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Research Article

ISOLATION, SCREENING AND CHARACTERIZATION OF POTENT MARINE *STREPTOMYCES* SP. PM105 AGAINST ANTIBIOTIC RESISTANT PATHOGENS

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ABSTRACT

Objectives: The present study aims to screen and isolate a promising strain of actinobacteria for the development of new antibiotic to fight against life-threatening infectious diseases.

Methods: A total of 59 marine samples were collected from various marine ecosystem of India. About 242 actinobacterial strains were isolated by using starch casein agar medium. Preliminary screening of actinobacteria against eight antibiotic resistant pathogens was done using agar plug and cross streak method. Secondary screening was performed by well diffusion method. The potential actinobacteria were selected for bioactive metabolites production and it was partially purified by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Further, it was morphologically and physiologically characterized.

Results: In preliminary screening, 18 actinobacterial strains were selected for their antibacterial activity against eight drug resistant pathogens adopting agar plug and cross streak method. The most promising strain PM105 was selected in secondary screening by performing agar well diffusion method. Actinobacterial strain PM105 showed most significant activity against both Gram-positive and Gram-negative antibiotic resistant pathogens with the concentration of 12.5 μg/ml. The maximum zone of inhibition was observed against three Gram-negative bacteria, such as *Klebsiella pneumoniae* (18.6±0.57), *Pseudomonas aeruginosa* (18.5±0.57) and *Escherichia coli* (18.4±0.57). Based on the morphological and physiological characteristics, potential strain PM105 was tentatively identified as *Streptomces* sp. Active compound was separated by TLC with R_f value of 0.7, and analyzed by HPLC, a major peak was observed at retention time of 4.779 minutes.

Conclusion: The above findings revealed that antibacterial potential of actinobacteria from the marine sediment of India.

Keywords: Marine sources, Actinobacteria, Antibiotic resistant pathogens, Bioactive metabolites, High-performance liquid chromatography.

INTRODUCTION

Infectious diseases are the most significant threat for human beings with high morbidity and mortality throughout the world, due to the rapid emergence of multiple drug resistance (MDR) and it creates a major health illness in the medication of infectious diseases spread by pathogenic microbes. It was estimated about 2 million infections and 23,000 deaths caused by antibiotic resistance pathogens per year in United States [1]. In Europe 25,000 people die every year due to antibiotic resistant bacteria [2]. About 0.5 million deaths caused by *Vibrio cholerae*, *Salmonella*, *Shigella*, *Escherichia coli*, and *Campylobacter* throughout the world [3].

It is very important to deliberate research and development of new, safe and effective antibiotics to combat the menace of concomitant MDR pathogens [4]. Natural products have a novel structure, which remains to be the major promising source of secondary metabolites [5].

The microbial diversity of the marine ecosystem in Indian peninsula is vast. However, hitherto the prosperity of marine micro-flora has not been fully investigated [6]. Marine microbes have an extensive surrounding area, and it is anticipated to discover novel metabolites for trapping their potentiality. The microbes growing in marine environments are metabolically and physiologically different from terrestrial organisms [7].

Actinobacteria are free-living, saprophytic, spore-forming filamentous Gram-positive bacteria with high G+C content (69-73%) in their DNA. They form extensive branching substrate, aerial mycelia and widely distributed in soil [8]. They produce a wide variety of secondary metabolites with varying biological activities such as antibacterial, antifungal, antiviral, anticancer, enzyme, immunosuppressant and other industrially useful compounds [9,10]. Antibiotics derived from the marine ecosystem are more effective against microbial infections due to resistance, and it is not developed in the terrestrial bacteria [11]. Around 13,700 bioactive secondary metabolites are produced from actinobacteria in which *Streptomyces* sp. alone comprised of ~10,400 (75%) and 39% of entire microbial products [9].

The present study aims to screen and isolate a promising strain of actinobacteria for the development of new antibiotic to fight against life-threatening infectious diseases.

METHODS

Sample collection

The marine sediment samples were collected from 18 different locations in India. A total of 26 marine sediments, 5 marine sponges and 4 marine animal samples were collected from different locations in South India along the coast of the Bay of Bengal, Palk Strait, Gulf of Mannar and Lakshadweep. Totally 4 mangrove sediments and 14 mangrove rhizosphere sediment samples were collected from Pitchavaram, Parangipettai, Killai, Karankadu and Manakudy mangroves in Tamil Nadu. Two estuarine sediments were collected from Vellar estuary at Parangipettai. In addition, 4 salt pan samples were collected from Sikkal in Tamil Nadu (Tables 1 and 2). All the samples were collected in a sterile container and carefully transported to the laboratory for further analysis.

