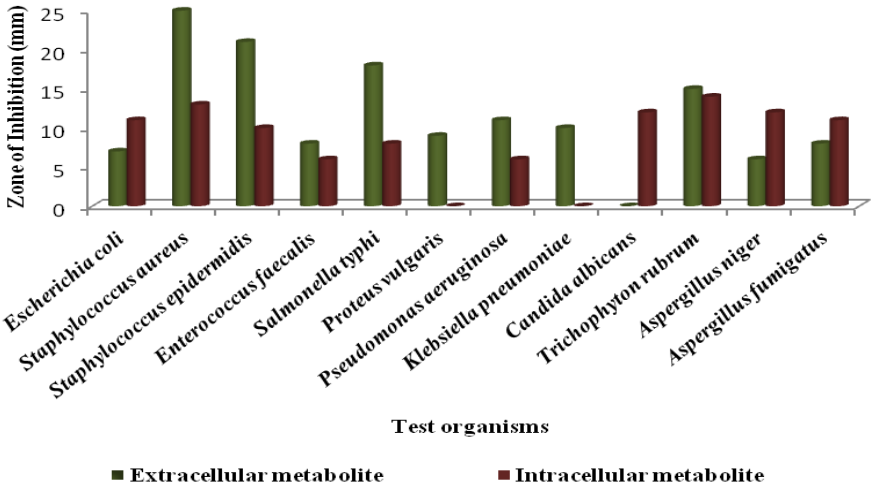


Figure 3: Antimicrobial activity profile for extracellular and intracellular metabolites

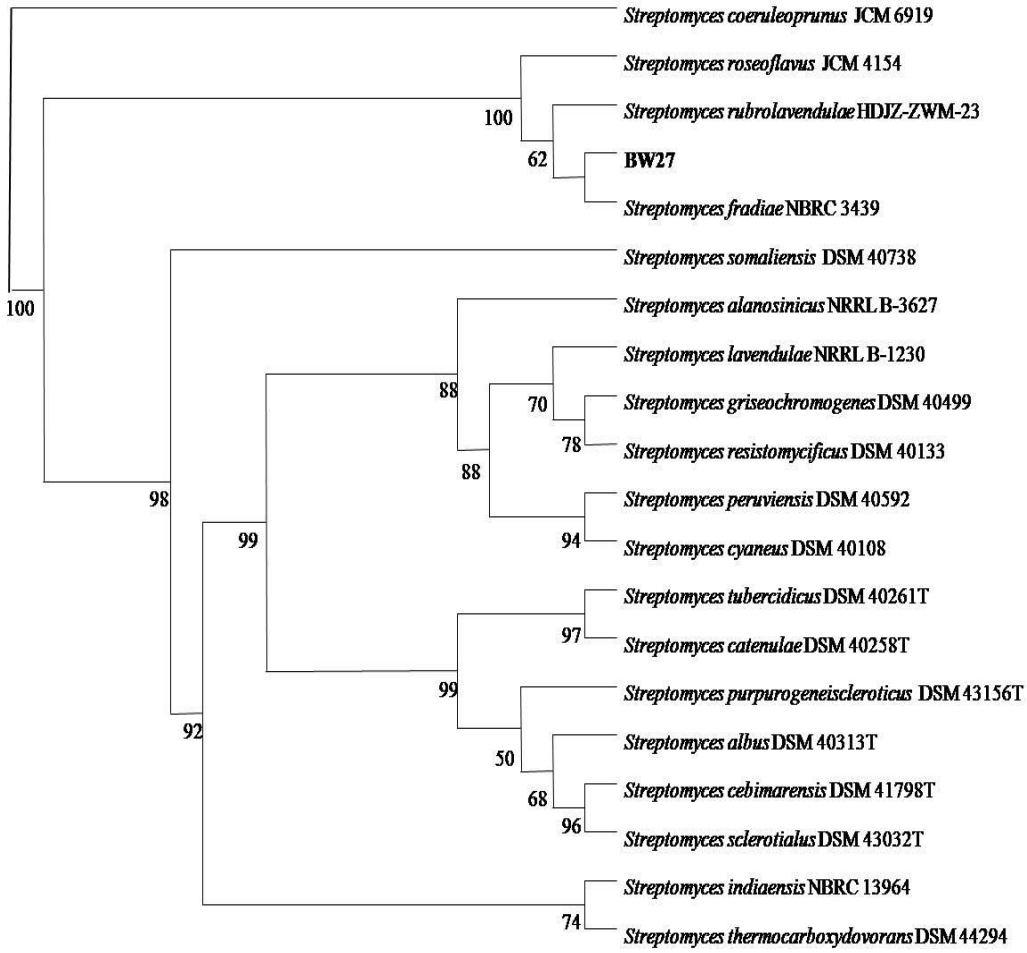


Figure 4: Phylogenetic tree analysis based on Neighbour-Joining method

The phylogram showing the position of strain BW2-7 with other *Streptomyces* based on 16S rRNA gene sequence. Phylogenetic tree based on neighbor joining analysis of 1000 resampled data. Number at nodes indicates the percent level bootstrap support. Score bar represents one nucleotide substitution per 100 nucleotides. Bootstrap values of 50 and above only are shown.

