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Original Article

ISOLATION, SCREENING AND CHARACTERIZATION OF ANTIBIOTIC PRODUCING ACTINOMYCETES FROM KAPULUPPADA PLASTIC WASTE DUMPING YARD, VISAKHAPATNAM

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ABSTRACT

Objective: To isolate, screen and characterize antibiotic producing actinomycetes from Kapuluppada plastic waste dumping yard, Visakhapatnam.

Methods: A total of 12 soil samples were collected, serially diluted and spread on starch casein agar supplemented with Rifampicin and Cycloheximide for inhibition of bacteria and fungi, respectively. Cross-streak method was used to check the antagonistic activity of isolated actinomycetes against bacteria and fungi. Crude extracts from submerged state fermentation were used for the production of antimicrobial compounds. Agar well diffusion method was used for antimicrobial activity of crude extracts against test organisms. The isolates were characterized by morphological, physiological and biochemical methods.

Results: A total of 110 actinomycete isolates were isolated from plastic waste dumping yard. All isolates had shown antimicrobial activity against one or more tested bacteria/fungi. The crude extract of the isolates PD66 (12.2 mm), PD85 (11.5 mm) were most active against methicillin-resistant *Staphylococcus aureus*, PD4 (14.1 mm), PD66 (15.6 mm) were active against *Pseudomonas aeruginosa*, whereas the extracts of PD10 (19.2 mm), PD47 (19.8 mm), PD106 (19.1 mm) were active against *Candida albicans,* PD10 (14.6 mm), PD82 (15.7 mm) active against *Saccharomyces cereviciae*. The isolates had shown varying morphological, physiological and biochemical characteristics.

Conclusion: The actinomycetes isolated from Kapuluppada plastic waste dumping yard were found to be most promising microorganisms for the production of antibacterial and antifungal antibiotics.

Keywords: Actinomycetes, Cross-streak method, Agar well diffusion method, Crude extracts, Antibiotics, Antibacterial activity

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INTRODUCTION

Bioactive secondary metabolites are produced by microorganisms such as bacteria, fungi, actinomycetes. Of these, actinomycetes play an important role [1-3]. Actinomycetes are Gram-positive bacteria but are distinguished from other bacteria by their morphology and DNA (deoxyribonucleic acid) rich in guanine plus cytosine (G+C) [4-6]. Actinomycetes are of universal occurrence in nature and are widely distributed. They are found in soils, fresh water, marine water and mangrove sediments, lake, manures, composts and dust as well as on plastic waste areas and in food products, etc. However, the diversity and distribution of actinomycetes that produce bioactive secondary metabolites can be determined by physical, chemical and geographical means [5, 6]. They produce many important bioactive substances having commercial value and their ability to produce a variety of bioactive substances has been utilized in numerous research and industrial laboratories. This has resulted in the isolation of metabolites from actinomycetes, which have found application in treating a variety of human infections [7]. Because of this reason, 70% of naturally occurring antibiotics had been isolated from a different genus of actinomycetes [8]. Among them, *Streptomyces* is the largest genus known for the production of many bioactive secondary metabolites [9], which had antibacterial, antifungal, antiparasitic, anticancer and immunosuppressive activities [1, 10, 11]. Some antibiotics such as penicillin, erythromycin, and methicillin which were effective against infectious diseases [12, 13], are now less effective because bacteria had become resistant to such antibiotics. Methicillin resistant pathogen such as methicillin-resistant *Staphylococcus aureus* and others cause an enormous threat to the treatment of serious infections. To overcome this problem, immediate replacement of the existing antibiotic is necessary [13], and the production of novel drugs against drug-resistant pathogens is important. Thus, producing novel antibiotics as well as using combinatorial therapy are effective in the treatment of resistant pathogenic infections. Antibiotic synergism between known antibiotics is a novel approach and has an important activity against pathogens and host cells [14]. The research focused mainly in finding novel antibiotics and increasing the productivity of antibiotics has been a very important activity [3, 7]. This is because some biologically important drugs are expensive and have adverse effects to the host, some microorganisms have no successful antimicrobial agents, and others are developing multidrug resistance. It requires more attention to find solutions by searching and producing novel and more effective antibiotics from actinomycetes. However, there is no such scientific report on antibiotics producing actinomycetes from soil samples collected in Kapuluppada plastic waste dumping yard, Visakhapatnam, Andhra Pradesh (India). Therefore, the objective of the present study was to isolate, screen and characterize antibiotic producing actinomycetes from plastic waste dumping yard. The outcome of this finding may be important to give direction for researchers and for future treatment of multidrug-resistant human pathogens.

MATERIALS AND METHODS

Study area and samples collection

The soil samples were collected from Kapuluppada plastic waste dumping yard near Kommadi (Madhurawada), Visakhapatnam, Andhra Pradesh (India). The 80-acre dump yard is about 20 km away from Visakhapatnam and is being dumped at the yard for nearly 17 y. Within a short distance of this yard, there are around 8,000 housing units constructed under Rajiv Gruhakalpa, while Marikavalasa, Boruvanipalem, Drivers' colony, Saradanagar, Pata Paradesipalem, Kotta Paradesipalem and Kapuluppda are in the vicinity. About 30-40 percent living in the housing colonies have vacated because of the pollution caused by this yard.