Isolation and enumeration of marine actinobacteria

All the samples were air-dried at 30°C for 5 days and 5 g of dried samples were pretreated by heating at 55°C for 10 minutes, which favors the isolation of actinobacteria by reducing most unwanted Gram-negative and other spore-forming bacteria. Isolation and enumeration of marine actinobacteria were performed by serial dilution and spread plate techniques [12]. Among the total isolated colonies, the strains with the same size, color, morphology and reverse side pigments were eliminated. Finally, 104 strains were selected from 242 isolates for the antibacterial activity. The pure actinobacteria colonies were selected and subcultured on yeast extract malt extract agar medium (ISP2) and 30% glycerol stored at −20°C [13].

Preliminary screening of actinobacteria for antagonistic activity

Agar plug method

The antibiotic resistant pathogens namely *E. coli*, *Pseudomonas aeruginosa*, *V. cholerae, Klebsiella pneumoniae, Salmonella typhi, Bacillus subtilis, Corynebacterium diptheriae* and *Staphylococcus aureus* were inoculated into sterile nutrient broth medium (Hi-Media - Mumbai). It was incubated at 37°C for 12 hrs to reach the visible turbidity and density equal to 0.5 McFarland standards. After incubation, the broth cultures were seeded individually using sterile cotton swab on nutrient agar plates (all the antibiotic resistant pathogens such as *E. coli, P. aeruginosa, V. cholerae, K. pneumoniae, S. typhi, B. subtilis, C. diptheriae* and *S. aureus* were obtained from the Department of Microbiology, Periyar University). Antibacterial activity of marine actinobacterial isolates were done by adopting agar plug method [14].

Cross streak method

The antibacterial activity of selected actinobacterial isolates were confirmed by using cross streak method [15]. The zone of inhibition was calculated after 24 and 48 hrs. Actinobacteria isolates, which showed maximum inhibition, were selected for secondary screening.

Production and extraction of antibacterial metabolites

Based on the preliminary screening, four actinobacterial strains PM105, TMS 7, M 5 and TMM 4 were selected for further studies. All the selected actinobacterial strains were inoculated into fresh yeast extract-malt extract (ISP2) agar plates (25 ml/plate) and incubated at 28°C for 7 days. After incubation, aerial and substrate mycelial growth was detached aseptically using a sterile spatula. The pigment produced agar medium was sliced into small pieces and extracted using ethyl acetate for overnight at room temperature [16]. The solvent extracts were concentrated under reduced pressure, quantified and were redissolved in 10% dimethyl sulfoxide (DMSO) (Merck, Mumbai) further filter sterilized by using 0.22 µm filter (Hi-Media, Mumbai).

Secondary screening of actinobacteria for antagonistic activity

Well diffusion method

All the antibiotic resistant pathogens as mentioned in preliminary screening were inoculated onto Muller Hinton agar medium (Hi-Media, Mumbai) (25 ml/plate) by using sterile cotton swabs. About 5 mm diameter wells were made on all the inoculated plates using sterile cork borer. Further, each well were loaded with 12.5, 25, 50, 75 and 100 µg/ml of crude extracts and incubated at 37°C for 24 hrs. The antibacterial activity of extracts were compared with known antibiotics penicillin (2 units/disc), methicillin (10 µg/disc), vancomycin (10 µg/disc) as positive control and 10% DMSO 25 µl as negative control. After incubation, the zone of inhibition was evaluated and expressed as mm in diameter (Figs. 1 and 2). The results were presented as mean±standard deviation with triplicates. Based on the results of secondary screening, the potential actinobacterial strain was selected for morphological and physiological characterization.

Characterization of potential actinobacterium

Potential actinobacterial strain PM105 was selected based on the results of secondary screening. It was characterized by cultural morphology with the substrate mycelium and aerial mycelium under the light microscope (Optika, Italy). Carbohydrate utilization was determined by growth on different carbon sources such as glucose, arabinose, sucrose, xylose, inositol, mannitol, fructose, rhamnose, raffinose, cellulose and nitrogen sources namely L-asparagine, L-glutamine and L-tyrosine. Minerals utilization was done by using K_2HPO_4, KNO_3, $MgSO_4$ and $CaCO_3$. Physiological characteristics like pH, temperature and NaCl (%) were also determined [13].