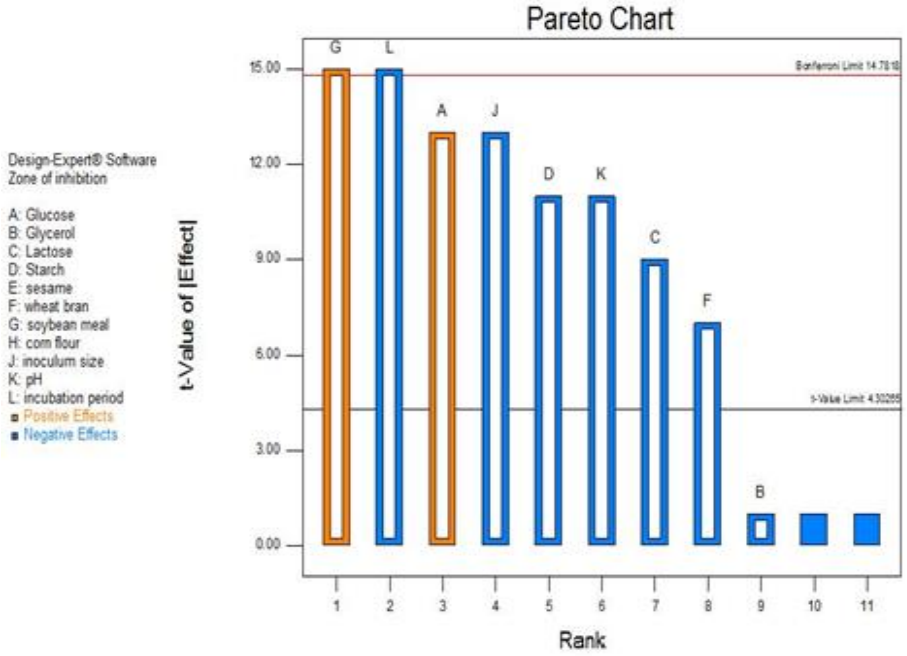
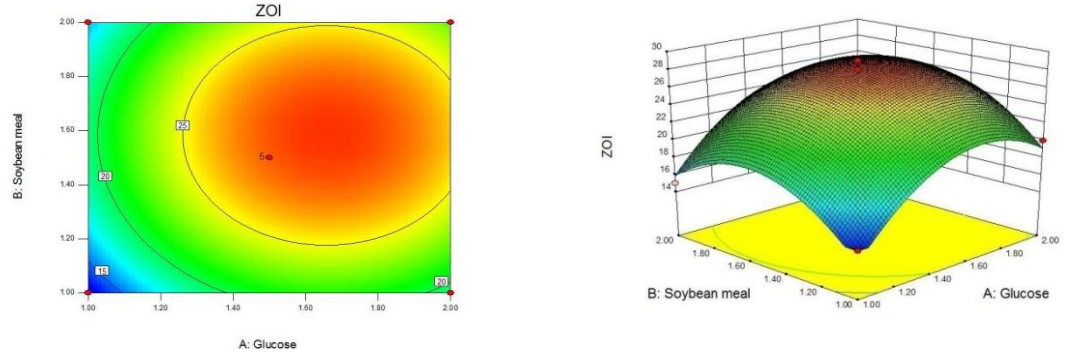
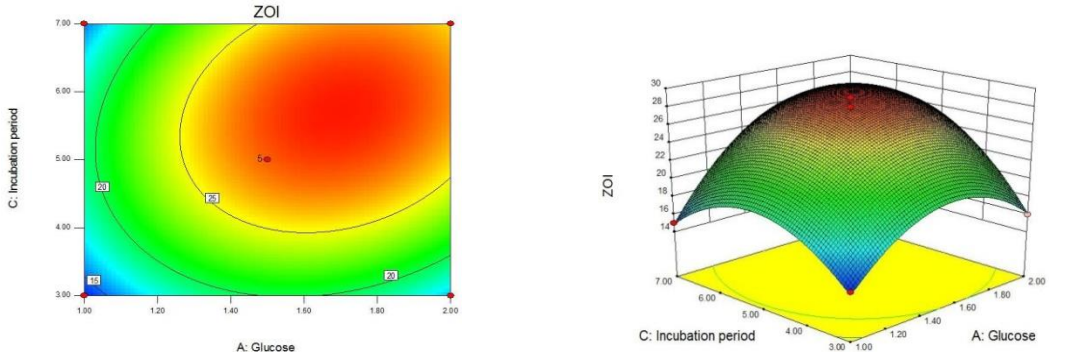


