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BIOPROSPECT STUDY OF *STREPTOMYCES* SP. ISOLATED FROM COASTAL SAND DUNES

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ABSTRACT

Coastal sand dune (CSD) habitat possesses a stressed and harsher environment. This could act as a potential source for obtaining resourceful novel microorganisms. Actinobacteria from the stressed environment including marine are known to have higher antimicrobial activity. Hence the present study proposes to explore CSD for obtaining potential Actinobacterial strains. Sand sample from a non-rhizosphere zone in Keri Goa was processed for obtaining two strains of *Streptomyces* spp. namely K2NRBAY005 and K2NRBAW008. These strains showed the closest match with *Streptomyces variabilis* and *Streptomyces albofaciens*, respectively after morphological, biochemical, and molecular characterization. These strains were screened for various biotechnological potential. These strains showed antibacterial activity against *Staphylococcus aureus* and *Proteus vulgaris* human pathogens. *Streptomyces* sp. K2NRBAY005 showed the production of 4 hydrolytic enzymes out of 5 tested. *Streptomyces* sp. K2NRBAW008 showed the only production of amylase. Further, these strains elaborated the various plant growth-promoting factors visually IAA, EPS, ammonia, siderophore production, and phosphate solubilization. Screening of these strains clearly demonstrated the industrial potential. Therefore, the current study proposes CSD as a potential and resourceful habitat for exploring the vital novel microorganisms that could have multipurpose prospectives.

Keywords: Antimicrobial activity; Bioprospecting; Coastal Sand Dunes; Hydrolytic enzymes; plant growth-promotion; *Streptomyces*

INTRODUCTION

Coastal sand dune (CSD) habitat is located in between marine and terrestrial ecosystems. CSD possesses several harsh environmental conditions including low nutrients, high salt concentration, high UV rays, high wind, tidal effect, unstable ground, constant sand burial, fluctuation in pH, and temperature. This makes the life form at CSD difficult to grow including plants and microbes. Microbial fauna inhabiting CSD is known to help the plant growth by producing various plant growth-promoting factors including the production of phytohormones (Indole-3-Acetic acid), hydrogen cyanide (HCN), 1-aminocyclopropanecarboxylic acid (ACC) deaminase, solubilizing inorganic phosphate, protection against phytopathogens, exopolysaccharides (EPS), ammonia, siderophores and extracellular hydrolytic enzymes **[1]**. CSD microbes are capable of producing various hydrolytic enzymes such as lipase, amylase, protease, cellulase, pectinase, and chitinase **[2]**. These enzymes can be harnessed by many industries such as detergent, textiles, and others for commercial use. Major industries rely on the microbial enzymes for the conversion of biological waste residue such as agro-waste into useful products **[2]**. Microbial products by actinobacteria have tremendous applications in various industries including pharmaceutical, agriculture, textile, and detergent industries **[3]**. Actinobacteria are the most important group of microorganisms capable of producing antioxidant, antifungal, antibacterial, and antiviral compounds **[4]**. About 60-70 % of antibiotics are produced by *Streptomyces* **[5]**. Species from Genus *Streptomyces* alone produces 600 different types of antibiotics **[6]**. Although there is extensive research done on antibiotics from *Streptomyces* and the fact that it is responsible for the multi-billion-dollar-a-year industry, the ecology of *Streptomyces* are poorly studied **[7]**. The most common hypothesis responsible for the production of antibiotics by *Streptomyces* is linked to the process of sporulation triggered due to stresses and nutrient depletion. To compete for the limiting nutrients *Streptomyces* produces antibiotics to inhibit competitors **[8]**. Actinobacteria from the marine environment have higher activity as compared to the terrestrial strains due to the various stress conditions present in this habitat for the lifeforms **[9]**. CSD habitat is both stressed and limited nutrient-containing, therefore the present study focuses on obtaining the isolates from CSD habitat and to check their capability for the production of

a potent antimicrobial agent against various pathogens, capability in promoting plant growth and producing hydrolytic enzymes such as amylase, protease, lipase, cellulase, and xylanase.