A total of 12 soil samples were collected from 5-10 cm depth during the month of March 2014 by inserting sterile corer into the soil. The

samples were transferred to a sterile polythene bag and transported immediately to the laboratory for further processing. The soil samples were air-dried at room temperature, crushed in mortar and pestle to make fine particles, sieved and used for actinomycetes isolation [15].

Sampling and isolation of actinomycetes

From each sample collected above, 1 g of soil sample was added in different test tubes containing 10 ml sterile distilled water and shaken well using vortex mixer. They were considered as stock samples for different soil sample sites. From the stock samples, a volume of 1 ml was transferred aseptically and added to a test tube containing 9 ml of sterile distilled water and mixed well. From this test tube, 1 ml of the aliquot was again transferred and mixed with another 9 ml of sterile distilled water to make 10^{-2} dilution factor. Similarly, dilutions up to 10^{-8} were made using serial dilution technique for all soil samples. A volume of 1 ml of suspension from 10^{-7} and 10^{-8} dilutions was taken and spread evenly with sterile glass rod over the surface of sterile starch casein agar plates aseptically using spread plating technique. Cycloheximide (50 µg/ml) and Rifampicin (25 µg/ml) were added in medium to inhibit fungal and bacterial contamination, respectively. The plates were incubated aerobically at 27 °C up to 7-10 d and observed intermittently during incubation [16]. After incubation, morphologically distinct actinomycetes colonies on the starch casein agar plates were picked and further sub-cultured onto their respective isolation media. The actinomycete colonies were purified by streak plate method [17, 18]. Once the pure colonies were obtained, each colony was further identified based on its earthy like smell, colony morphology, the colour of hyphae and the presence or absence of aerial and substrate mycelium. Then, selected and identified colonies of actinomycetes were transferred from the plate to starch casein agar slant and incubated at 27 °C for their growth. After incubation, the slants containing pure isolated actinomycetes were stored at 4 °C and preserved as glycerol stocks at-20 °C for further studies.

Preliminary screening

Actinomycetes isolated above from different soil samples were screened for their antimicrobial activity against bacteria and fungi. The test bacteria used for primary screening were Methicillin-resistant *Staphylococcus aureus* (clinical isolate, King George Hospital, Visakhapatnam), *Pseudomonas aeruginosa* (clinical isolate from King George Hospital (KGH)), *Escherichia coli* NCIM2065, *Klebsiella pneumonia*, *Bacillus subtilis* NCIM2063, and *Bacillus pumilis* NCIM2327. Antifungal activity of these actinomycetes was determined using *Saccharomyces cerevisiae* MTCC170, *Candida albicans* MTCC183, *Aspergillus niger* NCIM 548, *Penicillium chrysogenum* NCIM 738 and *Aspergillus oryzae* NCIM 643 as test organisms. Activities were performed using nutrient agar for bacteria and potato dextrose agar for fungi by cross streak method [19] and agar overlay method [20-22]. Each plate was streaked and stabbed with each isolate at the center of a plate and incubated at 27 °C for approximately 6-7 d. Then, 24 h subcultured bacteria, and 48 h old culture of fungi were streaked perpendicular to the actinomycete isolate and also by agar overlay method. Then, the plates were incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. After incubation, the zone of inhibition was measured and recorded.

Extraction of antimicrobial compounds

Based on the results of primary screening by streak plate and agar overlay methods, actinomycete isolates with potential antimicrobial activity were selected for submerged fermentation and extraction of antimicrobial compounds, and then the crude extracts were assessed following agar well diffusion method [23]. The selected antagonistic actinomycete isolates against bacteria and fungi were inoculated into starch casein broth separately and incubated at room temperature in a shaker at 180 rpm for seven days. After incubation, the broths were filtered through Whatman No. 1 filter paper. Then, the filtrates were centrifuged separately at 5000 rpm for 10 min to extract the antimicrobial compounds. The supernatant was transferred aseptically into a screw-capped bottles and stored at 4 °C for further study [24]. The wells of 6 mm diameter were cut using a sterile borer on nutrient agar for bacteria and potato dextrose agar for fungi. Twenty-four hours cultures of methicillin-resistant *Staphylococcus aureus*, *Psedomonas aeruginosa* (clinical isolates from KGH), *Escherichia coli* NCIM2065, *Klebsiella pneumonia*, *Bacillus subtilis* NCIM2063, and *Bacillus pumilis* NCIM2327 and 48 h old cultures of *Saccharomyces cerevisiae* MTCC170, *Candida albicans* MTCC183, *Aspergillus niger* NCIM 548, *Penicillium chrysogenum* NCIM 738 and *Aspergillus oryzae* NCIM 643 were swabbed with sterilized cotton swab on the surface of prepared nutrient agar for bacteria and potato dextrose agar for fungi. Fifty microliters of crude extract was loaded into each well and left for 30 min until the metabolite was diffused. Then the plates were incubated for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. After incubation, the zone of inhibition was measured and recorded. The antibiotics such as Ciprofloxacin, Cyclohexamide were used as antibacterial and antifungal agents, respectively.

Characterization of actinomycetes

The potential isolates selected from the primary and secondary screening for the production of antimicrobial agents were characterized by morphological, biochemical and physiological methods [25].

Morphological characterization

Macroscopic method

Morphological characters of the selected isolates were studied by inoculating into sterile media such as glycerol yeast extract agar, oatmeal agar, mineral salts agar, and starch casein agar [26]. The media was sterilized and poured into sterile Petri dishes. After solidification, selected isolates were streaked aseptically in the laminar air flow unit and incubated at 27 °C for 7 d. Morphological characters such as colony characteristics, pigment production, presence or absence of aerial and substrate mycelium were observed.