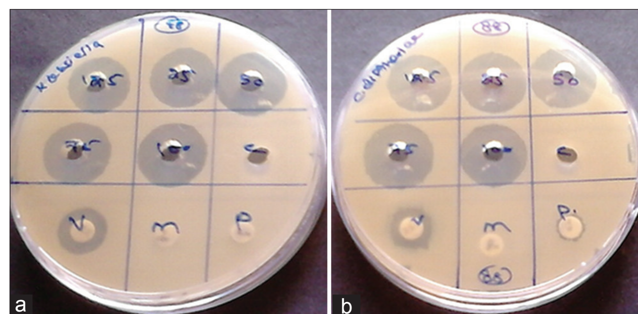

Fig. 1: Antibacterial activity of *Streptomyces* sp. PM105 against (a) *Klebsiella pneumoniae* and (b) Corynebacterium diptheriae

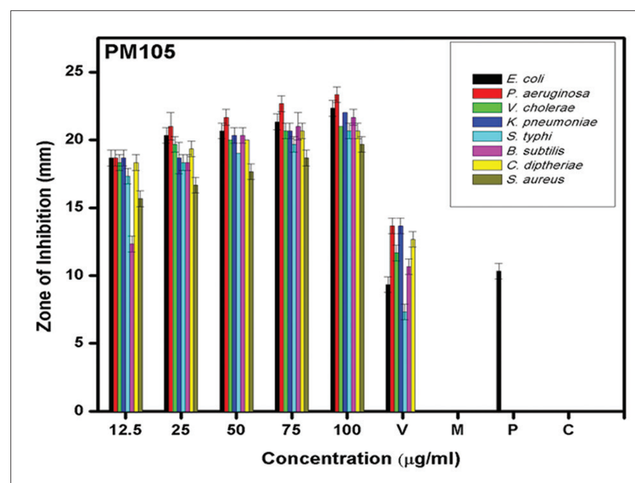

Fig. 2: Antibacterial activity of *Streptomyces* sp., PM105 against antibiotic resistant pathogens

Screening of extracellular enzyme production from marine actinobacteria

Potential strain PM105 was inoculated on the agar medium incorporated with substrates such as carboxyl methyl cellulose, starch casein agar, skim milk and tween 20, for the production of enzymes such as cellulose, amylase, protease and lipase respectively. Plates were incubated at 28°C for 7 days. Appropriate indicator solutions were flooded for each enzyme activity. The development of clear zone around the growth of actinobacterial strain was considered as positive enzyme production [6].

Thin layer chromatography (TLC)

Crude ethyl acetate extracts were used for TLC, which was done using silica gel sheets (Merck-F 254) with chloroform-methanol (9:1, v/v) as solvent system (Fig. 3). The chromatogram was observed under ultraviolet (UV) light and iodine vapors [17]. Based on the separation of bands the R_f value was calculated.

Bio autography

Antibacterial compounds separated in TLC sheets were placed into sterile petriplate, in which 15 ml of sterile nutrient agar seeded with 2% of antibiotic-resistant pathogen *S. aureus* was poured and it was incubated at 37°C for 24 hrs. After incubation, the zone of inhibition

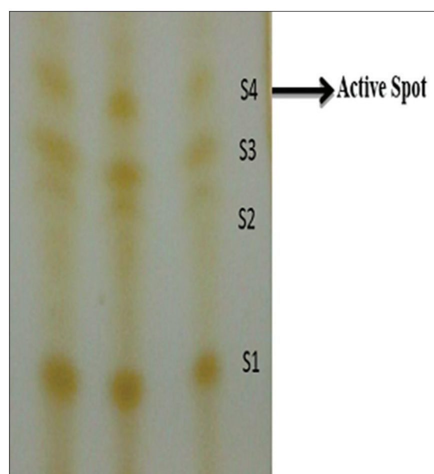

Fig.3: Thin layer chromatography chromatogram of ethyl acetate extract of *Streptomyces* sp. PM105

around the band was observed [18]. The clear zone around the band confirmed the presence of active antibacterial components. The band present in the clear zone was noted based on the R_f values.