MATERIALS AND METHODS

Sampling

The sand sample from a non-rhizosphere area of Keri beach Goa-India (15˚42'39.84" N; 73˚41'41.29" E) was collected during low tide using a sterile spatula in a sterile ziplock bag. This was stored at 4˚C until further processing. The temperature of the sand was recorded on the site using a thermometer. The pH of 10% suspension of the sample was recorded using a pH meter (Eutech Instruments, pH 700).

Isolation and characterization

Ten percent of the sand suspension prepared in sterile physiological saline was kept on the shaker for proper mixing for 2 hrs at 150 rpm, from this serial dilutions were prepared up to 10^{-6} and spread plated on Bannett's agar (1g yeast extract, 1g beef extract, 2g casein, 10ml glycerol, 20g/l agar, pH 7.2 + 0.2). The plates were incubated at room temperature for upto 7 days. The leathery appeared colonies were picked and maintained by sub-culturing every month on a Bannett's agar medium plate. The colony morphology of the isolates was recorded. Gram's staining and biochemical tests were performed. Scanning electron microscopy (SEM) was performed to study the mycelial and spore structure of isolates. A sterile coverslip was placed on the growth medium plate. A loopful of culture was streaked onto the edges of the coverslip and the plate was incubated at 28^0C for 7 days. The coverslip containing the growth of mycelia was removed from the agar plate. The coverslip was processed for fixation and dehydration as described by Murtey and Ramasamy (2016) **[10]**. The coverslip was sputter-coated with thin gold film using spi-module sputter and observed under a scanning electron microscope (ZEISS E VO 18 Special Edition). Magnification used in the SEM ranged from 3,000 to 50,000 X. The morphology of the mycelia cell was observed at lower magnifications (~5,000 X), whereas the surface of the spores was observed at higher magnifications (~50,000 X).

For further confirmation on the identity of the isolates, the DNA was extracted using the protocols described in Malke (1990) **[11]**. The 16S rRNA gene sequence of the bacteria was used for molecular identification as per the protocol explained by Nayak *et al*. (2013) **[12]**. The sequences obtained were analyzed through a BLAST search against the type species on

National Center for Biotechnology Information Nucleotide database. The alignment was performed using the Clustal W program **[12]**. The phylogenetic tree was constructed using MEGA X software and the neighbor-joining method. The sequences of the actinobacterial strains have been submitted to GenBank and accession numbers have been provided to the strains.

Clinical cultures

Bacterial and fungal pathogens namely *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Proteus vulgaris*, *Salmonella typhi,* and *Candida albicans* were collected from Goa Medical College Hospital, Bambolim, Panjim-Goa, India.

Screening for antimicrobial activity

Screening for antimicrobial activity was performed using a well-diffusion method **[13]**. Pathogens used for this test are listed earlier. Pathogens were grown in a nutrient broth for 24 hrs at 37 ˚C and were swabbed onto the Muller-Hinton agar medium. The swabbed plates were allowed to dry. Using a cork borer wells of 6 mm in diameter were bored on the medium. Test isolates were grown in nutrient broth at room temperature for 48hrs. The cell-free supernatant (50µl) was added into the wells and incubated for 24hrs at 37˚C. After 24hrs the plates were observed for the zone of inhibition.

Screening for hydrolytic activity

The isolates were screened for the production of hydrolytic enzymes, protease, amylase, lipase, cellulase, and xylanase based on Shin *et al*. (2007), Shaw *et al*. (1995), Kumar *et al*. (2012) and Wood *et al*. (1988) methods, respectively **[14-17]**.

Screening for plant growth-promoting potential

The isolates were screened for factors that contribute to plant growth promotion such as IAA production, phosphate solubilisation, siderophore production, EPS and ammonia production using the protocol described by Brick *et al*. (1991), Gaur *et al*. (1990), Schwyn and Neiland (1987), Van Geel-Schutten *et al*. (1998) and Cappuccino and Sherman (1992), respectively **[18-22]**.