Morphological characterization by microscopic coverslip culture method

The arrangement of spores and sporulating properties of the selected isolates were examined microscopically by using cover slip culture method by inserting sterile cover slip at an angle of 45 °C in the starch casein agar medium [27,28]. A loop full of each isolate was taken separately from 7 d old culture, inoculated at the insertion of coverslip and incubated at 27 °C for 7 d. Then, the coverslip was removed by using sterile forceps and placed upward on a clean glass slide. Finally, the cover slip was observed for the morphology of isolates under the trinocular microscope (America Inc., LABOMED USA CXR3 9122100) at 400 magnification.

Physiological and biochemical characterization

Effect of temperature on growth

The ability of the selected isolates to grow at different temperatures was studied at 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C and 50 °C. The isolates were streaked on starch casein agar slants and incubated at different temperatures [29] and after 7 d observed their growth.

Melanin production

The production of melanin is an important characteristic for the identification and classification of actinomycetes, especially Streptomyces. Melanin is a dark pigment produced by the microorganisms on tyrosine containing a medium. The tryptone yeast extract broth (ISP-1), yeast extract iron agar (ISP-6), tyrosine agar (ISP-7) were used for testing melanin production by the actinomycete isolates [18]. Seven-day old culture of actinomycete isolates was inoculated on each media and incubated at 27 °C for 4 d and the uninoculated media serves as a control. The color change was observed after 2 d and 4 d. Greenish brown and brown to black diffusible pigments were considered as positive for melanin production.

Gelatin hydrolysis

Gelatin is a protein produced by the hydrolysis of collagen. Microorganisms can produce gelatinase that can hydrolyze gelatin to amino-acids. For this, the actinomycete isolates were streaked on gelatin agar and incubated at 27 °C for 7 d. Following incubation, the

plates were flooded with 10 ml of mercuric chloride solution and observed for the zone of hydrolysis [30].

Peptonization and coagulation of milk

Milk coagulation and peptonization test were carried out with skim milk. The skim milk containing test tubes were inoculated with isolates and incubated at 27 °C for 7 d. The extent of coagulation and peptonization was recorded on 3rd and 8th d [31].

Casein hydrolysis

The proteolytic activity of the selected isolates was studied using milk casein agar. The isolates were streaked on milk casein agar and incubated at 27 °C for 5-7 d. Following incubation, isolates secreting protease enzyme will exhibit a zone of proteolysis, which is demonstrated by clear zone surrounding their growth. This loss of opacity is the result of a hydrolytic reaction yielding soluble, non-colloidal amino acids, and it represents a positive for casein hydrolysis [31].

Starch hydrolysis

The isolates were streaked on starch agar plates and incubated at 27 °C for 5-7 d. Then, the plates were flooded with iodine solution. Starch in the presence of iodine will impart a blue-black color to the medium indicating the absence of starch-splitting enzymes and representing a negative result. However, the presence of clear colorless zone surrounding the growth of the microbe represents a positive result for starch hydrolysis [31].

Urea hydrolysis

For this, isolates were inoculated into sterile urea agar slants and incubated at 30 °C for 7 d and a change in color was observed.

Nitrate reduction test

The selected isolates were inoculated into 5 ml of nitrate broth and incubated at 27 °C for 7 d and uninoculated nitrate broth serves as a control. After incubation, the ability of isolates to reduce nitrates to nitrites was determined by the addition of sulfanilic acid followed by α-naphthylamine and produces an immediate cherry red color, which was taken as positive for nitrate reduction [32].

Use of carbon and nitration sources

The ability of the isolates utilizing various carbon sources for energy was studied by following the method recommended by International *Streptomyces* Project (ISP) [33]. The carbon sources (1% w/v) such as D-Glucose, D-Xylose, L-Arabinose, D-Fructose, D-Galactose, Raffinose, D-Mannitol, Sucrose, Maltose, Lactose, Cellulose were used in the basal medium and the isolates were streaked on the media and then the observations were noted after 7th, 14th and 21st d by comparing with that of non-supplemented basal medium and on positive control containing D-glucose.

The nitrogen sources such as L-Histidine, Potassium nitrate, L-Valine, L-Phenylalanine, L-Arginine, and L-Threonine were used to test the ability of the isolates to utilize as nitrogen sources for energy. Each 0.1% was incorporated in basal media and isolates were streaked on them. After incubation at 27 °C for 7 d, the growth was observed.

RESULTS

Sampling and isolation of actinomycetes

From a total of 12 plastic waste dumped soil samples of Kapuluppada dumping yard, Visakhapatnam (fig. 1), 110 morphologically distinct colonies were obtained on starch casein agar plates. The isolates from each sample collected and their codes were depicted in table 1. To date, there is no report on the isolation of actinomycetes from this plastic waste dumping area. Hence, the soil samples were collected from this site and made an attempt to isolate actinomycete strains for polyethylene biodegradation.

