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BIOPROSPECTING FUNGICIDAL METABOLITE PRODUCING MARINE ACTINOMYCETES FROM SOUTHERN COASTAL REGIONS OF INDIA

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ABSTRACT

The fungal pathogens pose deleterious effects on the human beings, most of these diseases are not fatal, but once contracted, they may forever be a source of constant irritation. Microbial secondary metabolites are promising in this aspect by efficiently combating the pathogens. Actinomycetes are priceless prokaryotes which are reported to produce many of the fungicidal secondary metabolites. This investigation has been focused on isolating fungicidal metabolite producing actinomycetes strains from the southern coastal regions of India. A total of 30 actinomycetes isolates were obtained out of which five strains (PA2, KA2, NA2, AG1 and BW2-3) showed a good spectrum of fungicidal activity. These strains were identified as *Streptomyces* genera of actinomycetes by their morphological, biochemical and molecular characteristics, and their sequenceshave been submitted to the NCBI. Further, the secondary metabolites from these strains were extracted using acetone. These metabolites manifested a good spectrum of antifungal, antimicrobial activities against clinically important fungal and bacterial human pathogens. In addition, they contributed significant radical scavenging mechanism as evinced by DPPH and reducing power assays. Hence, this research explains the importance of marine actinomycetes and their prolific source of secondary metabolites with efficient biological activities that gain further research.

KEYWORDS:Antibacterial; Antifungal; Antioxidant; Marine actinomycetes; Metabolites

INTRODUCTION

Fungi are ubiquitous and most of which are opportunistic pathogens. The first exposure to fungi by humans occurs during birth, when they encounter the yeast *C. albicans* while passing through the vaginal canal.[1]The severity of infection depends upon the site and localization by the fungi. Not like the prokaryotes, the eukaryotic fungal eradication poses difficulty. This lightens up the new research focusing on microbial secondary metabolites mainly actinomycetes in combating the fungal pathogenesis with high target specificity without harming the mammalian cells. One such prolific source of actinomycetes is marine environment thatcovers almost 70% of the earth's surface for infinite chemically unique metabolite having a wide range of biological applications.[2,3] Marine ecosystems contain several unique features that set them apart from other aquatic ecosystems, the main factor being the presence of dissolved compounds in seawater. For marine microorganisms, their cellular adaptation to moderate and high-salt content is a fundamental biological process; hence produce different types of bioactive compounds needed for survival,[4] also pose ability to grow by adapting their osmoregulatory mechanisms that signal the production of osmolytes (e.g., polyols, amino acids) in conjunction with an increasing concentration of cytoplasmic ions.[5] Actinomycetes have provided many important bioactive compounds of high commercial value and continue to be routinely

screened for new bioactive compounds. The secondary metabolites produced from marine actinomycetes have distinct chemical structures, which may form the basis for the synthesis of new drugs.[6] Some of the novel metabolites isolated from marine actinomycetes are carcinostatic substances, fungicidal and aminoglycoside antibiotics, cytotoxic substances and other bioactive compounds.[7] In this work we aimed at isolating fungicidal metabolite producing actinomycetes strains from southern coastal areas of India and to investigate their biological properties which may lay a platform for employing them as therapeutic agents.

MATERIALS AND METHODS

Sample collection and Isolation

The soil samples were collected from the southern coastal regions of Tamil Nadu (Rameswaram, Kanniyakumari, Thirunelveli and Chennai), and Kerala (Cochin). From the areas with less human interference, 10cm depth of the top soil layer was removed and about 50g of soil samples were collected at a depth of 15-20cm from the top. The soil samples were brought to the laboratory in aseptic condition and were pretreated to remove moisture content. For isolating actinomycetes the soil samples were serially diluted with sterile distilled water, spread plated on Starch Casein Nitrate agar[8] and incubated for 7days at room temperature. Distinct colonies of actinomycetes were selected and streaked on a new plate, and were maintained in Starch-Casein agar slant, stored at 4°C and were revived prior usage.