RESULTS

Isolation and identification of the actinobacteria cultures

Leathery hard colonies with powdered spores on top of the colonies were selected for further studies. The colony of Isolate K2NRBAY005 showed a white powdery appearance with wavy margins having substrate pigment yellow aerial pigment as white **(Figure 1A)**. The SEM

analysis showed mycelia having monoverticillate structure with a spiral spore chains **(Figure 1B)**. The spores have spikes on their surface **(Figure 1C)**. The spore diameter ranged from 892 – 1000 nm. The colony of Isolate K2NRBAW008 appeared wrinkled with white spore powder on its surface and had a smooth margin. The substrate pigment of Isolate K2NRBAW008 was yellow and the aerial pigment was white **(Figure 2A)**. The SEM showed mycelia having an open spiral spore chains **(Figure 2B)**. The spore surface was smooth without any spikes or warts **(Figure 2C)**. The spore diameter ranged from 550 – 766 nm. Biochemical tests of isolates are provided in **Table 1**. The 16Sr RNA sequencing result gave the identity of isolates belonged to the genus *Streptomyces*. The sequences of Isolates K2NRBAY005 and K2NRBAW008 showed 99 % similarity with *Streptomyces variabilis* and *Streptomyces albofaciens*, respectively **(Figure 3)**. The sequences were submitted in GenBank for which accession numbers MK110490 and MK106260, respectively were provided.

Preliminary screening for antimicrobial activity, hydrolytic activity, and plant growth-promoting potential

Streptomyces sp. K2NRBAY005 and *Streptomyces* sp. K2NRBAW008 showed antimicrobial activity against *Proteus vulgaris* and *Staphylococcus aureus* **(Figure 4)**. The highest zone of inhibition was shown by *Streptomyces* sp. K2NRBAW008 against *P. vulgaris* i.e. 27 mm in diameter. *Streptomyces* sp. K2NRBAY005 showed a 20 mm diameter of zone of inhibition against this pathogen. Positive control, Penicillin showed a 9 mm zone of inhibition against *P. vulgaris*. Penicillin gave a 19 mm diameter of zone of inhibition against *S. aureus*. *Streptomyces* sp. K2NRBAY005 and *Streptomyces* sp. K2NRBAW008, showed a 15 and 11 mm inhibition zone, respectively around the well against *S. aureus*. No antimicrobial activity was shown by these strains against the rest of the tested pathogens namely *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi,* and *Candida albicans*.

Except for the xylanase enzyme, *Streptomyces* sp. K2NRBAY005 was able to produce amylase, protease, lipase, and cellulase **(Figure 5)**. *Streptomyces* sp. K2NRBAY005 showed the highest hydrolyzing zone of 18 mm for amylase followed by a 15 mm zone of hydrolysis for cellulase enzyme, 9 mm zone of hydrolysis for lipase, and 4 mm zone of hydrolysis for protease. *Streptomyces* sp. K2NRBAW008 was able to produce amylase and showed a

10 mm zone of hydrolysis. *Streptomyces* sp. K2NRBAW008 did not produce other tested enzymes.

Both the actinobacterial strains, *Streptomyces* sp. K2NRBAY005 and *Streptomyces* sp. K2NRBAY005 gave positive results for all the five tested plant growth-promoting factors namely the production of siderophores, EPS, ammonia, IAA, and Phosphate solubilization **(Table 2, Figure 6)**. *Streptomyces* sp. K2NRBAW008 showed the highest siderophore production with the zone size of 21 mm and *Streptomyces* sp. K2NRBAY005 showed a 10 mm zone size. *Streptomyces* sp. K2NRBAW008 showed the highest Phosphate Solubilization with a zone size of 7 mm followed by *Streptomyces* sp. K2NRBAY005 with a zone size of 5 mm. Both strains showed the production of IAA, EPS, and ammonia. IAA production by *Streptomyces* sp. K2NRBAW008 was higher than that of *Streptomyces* sp. K2NRBAY005.

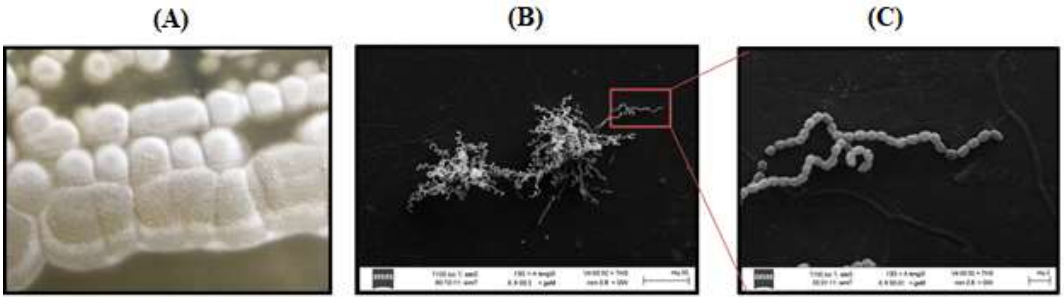

Figure 1: Morphological characteristics of Isolate K2NRBAY005 appeared on Bannett's agar (A), SEM of mycelia (B), and spore chains (C)