Fig. 1: Place where plastic dumped soil samples were collected

Table 1: Actinomycete isolates from each soil sample collected from Kapuluppada plastic waste dumping yard, near Kommadi, Visakhapatnam

Sample	Soil depth (cm)	No. of isolates	Isolate codes
1	4	8	PD1-PD8
2	7	11	PD9-PD19
3	10	5	PD20-PD24
4	9	6	PD25-PD30
5	6	13	PD31-PD43
6	4	9	PD44-PD52
7	7	9	PD53-PD61
8	10	10	PD62-PD71
9	5	7	PD72-PD78
10	5	9	PD79-PD87
11	4	12	PD88-PD99
12	7	11	PD100-PD110

Screening of isolated actinomycetes for their antimicrobial activities

Primary screening

As a result of primary screening, all actinomycete isolates were showed antimicrobial activity against one or more test bacteria and fungus (table 2-7). In this study, isolates PD10, PD22, PD36, PD47, PD66 and PD85 were active against both Gram-positive and Gram negative bacteria (table 2). The three isolates (PD5, PD47 and PD66) were active against all fungus used in this study. Among the 110 isolates, eight isolates had shown antagonistic activity against both *S. aureus* NCIM2079 and MRSA (clinical isolate from KGH, Visakhapatnam) (table 2). Moreover, eight isolates had been shown antagonistic activity against *C. albicans* MTCC183 (table 3).

The most promising isolates against human pathogen MRSA were PD66 (16 mm) and PD85 (15.6 mm) when compared to other isolates. The most promising isolates against human pathogen *Candida albicans*

were PD10 (20.4 mm), PD47 (22.9 mm) and PD106 (21.5 mm) when compared to other isolates. However, MRSA had shown resistant against PD2, PD4, PD36, PD47, PD79; PD93 isolates whereas *C. albicans* had shown resistance against PD15, PD35 isolates. The eight isolates, such as PD5 (17.6 mm), PD9 (10 mm), PD10 (16.2 mm) and PD15 (14.5 mm), PD35 (11 mm), PD47 (10), PD66 (8.9 mm) and PD82 (16) have shown the antagonistic activity against *S. cerevisiae* MTCC170 strain when compared to other isolates (table 3).

Table 2: Zone of inhibition (mm) of the Actinomycete Isolates against tested bacteria using perpendicular streak method

Isolates	*E. coli*	*B. Subtilis*	*S. aureus*	*B. pumilis*	*MRSA*	*P. aeruginosa*	*K. pneumonia*
PD2	-	15.4±0.3	7.5±0.3	11±0.3	-	12.6±0.6	8±0.5
PD4	8.5±0.2	12±0.3	5.4±0.2	10.2±0.2	-	16.2±0.5	6.7±0.4
PD9	6.8±0.3	11.4±0.3	7.2±0.3	4.8±0.3	14.2±0.3	9.6±0.3	-
PD10	21.2±0.2	9.6±0.2	9.4±0.2	15.5±0.5	12.8±0.5	6.4±0.3	11.9±0.6
PD22	9.2±0.2	15.8±0.7	10.3±0.2	-	10.8±0.6	11.6±0.2	9.5±0.3
PD36	11.4±0.2	14.4±0.6	9.7±0.5	9.4±0.5	-	10.5±0.3	-
PD47	19±0.6	18.3±0.3	-	9±0.6	-	7.5±0.4	9±0.4
PD51	9±0.5	8.4±0.2	7.5±0.6	9±0.2	8±0.3	9±0.2	7±0.6
PD66	13±0.2	18.6±0.3	16.5±0.5	14.9±0.6	16±0.2	21.5±0.3	14±0.3
PD70	9.8±0.3	-	6±0.2	-	5.5±0.4	8±0.2	5±0.4
PD79	10±0.2	8±0.3	6.4±0.3	8±0.3	-	7±0.5	7±0.3
PD85	6±0.3	5.2±0.3	4±0.5	7±0.2	15.6±0.6	7.5±0.6	6±0.3
PD93	4.3±0.3	-	-	-	-	5±0.4	7.2±0.2
PD101	6±0.3	8.2±0.2	6.3±0.3	8±0.5	4.5±0.6	17.5±0.4	-

Values are mean±SD of three replications; (-) denotes no zone of inhibition.

Table 3: Zone of inhibition (mm) of actinomycete isolates against tested fungi using perpendicular streak method

Isolates	*C. albicans*	*A. niger*	*A. oryzae*	*P. chrysogenum*	*S. cereviciae*
PD5	10±0.2	6.8±0.6	8±0.2	11±0.3	17.6±0.6
PD9	9.5±0.5	7±0.3	-	6.5±0.3	10±0.2
PD10	20.4±0.2	-	-	-	16.2±0.3
PD15	-	18.6±0.3	15.4±0.2	10±0.3	14.5±0.2
PD22	9±0.3	5.8±0.2	9.8±0.2	5.4±0.5	-
PD35	-	10.4±0.4	8.4±0.6	10.4±0.4	11±0.3
PD47	22.9±0.4	8.6±0.3	11.3±0.6	9.8±0.5	10±0.2
PD66	11.8±0.2	19.4±0.5	17.6±0.2	10±0.4	8.9±0.2
PD82	13.1±0.2	11.6±0.2	16.5±0.4	-	16±0.4
PD106	21.5±0.5	7.4±0.2	8±0.3	10±0.5	-

Values are mean±SD of three replications; (-) denotes no zone of inhibition.