Antagonistic Assessment

The isolated actinomycetes strains were checked for their antagonistic potentials against common human fungal and bacterial pathogens. The pathogens used in this study were procured from PSG Hospital, Coimbatore. Common nosocomial infectious pathogens were selected for this study. Fungal pathogens such as *Trichophyton rubrum* (cutaneous mycosis),*Aspergillus niger*, *Aspergillus clavatus* and *Candida albicans* (systemic mycosis), facultative anaerobic bacterial pathogens causing common infection, urinary tract infection and gastrointestinal infection such as *Staphylococcus aureus*, *Enterococcus* sp., *Shigella dystentriae*, *Salmonella typhimurium*, *Proteus vulgaris*, *Klebsiella pneumonia*, *Escherichiacoli, Pseudomonas* sp. were used for this study.

Primary Screening for Potential Strains

A modified 'cross-streak' method[9] was used for the primary screening of actinomycete isolates against fungal pathogens . Saboroud's Dextrose agar plates were inoculated at the center with a 5mm plug of the actinomycete isolate to be tested. The plates were incubated at 28±2°C for 5 days. Three replicates were made for each of the actinomycete isolates. To the 5-day-old plates, 4mm plugs of 4-day-old culture of fungal pathogens were 'cross plugged' 15mm away on either side to the original plug of actinomycete isolate. The control plates were also maintained with the cultures of the test fungi without the actinomycete isolates. The plates were then incubated at 28±2°C, and the zone of inhibition of fungal growth, if any, was measured at 72 h, and the strains showing higher inhibition have been selected for further assessment.

Identification of Potent Strains

Actinomycetes colonies were recognized on the basis of morphological characteristics following directions given by the International *Streptomyces* Project[10] and the Bergey's Manual of Systematic Bacteriology.[11] The cultural and morphological behaviors of the isolates were studied with different medium such as Starch casein nitrate agar(SCN), Streptomyces agar, kusters agar and ISP1-ISP7. The morphology of sporophore with the entire spore chain was observed with a Phase Contrast microscope (Lynx) and Scanning electron microscope using the cover-slip method. The biochemical characteristics such as MR-VP, Indole, H_2S, TSI, production of extracellular enzymes such as oxidase, catalase, amylase, citrase, esterase, gelatinase, caseinase, urease, nitrate reductase, sensitivity to pH, temperature and salinity were estimated followed by the methods of Berd[12] and Kurup *et al.*[13] with minor modifications. The extrachromosomal DNA was extracted based on modified Kutchma *et al*.[14] method, amplified using 16SrRNA gene primers and were submitted to NCBI(KJ921653, KJ921652, KJ921655, KJ921651 and KJ921654).

Production and Extraction of Bioactive Metabolite

The potent strains were inoculated in SCN broth and were incubated in an orbital shaker 180rpm at room temperature for 9 days. The bioactive metabolites from the culture filtrate were extracted using a range of polar and non-polar solvents such

as water, methanol, ethanol, acetone, ethyl acetate, acetonitrile, isopropanol, petroleum ether, dichloromethane, n-butyl alcohol, isoamyl alcohol, acetic acid and chloroform. The best solvent extract that had maximum inhibitory activity was further employed. After extraction, the solvent from the crude metabolites were concentrated, dried and were used for analyzing their bioactive potentials.

Assessment of Bioactive Potential of Crude Extract

Antifungal property

The fungicidal property of the crude extract was determined by well-diffusion assay technique. The above said fungal pathogens were swabbed on Muller Hinton Agar plates and Aliquots of 50µg crude extracts were filled in the previously cut wells on it. The positive control used was Fluconazole (15µg/mL). The plates were then incubated for 48h at room temperature, after which the zone of inhibition was measured. The minimal fungicidal concentration was also evaluated.

Antibacterial property

The antibacterial activity of the concentrated crude extract was determined by the well diffusion method[15] against the specified bacterial pathogens. With the help of a sterile cork borer, wells were punctured onto Mueller - Hinton agar plates previously seeded with one of the test organism. Fifty microgram of the crude extract was added to the wells. The positive control used was Streptomycin (15µg/mL). The diameter of inhibition zone was measured after 24h of incubation at 37^0C. Further, the minimal inhibitory concentrations against the pathogens were also evaluated.