Figure 5: Pareto chart for antibiotic production by *Streptomyces fradiae* BW2-7

Zone of inhibition Vs Glucose and Soybean meal



Zone of inhibition Vs Glucose and Incubation period



Zone of inhibition Vs Soybean meal and Incubation period

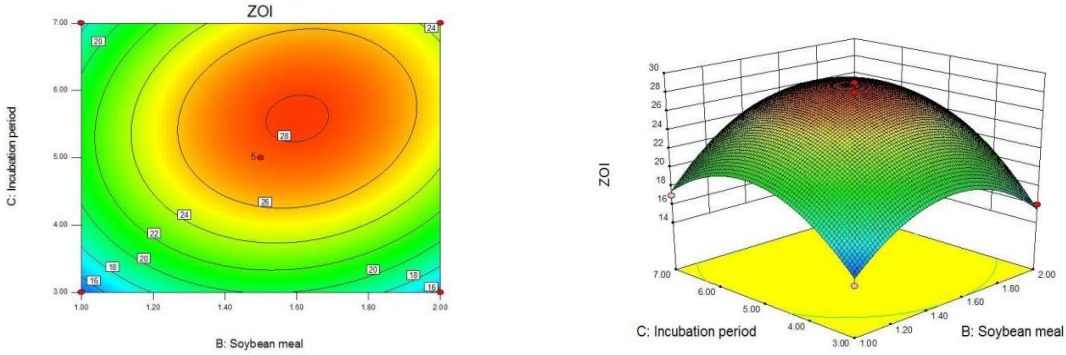


Figure 6: Contour plots and 3-D plots for antibiotic production by *S. fradiae* BW2-7

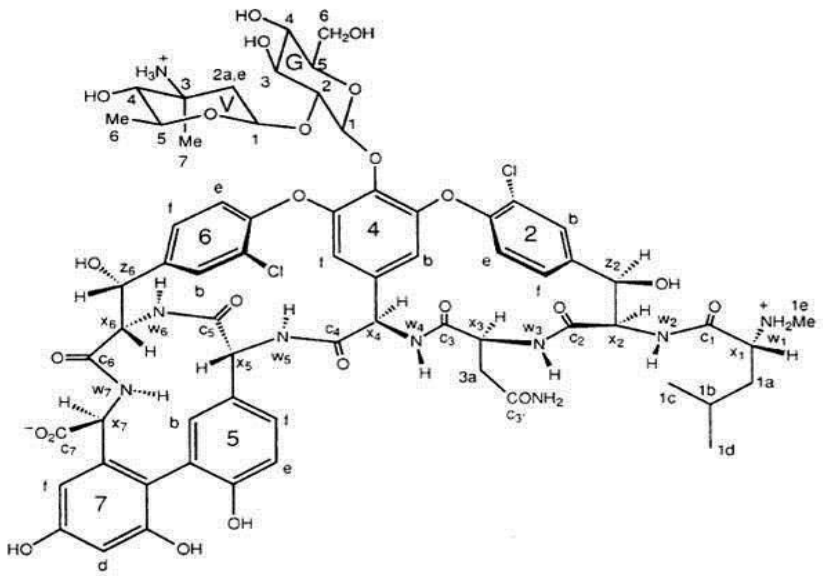


Figure 7: Structure of isolated compound Vancomycin (MRK1)

Table 1: Morphological, physiological and biochemical characteristics of *Streptomyces* sp. BW2-7

Character	Response
Morphological	
Cell shape	Rod
Sporophore morphology	Oval
Aerial mycelium colour	Peach
Colony appearance	Leathery
Pigment colour	Pink
Physiological	
Growth under anaerobic conditions	-
Gram reaction	+
Acid- fast reaction	-
Range of temperature for growth	25˚C - 40˚C
Optimum temperature for growth	30˚C
Range of pH for growth	6.0 – 9.0
Optimum pH for growth	8
NaCl tolerance	4%
Biochemical	
Catalase production	+
Oxidase production	-
Urease production	+
Hydrogen sulfide production	-
Nitrate reduction	+
Gelatin liquefaction	+
Methyl red test	+
Vogues proskauer test	-
Indole production	-
Citrate utilization	-
Amylase production	+
Protease production	+
Esterase production	+
Tyrosine degradation	+
Pectin degradation	-
TSI	AL/AL

Table 2: Culture characteristics of *Streptomyces* sp. BW2-7 on different media

S. No	Name of medium	Appearance
1	Malt extract yeast extract agar (ISP2) Aerial mycelium Substrate mycelium	 Peach Pale yellow
2	Oat meal agar (ISP-3) Aerial mycelium Substrate mycelium	 Light green White
3	Tryptone yeast Aerial mycelium Substrate mycelium	 Dull peach Dull pink
4	Glycerol asparagine agar (ISP-5) Aerial mycelium Substrate mycelium	 Light pink Pale yellow
5	Nutrient agar Aerial mycelium Substrate mycelium	 Dark peach Pale yellow
6	Starch casein nitrate agar Aerial mycelium Substrate mycelium	 Peach Pale yellow