Secondary screening

The crude extracts prepared from 10 potential isolates against bacteria and 9 isolates against fungi by using submerged state fermentation method were subjected to secondary screening by agar well diffusion method. The crude extract of PD2 (13.4 mm), PD66 (13.4 mm) were most active against *B. subtilis* NCIM2063, PD47 (14.3 mm) against *E. coli*, PD66 (10.4 mm) against *S. aureus* NCIM2079, PD66 (12.2 mm), PD85 (11.5 mm) were most active against methicillin-resistant *Staphylococcus aureus*, PD4 (14.1 mm), PD66 (15.6 mm) were active against *Pseudomonas aeruginosa* (table 4).

The crude extracts from potential isolates PD10 (19.2 mm), PD47 (19.8 mm), PD106 (19.1 mm) had shown very good activity against *C. albicans* whereas PD10 (14.6 mm), PD82 (15.7 mm) were active against *S. cereviciae* (table 5).

Table 4: Zone of inhibition (mm) in secondary screening of crude extracts (10 mg/ml) produced from submerged fermentation against bacteria by using agar well diffusion method

Bacteria/ Isolate	PD2	PD4	PD9	PD10	PD22	PD47	PD66	PD79	PD85	PD101	Ciprofloxacin
E. coli	-	6±0.2	-	13.7±0.6	4.8±0.2	14.3±0.2	9.8±0.4	6.5±0.6	-	-	16.5±0.3
B. subtilis	13.4±0.2	10.5±0.2	9.5±0.3	4.3±0.5	12.9±0.3	11±0.3	13.4±0.4	5±0.2	-	5.6±0.4	15.7±0.6
S. aureus	5±0.5	-	-	5.4±0.3	4.4±0.4	-	10.4±0.3	5.2±0.2	-	4±0.2	19.5±0.5
B. pumilis	10.1±0.5	7.9±0.3	-	12.8±0.2	-	5.6±0.4	9±0.4	6.3±0.2	-	5.9±0.2	13.2±0.4
MRSA	-	-	10.9±0.3	9.1±0.2	-	-	12.2±0.3	-	11.5±0.2	-	17.1±0.2
P. aeruginosa	11.8±0.4	14.1±0.5	5.6±0.4	-	6.7±0.3	-	15.6±0.3	-	-	14±0.3	16.6±0.5
K. pneumoniae	5.4±0.3	-	-	6±0.2	5.2±0.4	-	9.3±0.5	-	-	-	12.7±0.8

Control (50% of methanol). Values are mean±SD of three replications; (-) denotes no zone of inhibition.

Table 5: Zone of inhibition (mm) in secondary screening of crude extracts (10 mg/ml) produced from submerged fermentation against fungi by using agar well diffusion method

Fungi/Isolates	PD5	PD9	PD10	PD15	PD35	PD47	PD66	PD82	PD106	Cyclohexamide
C. albicans	7.2±0.2	8.8±0.3	19.2±0.4	-	-	19.8±0.2	9.6±0.4	10±0.2	19.1±0.5	25±1
A. niger	-	-	-	15.4±0.3	7.1±0.5	-	14.9±0.4	8.9±0.3	-	-
A. orizae	5.6±0.3	-	-	14±0.3	-	9±0.4	14±0.3	12.4±0.4	-	-
P. chrysogenum	10±0.5	-	-	6.9±0.3	6±0.2	-	8.4±0.2	-	-	-
S. cereviciae	17.3±0.2	8.6±0.2	14.6±0.4	11.9±0.2	8.7±0.4	6.6±0.3	-	15.7±0.5	-	-

Control (50% of methanol). Values are mean±SD of three replications; (-) denotes no zone of inhibition.