Antioxidant Property

DPPH assay

The antioxidant property of the crude extract was assessed by DPPH radical scavenging assay technique.[16]The reaction mixture contained 1mM DPPH (diphenyl 2-picryl hydrazyl radical) in 95% methanol. Crude extract at different concentrations (0.1mL) was mixed with 3.9 mL of DPPH (0.025g/L) and incubated in the dark for 30 min. Absorption of samples was measured at 517nm, and ascorbic acid was used as standard.

Reducing power

The reducing capacity of the compound may serve as a significant indicator of potential antioxidant activity. The reduction of ferrous ion ($Fe^{3+)}$ to ferric ion (Fe^{2+}) is measured by the intensity of the resultant Persian-blue solution at 700 nm. The Fe^{3+}-reducing power of the extractwas determined by the method of Oyaizu *et al*.[17] with slight modification. Different concentrations (20-100 µg/mL) of the extract (1ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium hexa cyanoferrate (1%), followed by incubation at 50°C in a water bath for 20 min. After incubation, 2.5 mL of trichloro acetic acid (TCA 10%) was added to terminate the reaction. The upper portion of the solution (2.5mL) was mixed with 2.5 mL distilled water, and 0.5 mL $FeCl_3$ solution (0.1%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. The higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

STATISTICAL ANALYSIS

All experiments were conducted as triplicates and data were expressed as mean ± standard error.

RESULTS

Isolation and Screening of Actinomycetes

A total of 30 different isolates were obtained from sediment samples of sea shore from different districts of southern coastal regions of India. The percentage of white-sporulated (40%) colonies were predominant when compared to the other colored colonies such as peach (20%), ash (14%), grey (13%), brown (7%), Yellow (3%) and pale yellow (3%) according to the standard actinomycetes color charts (Fig.1.).

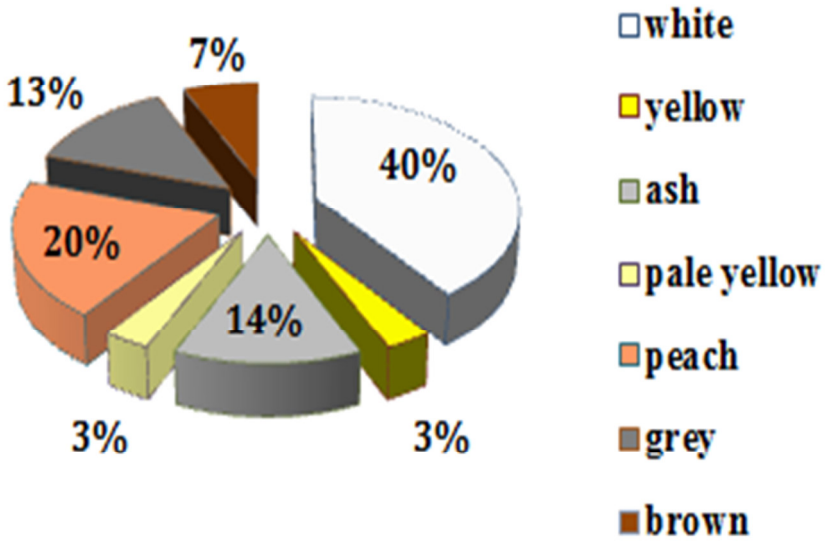

Figure 1
Percentage of colonies obtained

These isolates were screened for fungicidal potency against cutaneous and systemic mycosis causing pathogens. Five of the strains such as PA2, KA2, NA2, AG1 and BW2-3 showed potent activities and were further characterized (Table.1).

Table 1
Primary Screening of actinomycetes

Isolates	Primary Screening (zone of inhibition in mm)			
	Trichophyton rubrum	*Aspergillus niger*	*Aspergillus clavates*	*Candida albicans*
PA2	9 ± 0.06	19 ± 0.03	10 ± 0.05	11 ±0.03
KA2	17.2 ± 0.25	18.1 ± 0.27	16 ±0.11	18 ±0.12
NA2	5.9 ± 0.11	7 ± 0.0	10.3 ±0.33	9 ±0.05
AG1	19.3 ± 0.3	11.2 ± 0.39	15.5 ±0.20	13.7 ±0.23
BW2-3	17.2 ± 0.25	10 ± 0.05	11.5 ±0.11	12 ±0.05

Morphological studies of the screened five isolates revealed that all the isolates were gram positive and grew well on various media and showed leathery, lichnoid to butyrous texture morphology. Growth on the ISP media 1to7 was from moderate to good for BW2-3 isolate, and other four isolates (PA2, KA2, NA2 and AG1) showed similar growth on ISP media 1 to 7 except for ISP 4. The study of their sporophore and spore morphology ofthree isolates (PA2, KA2 and AG1) showed rectus whereas BW2-3 and NA2 showed flexibilis type of spore arrangement, and mature spores were of 0.4 – 0.7µm in diameter and 0.5-1.0µm in length (Fig.2).