Table 3: Plackett- Burman design and significance of medium components

Run	A	B	C	D	E	F	G	H	I	J	K	ZOI
1	2	1	2	2	2	1	1	1	7	6.5	5	14
2	2	2	2	1	1	1	1	2	7	7.5	1	20
3	2	1	2	2	1	2	2	2	3	6.5	1	21
4	1	2	2	2	1	1	2	1	3	7.5	5	13
5	1	1	1	1	1	1	1	1	3	6.5	1	21
6	2	1	1	1	2	1	2	2	3	7.5	5	21
7	1	2	2	1	2	2	1	2	3	6.5	5	18
8	1	1	1	2	1	2	1	2	7	7.5	5	14
9	2	2	1	2	2	2	1	1	3	7.5	1	18
10	2	2	1	1	1	2	2	1	7	6.5	5	17
11	1	1	2	1	2	2	2	1	7	7.5	1	14
12	1	2	1	2	2	1	2	2	7	6.5	1	19
SE	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	
***p*-value**	0.0059	0.4226	0.0121	0.0082	-	0.0198	0.0044	-	0.0059	0.0082	0.0044	

A-Glucose (%), B- Glycerol (%), C- Lactose (%), D- Starch (%), E- Sesame oil cake (%), F- Wheat bran (%), G- Soybean meal (%), H- Corn flour (%), I-Inoculum size (%), J- pH, K- Incubation period (days), ZOI- Zone of inhibition in diameter (mm).

Table 4: ANOVA for response surface quadratic model for antibiotic production by *S. fradiae* BW2-7

Source	Sum of Squares	Df	Mean Square	F Value	p- Value Prob > F	
Model	537.2853	9	59.69837	46.69146	< 0.0001	**Significant**
A-Glucose	84.5	1	84.5	66.08939	< 0.0001	
B-Soybean meal	21.125	1	21.125	16.52235	0.0048	
C-Incubation period	55.125	1	55.125	43.11453	0.0003	
AB	0	1	0	0	1.0000	
AC	16	1	16	12.51397	0.0095	
BC	6.25	1	6.25	4.888268	0.0627	
A^2	112.7605	1	112.7605	88.19259	< 0.0001	
B^2	102.1289	1	102.1289	79.87739	< 0.0001	
C^2	102.1289	1	102.1289	79.87739	< 0.0001	
Residual	8.95	7	1.278571			
Lack of Fit	3.75	3	1.25	0.961538	0.4924	**not significant**
Pure Error	5.2	4	1.3			
Cor Total	546.2353	16				

Standard deviation - 1.13; Mean - 20.53; C.V. % - 5.51; PRESS - 68.13; R-squared - 0.9836; Adjusted R-squared - 0.9625; Predicted R-squared - 0.8753; Adequate precision - 17.267