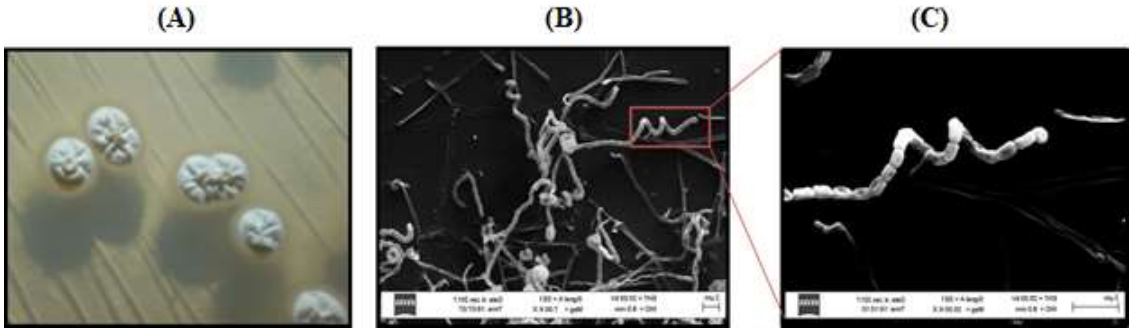

Figure 2: Morphological characteristics of Isolate K2NRBAW008 appeared on Bannett's agar (A), SEM of mycelia (B), and spore chains (C)

Table 1: Biochemical tests of Isolates K2NRBAY005 and K2NRBAW008

Biochemical tests	Isolate K2NRBAY005	Isolate K2NRBAW008
Colony size	10 mm	7 mm
Colony pigment (substrate)	Yellow	Yellow
Colony pigment (aerial)	White	White
Catalase	+	+
Sugar fermentation:		
Arabinose	-	+
Fructose	-	+
Glucose	+	+
Galactose	-	-
Glycerol	-	-
Lactose	+	+
Mannitol	-	+
Mannose	+	-
Maltose	+	+
Inositol	-	+
Sucrose	+	+
Xylose	-	+
Hydrolysis of:		
Starch	+	+
Casein	+	+
Urea	+	+
Esculin	-	-
Citrate Utilization	-	-
Production of:		
H_2S	-	-
Melanin	-	-
Extracellular Pigment	-	-