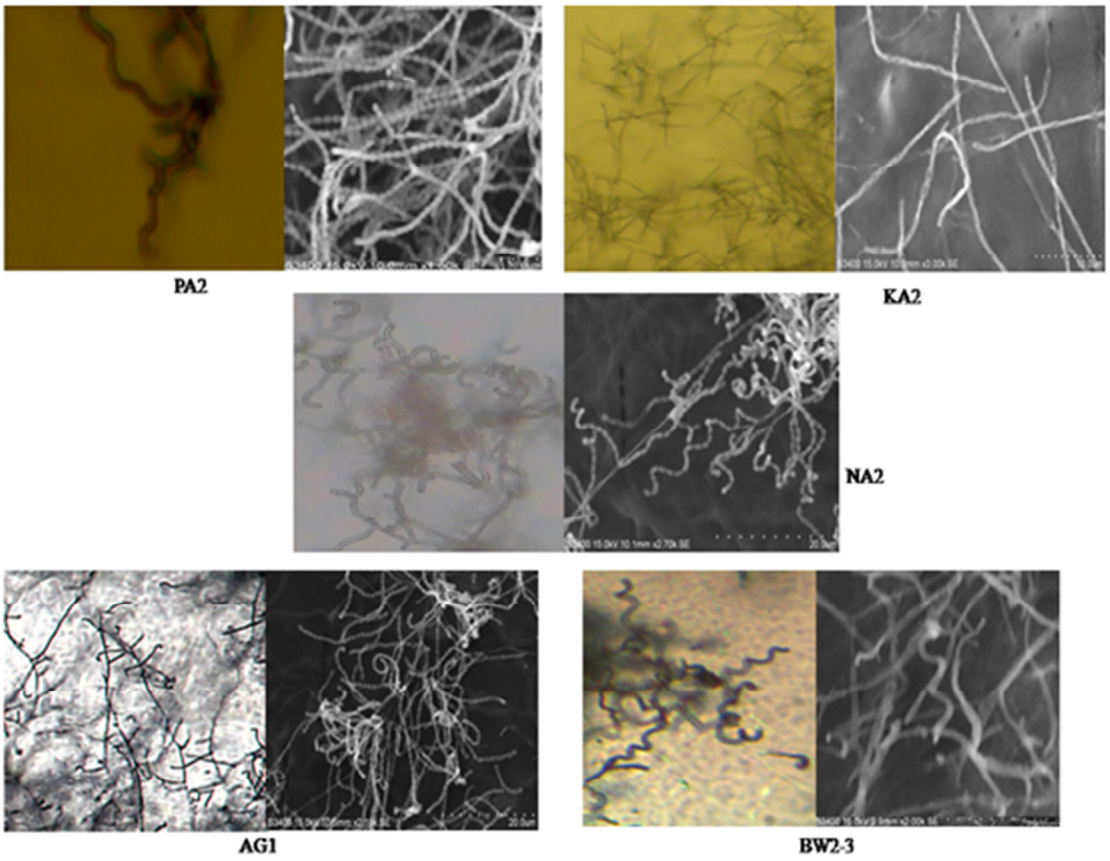

Figure 2
Spore morphology

The biochemical studies showed that they have reacted positive to negative in a wide range of tests used. All grew moderate to good in temperatures 4°C, 27°C, 37°C; pH range from 3 to 11 and tolerated upto 9% of salinity (Table 2.). The results of the morphological and biochemical studies were in congruence with the molecular studies evinced by the results accommodating the five strains in Streptomyces genera of Actinomycetes. The sequences of the five strains were submitted to the NCBI and their accession numbers are PA2- *Streptomyces sampsonii* (KJ921653), KA2- *Streptomyces tanashiensis* (KJ921652), NA2- *Streptomyces sp.* (KJ921655), AG1- *Actinoalloteichus cyanogriseus* (KJ921651) and BW2-3 - *Streptomyces griseus* (KJ921654).