Table 5: ^{1}H and ^{13}C NMR data for MRK1 in DMSO

S.No	Proton	value	Carbon	Value
1	**OH**	9.46(br s)	C_1	173.1
2	**OH**	9.15 (v br s)	C_7	172.4
3	**W_5**	8.63 (br d, 2.6)	$C_3(C_{3'})$	171.1
4	**W_7**	8.49 (br d, 5.7)	$C_{3'}(C_2)$	170.5
5	**W_4**	8.22 (v br s)	C_4	169.6
6	**W_2**	7.99 (v br s)	C_5	168.2
7	**6b**	7.86 (s)	C_6	167.5
8	**2f**	7.53(d, 8.5)	$C_2(C_3)$	167.0
9	**6f**	7.47(d, 8.5)	7e	157.6
10	**2b**	7.40 (br s)	7c	156.9
11	**$CONH_2$**	7.36 (o)	5d	155.5
12	**6e**	7.34 (d, 8.5)	4c	152.7
13	**2e**	7.24 (d, 8.0)	4e	151.7
14	**5b**	7.17 (s)	2d	149.9
15	**$CONH_2$**	6.94 (br s)	6d	148.7
16	**5f**	6.77 (d, 8.5)	6a	143.0
17	**5e**	6.72 (d, 8.0)	2a	139.9
18	**W_6**	6.69 (s)	7a	136.7
19	**W_3**	6.67 (s)	5b	136.1
20	**7d**	6.4 (d, 1.5)	4a	134.4
21	**7f**	6.26 (d, 1.5)	4d	132.3
22	**Z_6-OH**	5.98 (d, 5.5)	2b	128.7
23	**Z_2-OH**	5.88 (br s)	6b	127.8
24	**X_4**	5.75 (d, 8.0)	6f(2f/6f)	127.8
25	**4b**	5.55 (br s)	2f(5a)	127.7
26	**V_4-OH**	5.43 (br s)	2c (2f/6f)	127.6
27	**G_3-OH**	5.39 (d, 4.5)	6c	126.6
28	**G_1**	5.27 (d, 7.5)	5a (2c)	126.6
29	**V_1**	5.24 (d, 3.2)	5f	125.9
30	**4f**	5.21 (s)	2e	124.1
31	**Z_2**	5.17 (br s)	6e	123.1
32	**Z_6**	5.14 (br s)	5c	122.1
33	**G_4-OH**	5.11 (s)	7d	118.4
34	**X_2**	4.89 (br m)	5e	116.1
35	**V_5**	4.68(q, 6.5)	4b	107.0
36	**X_5**	4.43 (d, 2.2)	7f	105.0
37	**X_7**	4.42 (d, 5.5)	4f	104.7
38	**X_3**	4.34 (v br q, 5.5)	7d	102.2
39	**X_6**	4.19 (d, 10.5)	G_1	101.7
40	**G_6-OH**	4.07 (t, 5.2)	V_1	97.2
41	**G_{6a}**	3.66 (d, 10.0)	G_2	77.4
42	**G_2**	3.57 (t,8.0)	$G_3(G_5)$	77.1
43	**$G_{6a'}$**	3.53 (o)	G_5 (G_3)	76.6
44	**G_3**	3.40 (t, 8.0)	$Z_6(Z_2)$	72.0
45	**G_5**	2.8 (o)	$Z_2(Z_6)$	71.1
46	**G_4**	2.8 (o)	V_4	70.6
47	**X_1**	2.8 (o)	G_4	70.0
48	**V_4**	3.19 (s)	V_5	63.5
49	**3a**	2.50 (o)	X_6	61.6
50	**1e**	2.43(s)	X_1	61.5
51	**3a'**	2.14 (d, 10.0)	G_6	61.1
52	**V_{2ax}**	1.90 (br d, 9.5)	X_2	58.7
53	**V_{2eq}**	1.76 (br d, 12.5)	X_7	57.2
54	**1b**	1.70 (non, 6.5)	X_4	55.3
55	**1a**	1.50 (quin, 6.5)	V_3	54.4
56	**1a'**	1.49 (quin, 6.5)	X_5	54.2
57	**V_7**	1.32(s)	X_3	51.4
58	**V_6**	1.07(d, 6.5)	1a	40.5
59	**1c**	0.91(d, 6.5)	3a	38.1
60	**1d**	0.86(d, 6.5)	V_2	33.6
61	**-**	-	1e	33.2
62	**-**	-	1d	24.4
63	**-**	-	1c	23.2
64	**-**	-	1d(V_7)	23.1
65	**-**	-	V_7(1d)	22.7
66	**-**	-	V_6	17.3

a Multiplicity abbreviations: br = broad; d = doublet; m = multiplet; non = nonet; o = obscured; quin = quintet; **s** = singlet; t = triplet; v br = very broad; q = quartet.

DISCUSSION

The marine microorganisms are known to be rich sources of novel compounds and produce many pharmacologically potential compounds with antibiotic and antitumor properties. Actinomycetes have been proven as a potential source of bioactive compounds and richest source of secondary metabolites [23]. However, the research on marine actinomycetes from Indian peninsula is very scanty [43], hence east and west coasts of India were selected for this study. Our sampling sites of Bay of Bengal and Arabian Sea shore holds full of biological diversity and are rich in minerals. In the course of systematic screening of marine actinomycetes for antimicrobial activity from the coastal area resulted in isolation of 115 strains. Of all 115 actinomycetes strains, BW2-7 showed a broad-spectrum antibiotic activity (Fig.3) which was isolated from Kannamaly beach had broad spectral antimicrobial activity and was selected for further studies.

T. rubrum was susceptible to both intracellular and extracellular metabolites. So the extracellular metabolite was taken for both antibacterial and antifungal studies. Similarly, various solvents were used for the extraction of antibiotics from actinomycetes by many workers using ethyl acetate and methanol [44, 18] and chloroform [46]. *S. fradiae* BW2-7 isolate presented antibacterial spectrum, exhibiting inhibitory activity against several Gram-positive species such as *S. aureus* and *S. epidermidis* [25, 30]. Many strains of *Streptomyces* sp have been shown as potential biocontrol agents against fungal pathogens. It was reported that the principle mechanism of this antifungal activity involved the production of antibiotics [13]. In Indian peninsula only 41 species of actinomycetes were isolated and studied for its potential role [42]. There are reports on marine *Streptomyces* isolated from southeast coast of India; five antagonistic strains were reported in the Vellar estuary, Tamil Nadu against human bacterial pathogens [39]. Similarly, two *Streptomyces* strains showed antibacterial and antifungal activity collected from west coasts of India [35].