Keys: (+) Positive test; (-) Negative test

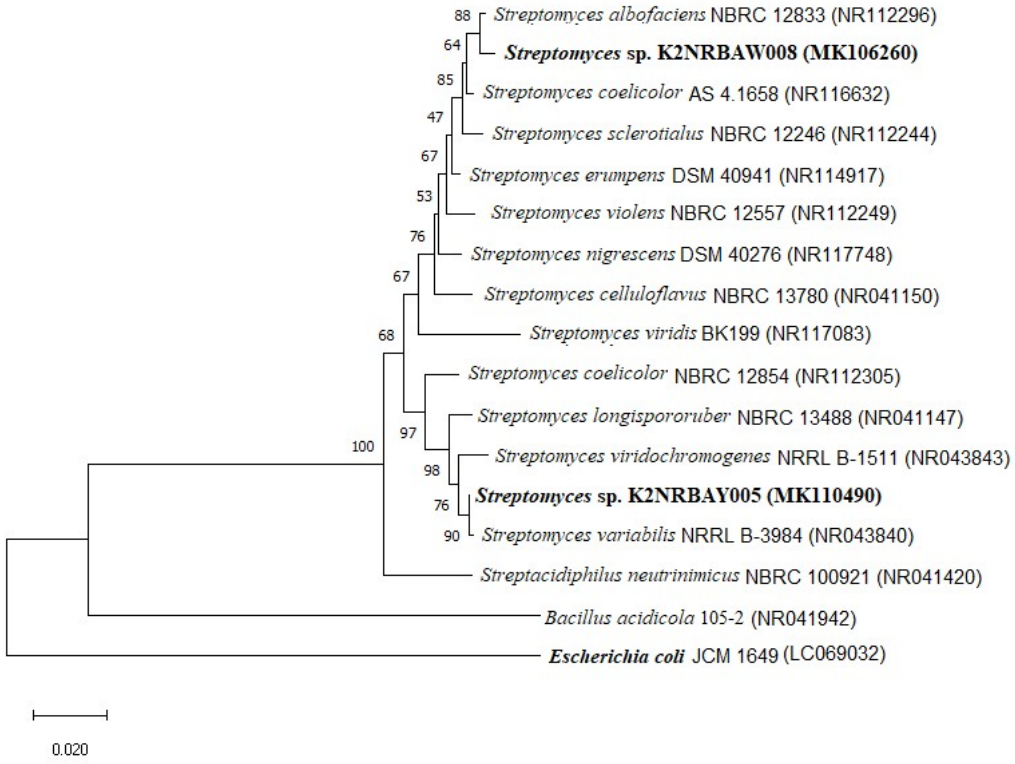

Figure 3: The phylogenetic tree of the 16S rRNA gene sequence of actinobacterial strains. The tree was constructed using 1000 bootstraps. The percentages of 1000 replicate trees are shown next to the branches. The bar is showing substitutions per nucleotide position. [Note: Bold- actinobacterial strains obtained from the present study and ( )- accession numbers]

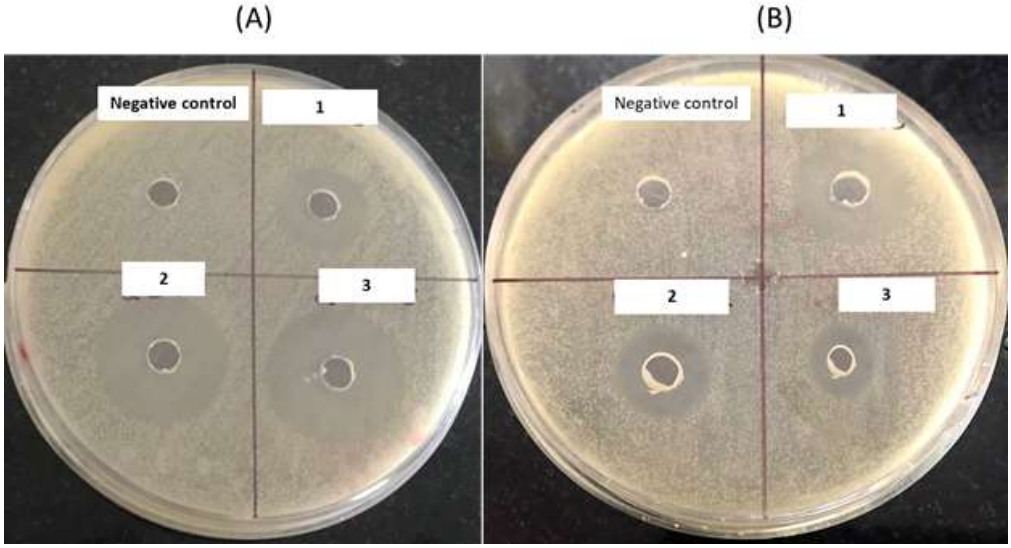

Figure 4: Muller-Hinton's agar plate showing antimicrobial activity against *Proteus vulgaris* (A) and *Staphylococcus aureus* (B). Keys: (1) Positive control (Penicillin); (2) Isolate K2NRBAY005; and (3) Isolate K2NRBAW008