Table 2
Potential strain characteristic

Strain	Species & Accession No.	Strain characteristics
PA2	*Streptomyces sampsonii* (KJ921653)	White colonies, extensively branched mycelium with Rectus sporophore, well grown except ISP4 medium, negative for MR-VP, H_2S, produces all extracellular enzymes except citrase, Mesophillic, withstand all pH ranges and upto 9% salinity
KA2	*Streptomyces tanashiensis*(KJ921652)	Peach colonies, extensively branched mycelium with Rectus sporophore, well grown except ISP4 medium, negative for VP, Indole, H_2S, not able to produce gelatinase, urease, oxidase, nitrate reductase and citrase, psychrophillic to Mesophillic, withstand all pH ranges and upto 2% salinity
NA2	*Streptomyces sp.* (KJ921655)	Grey colonies, extensively branched mycelium with Rectus sporophore, well grown except ISP4 medium, negative for MR-VP, Indole, H_2S, produces all extracellular enzymes except citrase and oxidase Mesophillic, withstand all pH ranges and upto 9% salinity
AG1	*Actinoalloteichus cyanogriseus* (KJ921651)	Grey colonies, extensively branched mycelium with Rectus sporophore, well grown except ISP4, ISP7 media, negative for MR-VP, H_2S, not able to produce caseinase, gelatinase, urease, nitrate reductase and citrase, Mesophillic, withstand all pH ranges and upto 7% salinity
BW2-3	*Streptomyces griseus* (KJ921654)	Peach colonies, extensively branched mycelium with Flexibilis sporophore, well grown in all media, negative for MR-VP, H_2S, not able to produce urease and citrase, psychrophillic to Mesophillic, withstand all pH ranges and upto 9% salinity

Production and Extraction of Bioactive Metabolites

The production medium for the bioactive metabolites used was SCN broth. After 7 days of incubation at shaking condition at room temperature, the metabolites were extracted with different solvents. However, the bioactive metabolites from the culture filtrate were well extracted with acetone. Once after extraction, the crude extracts were concentrated using the rotovapour, dried and weighed. The yield was about 1.08g, 1.5g, 1.3g, 1.1g and 1.7g of crude metabolites per litre from strains PA2, KA2, NA2, AG1 and BW2-3, respectively, which were higher than that of the other solvents employed, also the biopotency was retained which was evident from their biological activities. Hence acetone was used for the extraction of metabolites.

Assessment of Bioactive Potential of Crude Extract

Antimicrobial property

Antifungal property

The antifungal properties exhibited by the potential strains were displayed in Fig.3. All the strains showed good fungicidal activity (mm), strain PA2 (22±0.09) inhibited skin pathogen *Trichophyton rubrum* in a very efficient way followed by KA2 (20.2±0.2) and AG1 (20.3±0.04). The strain NA2 poses minimum activity towards *Trichophyton rubrum* whereas it proves its ability toward other fungal pathogens. AG1 showed maximum inhibiting activity towards *Aspergillus clavatus* (30.5±0.06) and *Aspergillus niger*(27.5±0.1) whereas BW2-3 was efficient in its activity towards *Candida albicans* (30.2±0.05) and *Aspergillus niger* (30±0.1). The minimal fungicidal concentrations of many of the strains were found to be 20µg against many of the fungal pathogens except for *Trichophytan rubrum*, 25µg of PA2 extract was sufficient to inhibit whereas the concentration of the other extracts was slightly higher.