A study on the production of antibiotics usually involves a search for optimal media. This is achieved by a systematic study of the suitability of a large number of carbon and nitrogen sources. Experiments were performed using different carbon and nitrogen sources to check their suitability for antibiotic production. Cultural characteristics and media composition were optimized by a systematic study and the suitability of number of carbon and nitrogen sources were evaluated and correlated. In fact it has been shown that the nature of carbon and nitrogen sources strongly affect antibiotic production in different organisms [17, 48]. The optimization of the environment parameter resulted not only the higher antibiotic activity but also in a reduced amount of the experiments [49]. In this paper, maximal production of antimicrobial compound by strain *S. fradiae* BW2-7 was obtained in a medium supplemented with glucose as carbon source and soybean meal as nitrogen source. But Selvin *et al*. [40] controversially reported that there was no production of antimicrobial compound by the strain when media was supplemented with different carbon sources at a different concentration. In other report, the addition of sucrose to the production medium favored the production of nisin antibiotic by *Lactococcus lactis* [47]. The supplementation of soybean meal as nitrogen source to the production medium resulted in enhanced antimicrobial activity. The production of antimicrobial compound in the media supplemented with soybean meal can be correlated to the presence of maximum growth in the supplemented media. Thus, the supplementation of soybean meal was found to be an essential factor for the production of antimicrobial compounds. A similar result was obtained in production of antimicrobial compounds with cotton seed meal as nitrogen source [8]. This study proves that the production of antibiotic as secondary metabolites is profoundly influenced by the kind and quality of nutritional elements available and environmental factors. After optimization the antibiotic production of *S. fradiae* BW2-7 was improved by 8% as compared with that obtained under unoptimized conditions. The antibiotic activity obtained from the bioreactor experiments was in close agreement with that of the model prediction and the flask experiments [10].

Vancomycin is a clinically important glycopeptide antibiotic. It is used to treat serious infections caused by Gram-positive bacteria and is considered a last line of defense against pathogens that are resistant to broad range of antibiotic agents. At the external surface of the bacterial cytoplasmic membrane, vancomycin's *N*-terminus binds to the D-alanine-D-alanine residues at the *C*-terminus of UDP-*N*-acetylmuramyl pentapeptide, the peptidoglycan precursor. In the absence of vancomycin, a transglycosylase enzyme adds the peptidoglycan precursor to the growing peptidoglycan chain. When vancomycin inhibits this glycosylation reaction, cell wall synthesis is inhibited [2, 26]. Vancomycin, which is produced by *Amycolatopsis orientalis* (previously designated *Nocardia orientalis* and *Streptomyces orientalis*), consists of a heptapeptide core ether linked through the hydroxyl group of its aromatic amino acids. TDP glucose, aglycosyl vancomycin glucosyltransferase catalyzes the addition of glucose to the peptide core of the antibiotic and uses TDP glucose and UDP-glucose. Further, vancomycin exhibited a strong inhibition effect on gram positive bacteria, such as *Streptococci*, *Staphylococci*, and *Clostridium difficile*, which are gram-positive bacteria resistant to penicillin and cephalosporin antibiotics [6, 4, 7, 11]. In addition, vancomycin has been known to have high treating effects on the diseases derived from methicillin-resistant *S. aureus* (MRSA), which is fatal to postoperation patients, elderly patients, and patients

having weak [7, 28]. This is the first report, where the compound was isolated from a new source *S. fradiae* and inhibiting the skin pathogens. To conclusion, the compound isolated from marine actinomycete *S. fradiae* BW2-7 will produce Vancomycin and it will be widely used for skin infections. These results are reported for the first time from these Islands, Gulf of Mannar.