Table 6: Morphological characteristics

Isolates/Media	Culture media	Growth	Aerial mycelium	Substrate mycelium	Pigments
PD2	Mineral agar	Good	White	Yellow	Yellowish
	Starch casein agar	Excellent	White	Yellow	Yellowish
	Glycerol yeast extract agar	Very good	White	Yellow	Yellowish
	Oatmeal agar	Good	White	Yellow	Yellowish
PD4	Mineral agar	Very good	White to ash	Dark black	Pale yellow
	Starch casein agar	Excellent	White to ash	Dark black	Pale yellow
	Glycerol yeast extract agar	Excellent	White to ash	Dark black	Pale yellow
	Oatmeal agar	Good	White to ash	Dark black	Pale yellow
PD5	Mineral agar	Very good	Dark ash	Brown	Yellow to brown
	Starch casein agar	Excellent	Dark ash	Brown	Yellow to brown
	Glycerol yeast extract agar	Excellent	Dark ash	Brown	Yellow to brown
	Oatmeal agar	Good	Dark ash	Brown	Yellow to brown
PD9	Mineral agar	Very good	Dark ash	Ash	Yellow to brown
	Starch casein agar	Excellent	Dark ash	Ash	Yellow to brown
	Glycerol yeast extract agar	Excellent	Dark ash	Ash	Yellow to brown
	Oatmeal agar	Very good	Dark ash	Ash	Yellow to brown
PD10	Mineral agar	Good	White	White	None
	Starch casein agar	Excellent	White	White	None
	Glycerol yeast extract agar	Excellent	White	White	None
	Oatmeal agar	Good	White	White	None
PD15	Mineral agar	Good	Pale red	Grey	None
	Starch casein agar	Excellent	Pale red	Grey	None
	Glycerol yeast extract agar	Very good	Pale red	Grey	None
	Oatmeal agar	Good	Pale red	Grey	None
PD22	Mineral agar	Good	White to ash	Brown	Brownish
	Starch casein agar	Excellent	White to ash	Brown	Brownish
	Glycerol yeast extract agar	Excellent	White to ash	Brown	Brownish
	Oatmeal agar	Good	White to ash	Brown	Brownish
PD35	Mineral agar	Good	White	White	None
	Starch casein agar	Excellent	White	White	None
	Glycerol yeast extract agar	Very good	White	White	None
	Oatmeal agar	Good	White	White	None
PD47	Mineral agar	Excellent	White	Yellow to brown	Pale yellow
	Starch casein agar	Excellent	White	Yellow to brown	Pale yellow
	Glycerol yeast extract agar	Excellent	White	Yellow to brown	Pale yellow
	Oatmeal agar	Good	White	Yellow to brown	Pale yellow
PD66	Mineral agar	Good	White	Yellow	Yellowish
	Starch casein agar	Excellent	White	Yellow	Yellowish
	Glycerol yeast extract agar	Excellent	White	Yellow	Yellowish
	Oatmeal agar	Good	White	Yellow	Yellowish
PD79	Mineral agar	Good	Pale yellow	White	None
	Starch casein agar	Excellent	White	White	None
	Glycerol yeast extract agar	Very good	White	White	None
	Oatmeal agar	Good	White	White	None
PD82	Mineral agar	Good	Chocolate	Black	None
	Starch casein agar	Excellent	Chocolate	Black	None
	Glycerol yeast extract agar	Very good	Chocolate	Black	None
	Oatmeal agar	Good	Grey	Black	None
PD85	Mineral agar	Good	Dark grey	Brown	None
	Starch casein agar	Excellent	Dark grey	Brown	None
	Glycerol yeast extract agar	Excellent	Dark grey	Brown	None
	Oatmeal agar	Good	Dark grey	Brown	None
PD101	Mineral agar	Good	White	Black	Dark blue
	Starch casein agar	Excellent	White	Black	Dark blue
	Glycerol yeast extract agar	Excellent	Pale white	Black	Dark blue
	Oatmeal agar	Good	Pale white	Black	Dark blue
PD106	Mineral agar	Good	Grey	Coffee	None
	Starch casein agar	Excellent	Grey	Coffee	None
	Glycerol yeast extract agar	Good	Grey	Coffee	None
	Oatmeal agar	Good	Grey	Coffee	None

Morphological characteristics of selected isolate

The growth of all the selected isolates was excellent in starch casein agar. The isolates PD4, PD5, PD9, PD10, PD22, PD47, PD66, PD85, and PD101 had shown excellent growth in starch casein agar as well as in glycerol yeast extract agar (table 6). The substrate and aerial mycelium color were varied among the selected isolates (table 6). The isolate PD2 had shown white colored aerial mycelium, yellow colored substrate mycelium, and yellowish diffusible pigments in mineral agar, starch casein agar, glycerol yeast extract agar, and oatmeal agar. The isolate PD4 had white to ash colored aerial mycelium and dark black colored substrate mycelium and pale yellow colored diffusible pigments. The isolate PD9 developed dark ash colored aerial mycelium and ash colored substrate mycelium and yellow to brownish colored diffusible pigments, the isolate PD22 developed white to ash colored aerial mycelium and brownish colored substrate mycelium and brown colored diffusible pigments, the isolate PD66 had developed white colored aerial mycelium and

yellow colored substrate mycelium and yellowish colored diffusible pigments (table 6). The morphology of the selected isolates was shown in fig. 2.

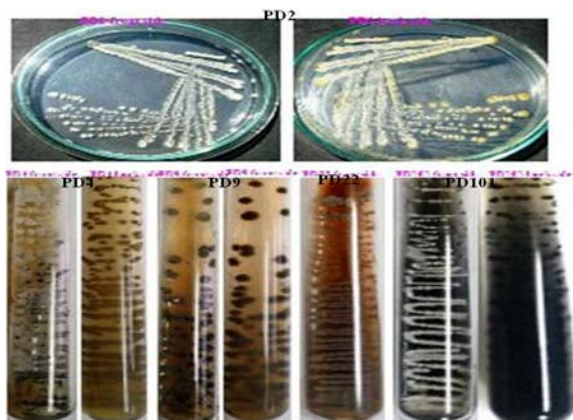

Fig. 2: Morphological appearance of actinomycete isolates

Biochemical characteristics of selected isolate

Results of the biochemical characteristics indicated that isolates PD4, PD15, PD82 had shown positive results for melanin production, and all the selected isolates showed the ability of starch hydrolysis. The isolates PD4, PD5, PD9, PD15, PD22, PD66, PD79, PD82, and PD101 were able to hydrolyze both casein and gelatin; the isolates PD2, PD4, PD5, PD9, PD10, PD47, PD66, PD79, and PD106 were shown a positive result for nitrate reduction. The detailed biochemical characteristics of the selected isolates were shown in table 7. The positive results of starch hydrolysis, casein hydrolysis, melanin production test and nitrate reduction test were depicted in fig. 3.