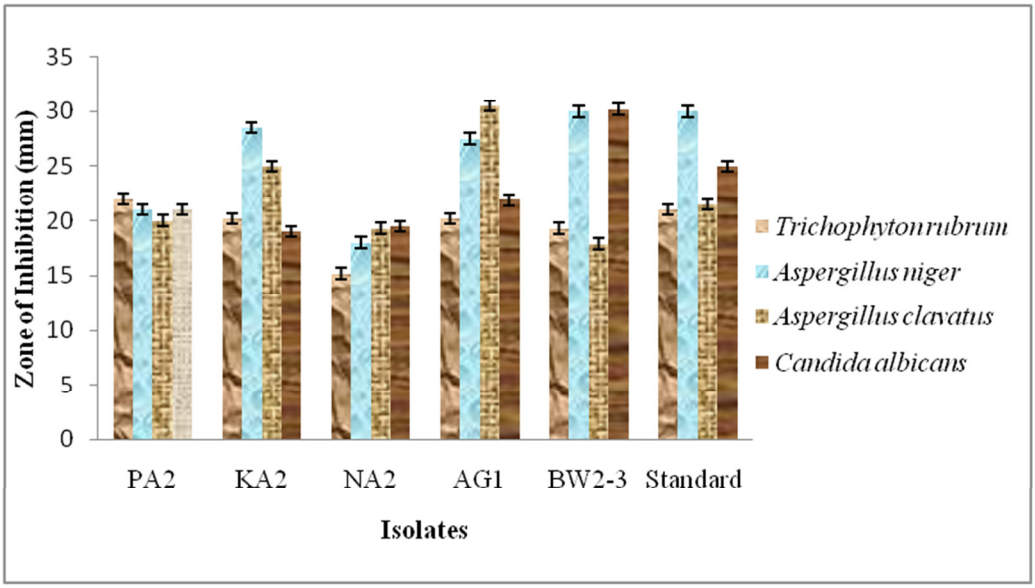

Figure 3
Antifungal property

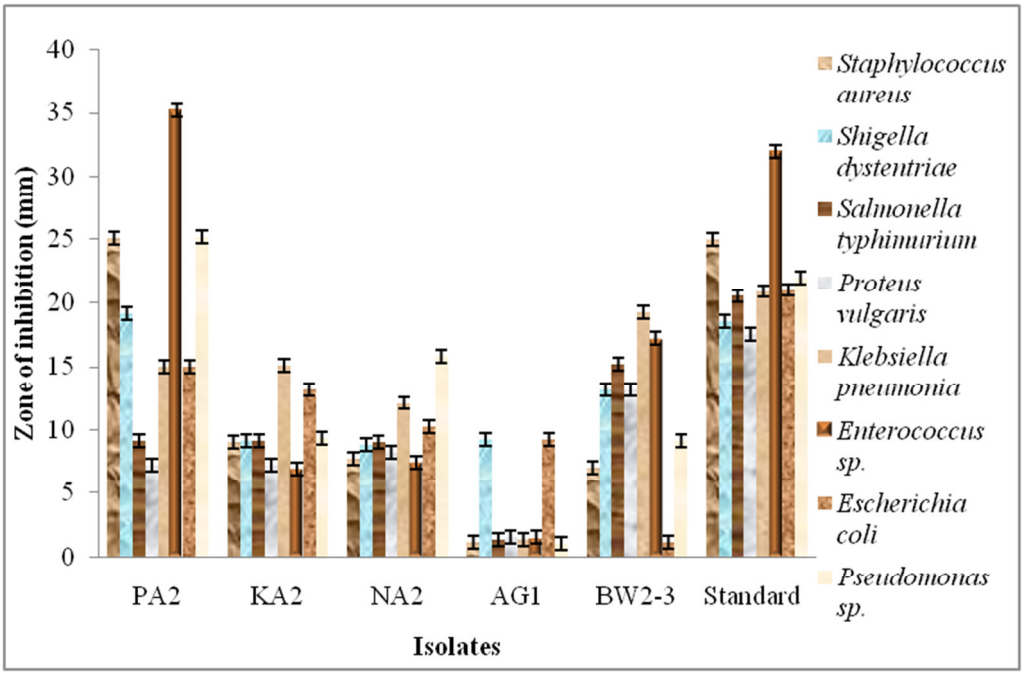

Figure 4
Antibacterial property

Antibacterial property

Many metabolites produced by actinomycetes were reported as antibiotic compounds exhibiting a wide range of the antagonistic spectrum against gram-positive and gram-negative bacteria. The new era of antibiotic resistance and specific targeting with less cumulative effects on other sites with easy removal from biological system kindles the new researches focusing on microbial metabolites. The metabolites from the previously specified five potent strains showed good antibacterial activity against the eight bacterial pathogens. Figure 4 shows their antibacterial spectrum. The strain PA2 showed maximum activity against all the pathogens and it had strong inhibition towards *Enterococcus sp.*(35.2±0.76). Strains KA2 and NA2 showed more or less similar activity towards the pathogens, whereas the strain AG1 exhibited good activity towards *Shigella dystentriae* and *Escherichia coli* but poor to inhibit the other pathogens. The strain BW2-3 had a good spectrum of inhibition against *Klebsiella pneumonia* (19.2±0.15) and *Enterococcus sp.* (17.2±0.17) and contributed less activity towards *Escherichia coli*. The inhibitory concentration was efficient even at 15µg to inhibit many of the bacterial pathogens by all the strains except for AG1.

Antioxidant Property

The multiple defense system present in our body against damaging free radicals is collectively called antioxidants.[18] Scientific evidence suggests that antioxidants reduce the risk of chronic diseases including cancer and heart disease.[19] In our study also the metabolites from the potent strains showed good antioxidant property evinced by their action towards DPPH and reducing power assays.

DPPH assay

Scavenging of DPPH represents the free radical scavenging activity of antioxidants based on a one electron reduction. The reduction in the number of DPPH molecules can be correlated with the number of available hydrogen groups. The activity was evident by the color change to yellow from purple during the assay. 50% of inhibition was contributed at the concentration range of 110µg, 160µg, 130µg, 121µg and 90µg by the strains PA2, KA2, NA2, and AG1and BW2-3, respectively, which proves their ability in scavenging free radical by hydrogen donation (Fig.5).

Reducing power assay

Reducing properties are generally associated with the presence of reductones. The antioxidant action of reductones is based on the breaking of the free radical chain by donating a hydrogen atom. The reducing power was measured by their increase in optical density. All the five strains showed a steady increase in their optical density at increasing concentrations, the strains AG1 and BW2-3 showed higher activity when compared with the other strains (Fig.6). The activity of BW2-3 was on par with the standard ascorbic acid used.