Acknowledgements

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REFERENCES

1. Augustine SK, Bhavsar SP, Kapadnis KP; A non-polyene antifungal antibiotic from *Streptomyces albidoflavus*. Journal of Biological Sciences, 2005; 30(2): 201-211.
2. Barna JCJ, Williams DH; The structure and mode of action of glycopeptides antibiotics of the vancomycin group. Annual Reviews of Microbiology, 1984; 38: 339-357.
3. Barry AL, Thornsberry C; Susceptibility tests: Diffusion test procedures. In Edwin.H.Lennette (Ed.) Annual of clinical microbiology 4th ed. ASM, Washington, DC, USA, 1985; 978-987.
4. Beam TR; Vancomycin therapy of experimental Pneumococcal meningitis caused by penicillin sensitive and resistant strains. Journal of Antimicrobial Chemotherapy, 1981; 7(1): 89-99.
5. Box GEP, Behnken DW; Some New Three-Level Designs for the Study of Quantitative Variables. Technometrics, 1960; 2: 455-475.
6. Cook FV, Farrar WE; Vancomycin revisited. Annals of Internal Medicine, 1978; 88(6): 813-818.
7. Cunha BA, Quintiliani R, Deglin JM, Izard MW, Nightingale CH; Pharmaco kinetics of vancomycin in Anuria. Review of Infectious Disease, 1981; 3: 269-272.
8. De Vuyst L, Vandamme EJ; Infuence of the phosphorus and nitrogen source on nisin production in *Lactococcus lactis* subsp. lactis batch fermentations using a complex medium. Applied Microbiology and Biotechnology, 1993; 40: 17-22.
9. Dutta JR, Dutta PK, Banerjee R; Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. Process Biochemistry, 2004; 39: 2193-2198.
10. Fang XL, Han LR, Cao XQ, Zhu MX, Zhang X, et al; Statistical Optimization of Process Variables for Antibiotic Activity of *Xenorhabdus bovienii*. PLoS ONE, 2012; 7(6): e38421.
11. Farber BF, Moellering RC, Bodey GP; Comparison of Vancomycin disposition in rats with normal and abnormal renal functions. Antimicrobial Agents and Chemotherapy, 1981; 20: 138-141.
12. Fenical W, Jensen PR; In: D.H. Attaway and O.R. Zaborsky (eds) Marine biotechnology I: Pharmaceutical and bioactive natural products. Plenum, New York, 1993; 419-459.
13. Fravel DR; Role of antibiosis in the biocontrol of diseases. Annual Reviews of Phytopathology, 1988; 26: 75-91.
14. Good fellow M, Williams E; Ecology of actinomycetes. Annu Review of Microbiology, 1983; 37: 189-216.
15. Good fellow M, Haynes JA; Actinomycetes in marine sediments. In Biological, Biochemical and Biomedical aspects of Actinomycetes, edited by Ortiz LO, Bojalil, L.F and Yakoleff, V. Academic press. Inc, Orlando Fl, 1984; 453.
16. Helme E, Weyland H; *Rhodococcus marinonascens* sp. Nov., an actinomycete from the sea. International of Journal of Systemic Bacteriology, 1984; 34: 127-138.
17. Holmalathi J, Raatikainen O, Wright A, Laatsch H, Spohr A, Lyngberg OK, Neilson J; Production of dihydro abikoviromycin by *Streptomyces anulatus*. Production parameters and chemical characterization of genotoxicity. Journal of Applied Microbiology, 1998; 85: 61-68.
18. Ilic SB, Kontantinovic SS, Todorovic ZB; UV/VIS analysis and antimicrobial activity of Streptomyces isolates. Facta Universitatis, 2005; 12: 44-46.
19. Janardhan A, Praveen Kumar A, Viswanath B, Saigopal DVR, Narasimha G; production of Bioactive compounds by Actinomycetes and their Antioxidant properties. Biotechnology Research Journal, 2014; Article ID 217030, 8 pages.
20. Jensen PR, Wight RD, Fenical W; Distribution of actinomycetes in near-shore tropical marine sediments. Applied Environmental Microbiology, 1991; 57: 1102-1108.
21. Kawato M, Shinolue R; A simple technique for the microscopical observation. In Memoirs of the Osaka university liberal arts and education.1-1 Yamadaoka Suita, Osaka Japan, 1959; 114.
22. Magarvey NA, Keller JM, Bernan V, Dworkin M, Sherman DH; Isolation and characterisation of novel marine derived actinomycete taxa rich in bioactive metabolites. Applied Environmental Microbiology, 2004; 70: 7520-7529.
23. Manivasagan P, Venkatesan J, Sivakumar K, Kim SK; Marine actinobacterial metabolites: Current status and future perspectives. Microbiological Research, 2013; 168: 311-332
24. Meena B, Anbu Rajan L, Vinith Kumar NV, Kirubagaran R; Novel marine actinobacteria from emerald Andaman & Nicobar Islands: a prospective source for industrial and pharmaceutical byproducts. BMC Microbiology, 2013; 13:145.
25. Mincer TJ, Jensen PR, Kauffman CA, Fenical W; Widespread and persistent populations of a major