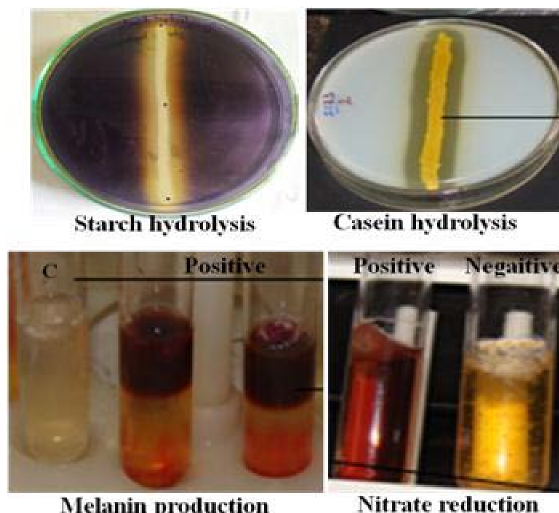

Fig. 3: Biochemical characteristics of the selected isolates

Table 7: Biochemical characteristics of the selected isolates

Test	Characteristics of isolates														
	PD2	**PD4**	**PD5**	**PD9**	**PD10**	**PD15**	**PD22**	**PD35**	**PD47**	**PD66**	**PD79**	**PD82**	**PD85**	**PD101**	**PD106**
Melanin production	-	+	-	-	-	+	-	-	-	-	-	+	-	-	-
Nitrate reduction	+	+	+	+	+	-	-	-	+	+	+	-	-	-	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	-	+	+	+	-	+	+	+	+	+	+	+	+	+	-
Gelatin hydrolysis	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Peptonization and coagulation of milk	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+
Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

'+' indicates positive result of the test; '-' indicates the negative result of the test

Table 8: Carbon source utilization

Carbon source	Isolates														
	PD2	**PD4**	**PD5**	**PD9**	**PD10**	**PD15**	**PD22**	**PD35**	**PD47**	**PD66**	**PD79**	**PD82**	**PD85**	**PD101**	**PD106**
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Meso-Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	-	-	-	+	+	-	-	-	-	-	+	-
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellulose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

'+' indicates carbon source utilization; '-' indicates non-utilization of the carbon source

Table 9: Nitrogen source utilization

Nitrogen source	Isolates														
	PD2	**PD4**	**PD5**	**PD9**	**PD10**	**PD15**	**PD22**	**PD35**	**PD47**	**PD66**	**PD79**	**PD82**	**PD85**	**PD101**	**PD106**
L-Histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potassium nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Valine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Threonine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

'+' indicates nitrogen source utilization; '-' indicates non-utilization of the nitrogen source

Carbon and nitrogen source utilization

All the selected isolates had been shown to utilize D-Glucose, D-Xylose, L-Arabinose, D-Fructose, D-Galactose, Sucrose, Lactose as their carbon source, whereas no isolate had shown to utilize the carbon sources such as Raffinose, Meso-Inositol, D-Mannitol, Salicin and cellulose (table 8). All the selected isolates in the present study had shown the utilization of nitrogen sources such as L-Histidine, Potassium nitrate, L-Valine, L-Arginine, L-Threonine (table 9).

Physiological characteristics

The results of physiological characteristics indicated that all the selected actinomycete isolates had shown excellent growth at 30 °C and 40 °C, good to moderate at 20 °C and 50 °C and at 10 °C the growth was poor. The isolate PD15 had no growth at 10 °C (table 10).

The actinomycete isolates had grown excellent at pH 7, good to excellent growth at pH 6, 8, 9, whereas poor to moderate growth at pH 5 was observed (table 11).

Table 10: Temperature

Temperature	Isolates														
	PD2	**PD4**	**PD5**	**PD9**	**PD10**	**PD15**	**PD22**	**PD35**	**PD47**	**PD66**	**PD79**	**PD82**	**PD85**	**PD101**	**PD106**
10 °C	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
20 °C	++	++	++	++	+++	++	+	+++	++	++	++	++	++	+++	++
30 °C	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
40 °C		++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
50 °C		++	++	++	++	++	++	++	++	++	++	++	++	++	++

++++Excellent; +++Good; ++Moderate; +Poor

Table 11: pH

pH	Isolates														
	PD2	**PD4**	**PD5**	**PD9**	**PD10**	**PD15**	**PD22**	**PD35**	**PD47**	**PD66**	**PD79**	**PD82**	**PD85**	**PD101**	**PD106**
5	+	++	++	+	+	+	+	+	+	+	++	++	+	+	+
6	++++	+++	+++	++++	+++	+++	++++	+++	+++	++++	++++	++++	++++	++++	++++
7	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
8	++++	++++	+++	+++	++++	++++	++++	++++	++++	+++	+++	++++	++++	++++	++++
9	+++	+++	+++	+++	++++	+++	++++	+++	+++	+++	++++	++++	++++	+++	+++