High-performance liquid chromatography (HPLC) analysis

The crude metabolites from potential actinobacterium were separated and analyzed by reverse phase HPLC-SPD-20A (Shimadzu-Japan). An aliquot of 20 µl sample was injected onto the HPLC column (C-18) and eluted by a linear gradient using methanol: Water (50:50 v/v) in 20 min at a flow rate of 1 ml/minute. The UV-visible spectrum was measured at 254 nm.

RESULTS

In the present study, 59 samples were collected from various marine ecosystems of South India during March 2013-January 2014 (Table 1). A total of 242 actinobacterial strains were isolated from 18 different collection sites of the marine ecosystem. Among them, a total of 151 actinobacterial strains were isolated from marine sediment, 5 from marine sponges and 8 from animals (molluscs). In the mangrove ecosystem, 60 and 6 actinobacterial strains were isolated from rhizosphere sediment and non-rhizosphere sediment, respectively. A total of 5 isolates were selected from estuarine sediment.

In addition, 7 strains were selected from salt pan sediments. In total, 242 strains were isolated of which 104 morphologically different actinobacterial colonies were selected for antibacterial studies (Table 2).

All the 104 actinobacterial strains were screened for preliminary screening by adopting agar plug method and cross streak method against 8 antibiotic resistant bacterial strains. Among them, 18 actinobacterial strains were showed antibacterial activity. Of 18 strains, 4 potential actinobacterial strains *viz*., PM105, M5, TMS7 and TMM4 were selected for secondary screening (Table 3) based on the effective activity.

In secondary screening, potent actinobacterial strain PM105 exhibited most significant activity against both Gram-positive and Gram-negative antibiotic resistant pathogens. At the concentration of 12.5 µg/ml, the maximum zone of inhibition have been observed against three predominant Gram-negative bacteria, such as *K. pneumoniae* (18.6±0.57), *P. aeruginosa* (18.5±0.57) and *E. coli* (18.4±0.57). *C. diphtheriae* and *V. cholerae* are also showed good level of inhibition with the same concentration such as (18.3±0.57), (18.4±0.58) respectively. In addition, *S. typhi, S. aureus* and *B. subtilis* were showed zone of inhibition with 17.33±0.57, 15.33±0.57, 12.33±0.57 respectively (Figs. 1 and 2).

Table 1: Location of sampling sites

Name of the station	Latitude	Longitude
Parangipettai	11°29’ N	79°45’ E
Killai	11°27’ N	79°46’ E
Pitchavaram	11°25’ N	79°46’ E
Tharangampadi	11°1’ N	79°51’ E
Adiramapattinam	10°18’ N	79°22’ E
Manora	10°15’ N	79°17’ E
S.P. Pattinam	9°49’ N	79°5’ E
Thondi	9°43’ N	79°1’ E
Karankadu	9°41’ N	78°59’ E
Mandapam	9°16’ N	79°9’ E
Pamban	9°16’ N	79°12’ E
Rameswaram	9°17’ N	79°19’ E
Katchatheevu	9°22’ N	79°31’ E
Tiruchendur	8°29’ N	78°7’ E
Manakudy	8°4’ N	77°29’ E
Port Blair	11°34’ N	92°45’ E
Agatti Island	10°51’ N	72°11’ E
Sikkal	9°14’ N	78°39’ E

Table 2: Sampling details of marine actinobacteria from various ecosystems

Ecosystem	Sample type	Number of sample	Total number of isolates	Number of isolates selected
Marine	Sediments	26	151	49
	Marine sponges	5	5	3
	Marine animals	4	8	2
Mangrove	Rhizosphere	14	60	40
	Non-rhizosphere	4	6	4
Estuary	Sediments	2	5	1
Salt pan	Sediments	4	7	5
Total		59	242	104

The standard antibiotic discs like methicillin - 10 µg, penicillin G 2 units and vancomycin - 10 µg (Hi-Media - Mumbai) were used as a positive control. Among the antibiotic resistant pathogens, except *S. aureus* all others were sensitive to vancomycin, similarly except *E. coli* remaining pathogens were resistant to penicillin-G and all the pathogens were resistant to methicillin (Fig. 2).

The potential marine actinobacterium PM105 formed grey color powdery colonies and produced aerial and substrate mycelium (Fig. 4). Good growth was observed on ISP1 - ISP5 medium, poor growth was observed on ISP6 and ISP7 medium. The ability of sugar utilization was observed on D-glucose, sucrose, D-xylose, inositol, D-mannitol, fructose, L-rhamnose, raffinose and cellulose. PM105 utilized the all sugars; good growth was produced except L-arabinose (Table 4).