new marine actinomycete taxon in ocean sediments. Applied Environmetal Microbiology, 2002; 68: 5005-5011.
26. Nagarajan R; Antibacterial activities and modes of action of vancomycin and related glycopeptides. Antimicrobal Agents and Chemotherapy, 1991; 35(4): 605-609.
27. Nolan RD, Cross T; Isolation and screening of actinomycetes. In: *Actinomycetes in biotechnology*, M. Goodfellow, S.T. Williams, M. Mordarski (Eds), San Diego: Academic Press, 1998; 1-32.
28. Norden CW, Shaffer M; Treatment of experimental chronic osteomyelitis due to *Staphylococcus aureus* with Vancomycin and Rifampicin. Journal of Infectious Disease, 1983; 147: 352-357.
29. Oskay M; Antifungal and antibacterial compounds from Streptomyces strains. African Journal of Biotechnology, 2009; 8: 3007-3017.
30. Parente E, Riccardi A; Production, recovery and purification of bacteriocins from lactic acid bacteria. Applied Microbiology Biotechnology, 1998; 52: 628-638.
31. Pearce CM, Williams DH; Complete assingment of the ^{13}C NMR specrum of vancomycin. Journal of Chemical Society Perkin Transactions, 1995; 2: 153-157.
32. Pridham TG, Lyons AJ; *Streptomyces albus* (Rossi Doria) Waksman. Henrici: Taxonomic study of strains labeled *Streptomyces albus*. Journal of Bacteriology, 1961; 81: 431-441.
33. Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E; The genus Nocardiopsis represents a phylogenetically coherent taxon and a distinct actinomycete lineage: Proposal of *Nocardiopsaceae fam*. nov. International Journal of Systemic Bacteriology, 1996; 46: 1088-1092.
34. Ravel J, Amoroso MJ, Colwell RR, Hill RT; Mercury- resistant actinomycetes from the Chesapeake Bay. FEMS Microbiology Letters, 1998; 162: 177-184.
35. Remya M, Vijayakumar R; Isolation and characterization of marine antagonistic actinomycetes from west coast of India. Facta uviversitatis, 2008; 15: 13-19.
36. Rao JK, Kim C, Rhee S; Statistical optimization of medium for the production of recombinant hirudin from *Saccharomyces cerevisiae* using response surface methodology. Process Biochemistry, 2000; 35: 639-647.
37. Roessner CA, Scott AI; Genetically engineered synthesis of natural products: from alkaloids to corrins. Annual Review of Microbiology, 1996; 50: 467-490.
38. Rosenthal A, Pyle DL, Niranjan K, Gilmour S, Trinca L; Combined effect of operational variables and enzyme activity on aqueous enzymatic extraction of oil and protein from soybean. Enzyme Microbial Technology, 2001; 28: 499-509.
39. Sahu MK, Sivakumar K, Kannan L; Isolation and characterization actinomycetes inhibitory to human pathogens. Geobios, 2006; 33(2-3): 105-109.
40. Selvin J, Shanmugapriya S, Gandhimathi R, Kiran GS, Ravji TR, Natarajaseenivasan K, Hema TA; Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes *Nocardiopsis dassonvelli* MAD08. Applied Microbiology and Biotechnology, 2009; 83(3):435-45.
41. Shirling EB, Gottlieb D; Methods for characterization of *Streptomyces* sp. International Journal of Systemic Bacteriology, 1966; 16: 312-340.
42. Sivakumar K, Sahu MK, Thangaradjou T, Kannan L; Research on Marine actinobacteria in India, Indian Journal of Microbiology, 2007; 47: 186-196.
43. Suthindhiran K, Kannabiran K; Cytotoxic and antimicrobial potential of actinomycete species *Saccharopolyspora salina* VITSDK4 isolated from the Bay of Bengal Coast of India. American Journal of Disease, 2009; 5: 90-98.
44. Taechowisan T, Lu C, Shen Y, Lumyong S; Secondary metabolites from endophytic *Streptomyces aureofaciens* eMUAc130 and their antifungal activity. Microbiology, 2005; 151: 1651-1695.
45. Takizawa M, Colwell RR, Hill RT; Isolation and diversity of actinomycetes in the Chesapeake Bay. Applied Environmental Microbiology, 1993; 59: 997-1002.
46. Thangadurai D, Murthy KSR, Prasad PJN, Pullaiah T; Antimicrobial screening of *Decalepis hamiltonii Wight* and Arn. (Asclepiadaceae) root extracts against food-related microorganisms. Journal of Food Safety, 2004; 24: 239-245.
47. Vessoni Penna, Moraes DA; Optimization of nisin production by *Lactococcus lactis.* Applied Biochemistry and Biotechnology, 2002; 98(100): 775-789.
48. Vilches C, Mendez C, Hardisson C, Salas JA; Biosynthesis of oleandomycin by *Streptomyces antibioticus*: Influence of nutritional conditions and development of resistance. Journal of General Microbiology, 1990; 136: 1447-1454.
49. Wang HS, Lu Y, Xue Y, Ruan ZY, Jiang RB, Xing XH, Lou K, Wei D; Separation, purification and structure identification of purple pigments from Duganella sp. B2. Journal of Indian Chemical Engineering, 2009; 59: 630-635.
50. Westley JW, Liu CM, Evans RH, Blount JF; Conglobatin, a novel macrolide dilactone from *Streptomyces conglobatus* ATCC 31005, Journal of Antibiotics, 1979; 32: 874-877.
51. Weyland H; Distribution of actinomycetes on the sea floor. Zentrabl. Bankeriol. Parasitenkd. Infektionskr. Hy.G. Abt.1, 1981; Suppl 11: 185-193.

52. Weyland H, Helmke E; Actinomycetes in the marine environment. In the biology of actinomycetes. Proceedings of the Biology of Actinomycetes, ed by Okami Y, Beppu J and Ogamura H. Japan Science Society Press, Tokyo, 1988; 294.
53. Williams ST; Streptomyces in soil ecosysytem. In Mordarski M, Kurylowicz W, Jeljaszewiezled J (eds) Nocardia and Streptomycetes. Warsaw. October. Gustav fischer verlag, Stuttgart, 1978; 137-142.
54. Xiong YH, Liu JZ, Song HY, Ji LN; Enhanced production of extracellular ribonuclease from *Aspergillus niger* by optimization of culture conditions using response surface methodology. Biochemical Engineering Journal, 2004; 21: 27-32.