Antioxidant property

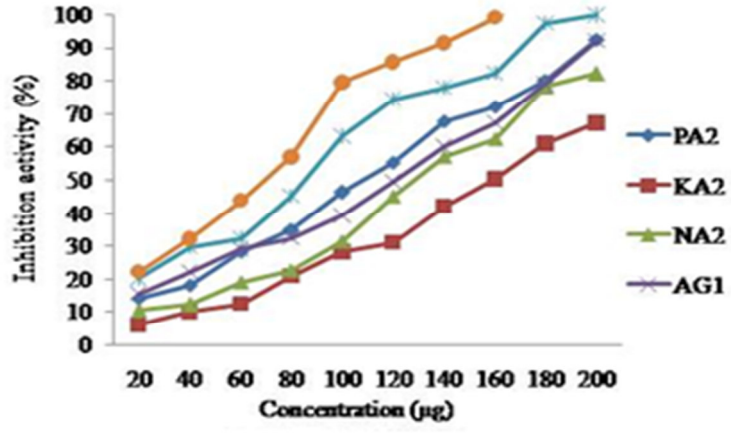

Figure 5
DPPH assay

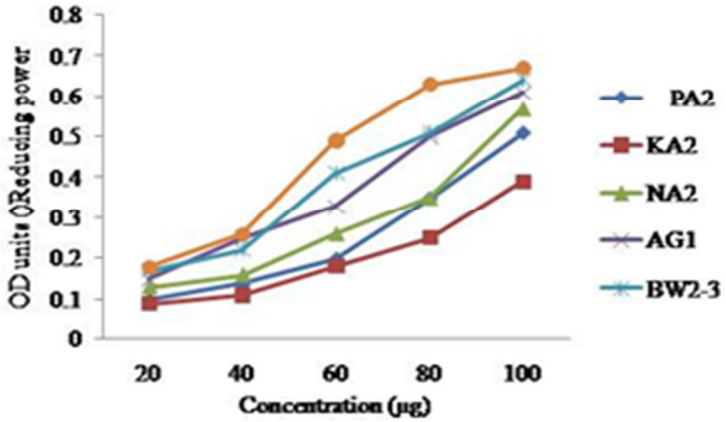

Figure 6
Reducing power assay

DISCUSSION

Many of the fungal metabolisms like protein and nucleic acid synthesis were much similar to that of the mammalian system, hence pose difficulties in eradicating. In this aspect, many microbial metabolites have been studied widely and actinomycetes were found to be promising with new novel metabolites with its potency towards fungal pathogens. In this work, we focused on the fungicidal metabolites from actinomycetes isolated from the southern coastal regions of India. The marine environment kindles the actinomycetes with many secondary metabolites for adaptive survival due to the stressed conditions posed by it. Surprisingly, the secondary metabolites produced by microbial adaptation to rough conditions are of industrially important due to their novel properties. Many of the actinomycetes are found to be promising contributors of economically important secondary metabolites, among which 80% were accounted from Streptomyces genera till date.[20] Similarly in our study, a total number of 30 different actinomycetes isolates were obtained among which the potent isolates were belonged to Streptomyces genera. On fungicidal screening against systemic and cutaneous mycotic pathogens, five of the strains (PA2, KA2, NA2, AG1 and BW2-3) showed good potentials and were identified as Streptomyces genera through their morphological and biochemical behaviours as per the directions of Bergey's manual and international streptomyces project. Further, it has been confirmed by the 16SrRNA sequencing and these strains were identified as PA2- *Streptomyces sampsonii* (KJ921653), KA2- *Streptomyces tanashiensis* (KJ921652), NA2- *Streptomyces sp.* (KJ921655), AG1- *Actinoalloteichus cyanogriseus* (KJ921651) and BW2-3 - *Streptomyces griseus* (KJ921654).The percentage of metabolically active isolates in terms of extracellular enzyme and bioactive secondary metabolite production was higher for those five screened isolates. Positive

reaction for the production of extracellular enzymes and other tests revealed that these isolates from the southern coastal regions were metabolically active. The growth of isolates in saline conditions (upto 9% NaCl) and at higher pH (pH 11) showed that these organisms were extremophilic with regard to their salt tolerance. Hence these metabolites were extracted with the help of solvent acetone and were taken to study their biological applications such as antibacterial, antifungal and antioxidants.All the five strains exemplified their potentiality by exhibiting strong inhibition activities towards fungal and bacterial pathogens with almost the least minimum fungicidal concentrations (20µg) and minimum inhibitory concentrations (15µg). The spectrum of their activities differed mainly due to the differences of their secondary metabolite production. The strain PA2 (*Streptomyces sampsonii*) exhibited a good spectrum of antibacterial activity when compared to the other four strains. Similar to the study by Jain *et al*.[21], this strain also proved its potency over the fungal strains used. The strains AG1 (*Actinoalloteichus cyanogriseus*) and BW2-3 (*Streptomyces griseus*) were found to be very effective against fungal pathogens. When compared to the previous studies by several researchers, the isolated strains proved their efficiency in combating human pathogens.For last few decades, the researchers revealed the importance of free radicals and their role in inducing several dreadful diseases in humans. The free radical generally arises during normal cell metabolism and when in intemperance, it leads to oxidative stress ensuing DNA damages and implicated in the pathogenesis of abundant disorders. The novel and highly potential antioxidants from the marine actinomycetes are promising in scavenging the free radicals and thus preventing the disorders.[22]Similarly in our studies also the isolated metabolites from the potent strains evinced their activity in scavenging the free radical by donating hydrogen atoms, and their antioxidant potential followed a dose-dependent phenomenon. The scavenging activities were prompt even with their lowest concentration as evident from these assays indicating their potential use as antioxidant compounds.

CONCLUSION

In conclusion, this research signifies the metabolites from the marine Streptomyces as potent antimicrobial and antioxidant compounds since these strains from an adaptive environment. Further from the results, it is understood that the metabolites obtained from the potent strains has the prospective to be exploited as the source of novel microbial bioactive compounds exhibiting good fungicidal, bactericidal,and anti-cancer compound since exhibiting potent antioxidants even at lower concentrations. Hence the further studies aimed at purifying potent biomolecules to study their chemical nature and employing them in antimicrobials as well as anticancer studies through *in vivo* and also as to exercise as therapeutics.

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