The potential strain PM105 moderately utilized L-tyrosine, whereas remaining nitrogen sources L-glutamine and L-asparagine were utilized poorly. Good growth was observed at pH range of 7-10 and temperature of 30°C and 40°C. The potential strain PM105 tolerated NaCl in series of 1-7.5%. Further it utilized various mineral sources such as K_2HPO_4, KNO_3 and $MgSO_4$. In addition to this, the potential strain PM105 produced industrially important enzymes like lipase and protease. Based on the morphological and physiological characteristics, potential strain PM105 was tentatively identified as *Streptomces* sp.

In the TLC study, four spots were visualized under UV light and iodine vapors. The R_f values were measured. In the bioautography study, 10 mm inhibition zone was observed against *S. aureus* for the spot 4 with 0.7 R_f value (Fig. 3). Further, the crude compound was analyzed by HPLC, a major peak was observed in a retention time of 4.779 minutes (Fig. 5).

Table 3: Primary screening of antibacterial activity of marine actinobacteria against antibiotic resistant pathogens using agar plug method

Strain no.	Zone of inhibition diameter in (mm)							
	E. coli	*P. aeruginosa*	*V. cholerae*	*K. pneumoniae*	*S. typhi*	*B. subtilis*	*C. diptheriae*	*S. aureus*
M5	8	7	7	7	7	6	7	6
SM1	7	-	6	7	7	6	-	6
ANS2	8	7	-	7	6	6	-	7
ANS 4	7	7	6	6	7	6	7	7
CE 1	8	-	-	-	-	8	-	6
MK6	7	7	-	-	-	7	-	-
P101	7	7	7	-	7	8	7	6
TCA3	9	8	7	7	6	7	-	-
TCA2	8	8	8	6	7	7	-	6
MK4	7	7	-	-	-	7	-	6
PM101	7	6	-	-	-	6	-	-
PM105	12	11	10	13	12	12	13	12
MA1	7	6	-	-	6	7	-	-
SSP2	7	-	-	7	-	7	8	-
TMM4	8	7	7	7	7	8	7	8
PV11	7	-	6	6	-	6	-	6
GM1	8	6	-	7	7	6	7	-
TMS7	9	8	7	7	6	7	8	6

E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa, V. cholera: Vibrio cholerae, K. pneumoniae: Klebsiella pneumoniae, S. typhi: Salmonella typhi, B. subtilis: Bacillus subtilis, C. diptheriae: Corynebacterium diptheriae, S. aureus: Staphylococcus aureus

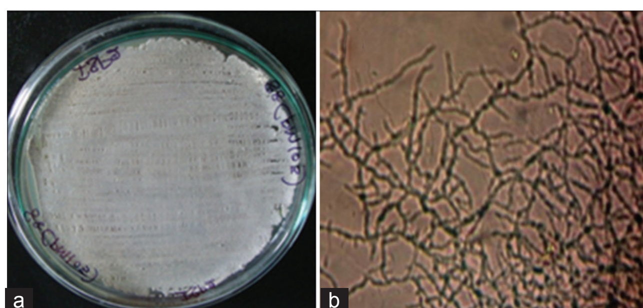

Fig. 4: Colony morphology (A) and substrate mycelium, (B) of *Streptomyces* sp. PM105 (×40)

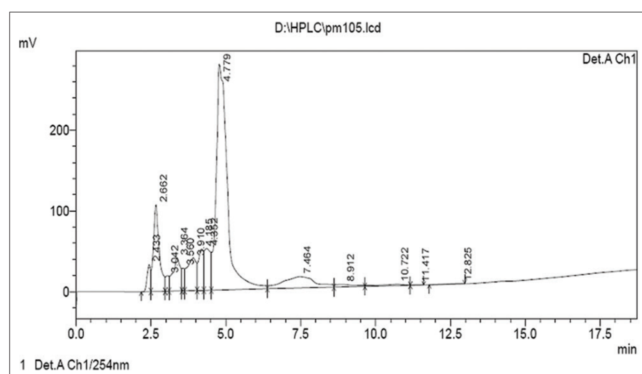

Fig. 5: High-performance liquid chromatography chromatogram of *Streptomyces* sp. PM1050

DISCUSSION

Nowadays, the occurrence of antibiotic resistance in bacteria that cause common infections (e.g. urinary tract infections, pneumonia, bloodstream infections) are emerging throughout the world, in addition to high prevalence of hospital-borne infections caused by methicillin-resistant *S. aureus* and *P. aeruginosa* particularly among injured and immuno-compromised patients [19]. Therefore, there is a crucial need for the production of novel antibiotics to treat MDR bacterial pathogens.