++++Excellent; +++Good; ++Moderate; +Poor

DISCUSSION

Antibiotics are the very important bioactive molecules for the treatment of various pathogenic and autoimmune diseases. Currently, because of the emergencies of multi-drug resistant pathogenic organisms, treatment of resistant pathogens is a challenging problem. For the treatment of multi-drug resistant pathogens, there has been increasing demand for effective antibiotics from soil actinomycetes [34, 35]. Actinomycetes account 70% of the earth's surface and are a good source for isolation and production of novel and potent bioactive secondary metabolites such as antibiotics [36, 37]. Isolation of novel actinomycetes from the soil is very interesting nowadays to isolate and produce novel bioactive metabolites from waste disposal areas had shown antimicrobial activity against bacteria and fungi [38]. These recent studies motivated the corresponding author of this article to isolate antibiotic producing actinomycetes from Kapuluppada plastic waste dumping yard, near Kommadi, Visakhapatnam (A. P.). This is the first report on the isolation of actinomycetes from plastic dumping area producing an anti-microbial compound against bacteria and fungi. In the present study, the randomly selected 12 plastic waste dumped soil samples were taken for isolation of potent actinomycetes of antimicrobial activity. From the soil samples, a total of 110 actinomycetes were isolated and were used for primary screening against bacteria and fungi using single streak methods. The results indicated that most of the actinomycete isolates showed potential antimicrobial activity against one or more test bacteria and/or fungus (table 2 and 3). Sharma *et al.* [38] isolated actinomycetes from Pulicat, Muttukadu, and Ennore estuaries and they had shown antibacterial and antifungal activity. Thakur *et al.* [39] reported that a total of 110 actinomycetes strains were isolated from the soil samples collected from the protected forest soil from two states in Northeast India. The isolates were then characterized by conventional methods and assessed for their antimicrobial activity against test microorganisms. The selected actinomycete isolates had shown dissimilar activities between Gram-positive and Gram-negative bacterial strains. From the results, it was understood that a Gram-positive bacterium was highly susceptible to the tested crude extracts compared to Gram-negative bacteria. This result was

in agreement with the previous report of Ilic *et al.*[40]. It has been suggested that different sensitivity between Gram positive and Gram negative bacteria could be ascribed to morphological differences such as, the outer membrane of Gram-negative bacteria having lipopolysaccharide which makes the cell wall impermeable to lipophilic extracts, whereas, a Gram-positive bacterium was more susceptible because of lack of outer membrane. A Clear zone of inhibition around the wells on the inoculated plates is an indication of antimicrobial activities of metabolites extracted from actinomycetes against test organisms. Smriti *et al.* [41] had shown that a maximum inhibition zone of 14 mm against tested bacteria. Gurung *et al.* [42] reported 0-18 mm inhibition zone of crude extracts against selected tested organisms. In the present study, 0-23 mm inhibition zone of crude extracts from isolated against test organisms was observed, which is higher than the previous reports.

The previous study indicated that the inhibition zone of crude extracts from isolated against MRSAs ranged from 0-15 mm [43]. Whereas in this study, inhibition zone of crude extracts from three isolates against MRSAs ranged from 0-23 mm which was found to be good when compared to Yucel and Yemac's results [43]. The results of the present study were interesting and encouraging because the crude extracts from the isolates may have promising antibiotics for treatment of pathogenic MRSA and *Candida albicans*. We have observed greater inhibition zone when compared to crude extracts from the actinomycete isolates from the previous studies. In order to get better inhibition zones, further purification of bioactive metabolite is necessary to get pure antibiotic substance for treatment of different pathogenic microorganisms and it has been suggested for intensive studies on the actinobacterial diversity of plastic waste dumping yard to establish the rich source of actinomycetes diversity and could put an important input into research and pharmaceutical industries.

The color of aerial mycelium, substrate mycelium and diffusible pigments showed distinct variation depending upon the culture medium, in which the selected isolates were grown. Among the four culture media (starch casein agar, glycerol yeast extract agar, mineral agar and oatmeal agar) used, the growth of all the selected isolates was excellent in starch casein. Valli *et al.* had also observed, white powdery, creamy, pinpoint colonies of actinomycetes [44]. Gebreselema *et al.* had also reported that the selected antimicrobial actinomycete isolates shown excellent growth in starch casein agar [30]. All the selected isolates in this study had the ability to hydrolyze starch but fail to hydrolyze urea. All the isolates have the ability utilize carbon sources such as D-Glucose, D-Xylose, L-Arabinose, D-Fructose, D-Galactose, Sucrose, Lactose but fail to utilize Raffinose, Meso-Inositol, D-Mannitol, Salicin, cellulose (table 8) and nitrogen sources such as L-Histidine, Potassium nitrate, L-Valine, L-Arginine, L-Threonine (table 9) for their cellular energy. The similar results were reported in the study conducted by Sharma and David, 2012 [38] in which actinomycetes were isolated from marine sediments of Pulicat estuary, Muttukadu estuary and Ennore estuary, Tamil Nadu, India. The selected isolates had shown excellent growth at temperature 30-40 °C, pH 7-8 (table 10 and 11). The actinomycetes isolated from Pulicat estuary, Muttukadu estuary, and Ennore estuary grow well at temperature 30 °C and 40 °C and pH 7.0 to 9.0 [38] whereas the actinomycetes isolated from water and sediments of Lake Tana, Ethiopia, the optimum temperature for the growth of isolates was ranged from 25 to 30 °C [30]. Therefore, based on the results the selected isolates obtained from Kapuluppada plastic waste dumping yard were grouped under the genera of *Streptomyces*.

The actinomycete isolates from Kapuluppada plastic waste dumping yard have significant activity against bacteria and fungi and could be useful for many applications such as control of infectious diseases and drug discovery and further studies are focused on standardization of fermentation conditions to improve effective antimicrobial production in liquid medium and isolation of antimicrobial compounds. Further detailed studies on these antimicrobial metabolites have to be carried out for biotechnological applications.

CONCLUSION

Till date, there is no report on actinomycetes producing antimicrobial compounds from the Kapuluppada plastic dumping yard, Visakhapatnam. Therefore, isolation and screening of actinomycetes may contribute to the discovery of new antibiotics and could fight against antibiotic resistance pathogens. Further, purification and characterization of antibiotics are required for the commercial value of these antibiotics.

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CONFLICT OF INTERESTS

Authors declare that we have no conflict of interest

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