Table 4: Morphological and physiological characteristics of *Streptomyces* sp. PM105

Characteristics	Variables	Result
Cultural characteristics	ISP1 (tryptone agar)	++
	ISP2 (yeast extract - malt extract agar)	++
	ISP3 (oat meal agar)	++
	ISP4 (inorganic salts - starch agar)	++
	ISP5 (glycerol - asparagine agar)	++
	ISP6 (peptone - yeast extract iron agar)	−−
	ISP7 (tyrosine agar)	−−
Utilization of carbon source	Glucose	++
	Arabinose	−−
	Sucrose	++
	Xylose	++
	Inositol	++
	Mannitol	++
	Fructose	++
	Rhamnose	++
	Raffinose	++
	Cellulose	++
Utilization of nitrogen source	L-asparagine	−−
	L-glutamine	−−
	L-tyrosine	+
pH	6	−−
	7	++
	8	++
	10	++
	12	−−
Temperature	20	−−
	30	+
	40	+
	50	+
NaCl tolerance (%)	1	++
	2.5	++
	5.0	++
	7.5	++
	10.0	+
Enzyme activity	Lipase	++
	Protease	++
	Amylase	−−
Mineral source	K_2HPO_4	++
	KNO_3	++
	$MgSO_4$	++
	$CaCO_3$	−−

++: Good, +: Moderate, −−: Poor

In recent years, marine actinobacteria have a great deal of attentions for the production of novel microbial products like antimicrobial, antiviral, anticancer, antimalarial, and anticoagulant etc., [9].

In the present study, screening of marine actinobacteria against antibiotic resistant pathogens resulted for the isolation and characterization of potential strain *Streptomyces* sp. PM105. Among 104 actinobacterial strains, 18 strains (17.3%) exhibited antibacterial activity against selected drug resistant pathogens, in which four strains namely PM105, M5, TMS7 and TMM4 were showed promising activity against all the antibiotic resistant pathogens. Similarly, earlier studies reported that actinobacteria isolated from marine sediments of Visakhapattinam showed 18% of antibacterial activity [20] and actinobacteria isolated from marine sediments from West coast of India exhibited 18.8% of antibacterial activity [21]. However, actinobacteria from marine sediments were well acknowledged, the ratio of indigenous marine micro flora are still remains unclear [22].

The potential strain *Streptomyces* sp. PM105 showed a significant antibacterial activity against all the resistant pathogens in minimum concentration of 12.5 µg/ml and produced 18.6±0.57 mm inhibition zone against *E. coli*, *P. aeruginosa* and *K. pneumoniae*. Similarly, *Nocardiopsis* sp. isolated from Puducherry coast was found to be 18 mm, 20 mm and 15 mm zone of inhibition against *E. coli*, *P. aeruginosa* and *K. pneumoniae* respectively [23]. Mohseni *et al*. (2013) reported that actinobacteria isolated from Caspian sea were exhibited zone of inhibition against *E. coli*, *P. aeruginosa* and *K. pneumoniae* with 17, 20 mm and 18 mm respectively [24].

Furthermore, *Streptomyces* sp. PM105 was produced extracellular enzymes lipase and protease. Similarly Ramesh (2009) reported lipase and protease producing marine actinobacteria from the Bay of Bengal [6] and *Actinopolyspora* sp. from West coast of India [25].

HPLC analysis reveals that, a major peak was observed in a retention time of 4.779 minutes, which virtually matches with the retention time of antibiotic rifampicin [26]. These above findings revealed that *Streptomces* sp. PM105 might be a potential bioactive metabolite to treat the diseases caused by drug resistant pathogens. Further purification, optimization and structure elucidation of bioactive compound and the identification of the potential isolate is under progress.

CONCLUSION

A search for novel, safe and effective antibiotics against MDR pathogens is the need of the hour. Findings of the present study reveals the *Streptomyces* sp. PM105 will be the potent source for producing effective antibacterial compounds to treat the diseases caused by resistant pathogens.

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