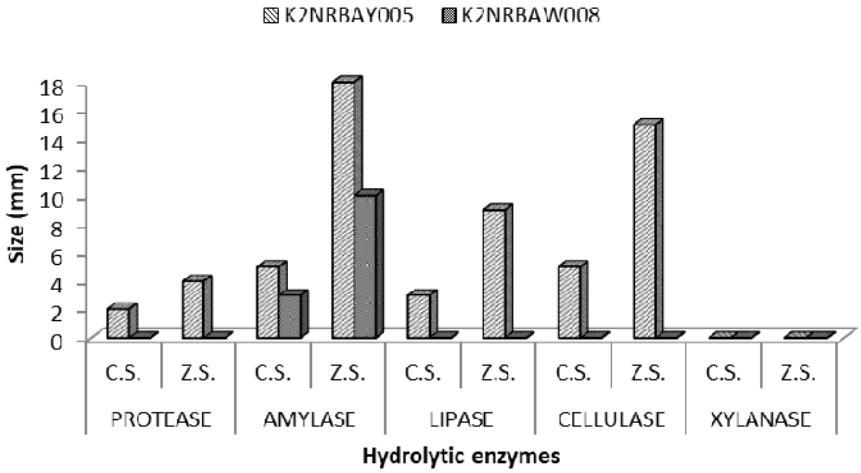

Figure 5: Production of hydrolytic enzymes by strains *Streptomyces* sp. K2NRBAY005 and *Streptomyces* sp. K2NRBAW008. Keys: CS- Colony Size; ZS- Zone Size

Table 2: Screening of the *Streptomyces* species for plant growth-promoting factors

Isolates	Phosphate Solubilization		Siderophore production		IAA	EPS	Ammonia
	CS(mm)	ZS(mm)	CS(mm)	ZS(mm)			
K2NRBAY005	3	5	3	10	+	+++	+++
K2NRBAW008	2	7	3	21	+++	+++	+++

Key: CS (Colony Size), ZS (Zone Size), mm (milimeter), IAA (Indole Acetic-Acid), EPS (Exopolysaccharides), (+) Positive, (++) moderate positive, (+++) strong positive

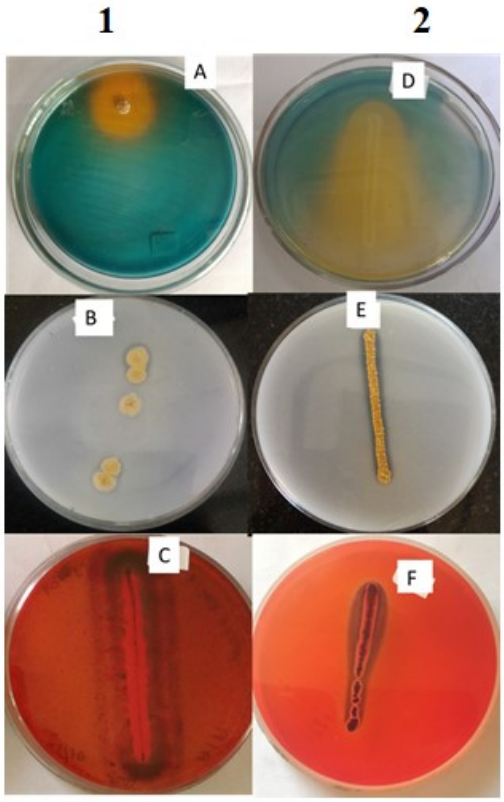

Figure 6: Screening of *Streptomyces* species K2NRBAY005 (1) and K2NRBAW008 (2) for Plant growth-promoting factors such as siderophore production (A and D); Phosphate solubilization (B and E); EPS production (C and F).

DISCUSSION

There are about 23,000 biologically active secondary metabolites from microorganisms. Actinobacteria are contributing two-thirds of it and among this *Streptomyces* alone is responsible for producing more than 70 % **[23]**. Streptomycetes produce secondary metabolites to adapt to various physical, chemical, and biological stresses. Actinobacteria produce antibiotics under limited nutrient conditions **[24]**. Many of these secondary metabolites have antibacterial, antiviral, and antitumor activities. Despite the increased number of anti-bacterial agents in the market, their usage is decreasing since the pathogens such as *Staphylococcus aureus* rapidly develops resistance to these drugs. CSD habitat constitutes challenging environmental stresses for the living organisms. These include low nutrients, low moisture, high salt, and high UV rays. The flora and fauna inhabiting CSD have well adapted to these conditions. Actinobacteria are most notably the dominant genus found in many of the ecosystems including CSD. Actinobacteria from the CSD environment could have great

potential since they have been adapted to survive extreme conditions. In the present study, two *Streptomyces* spp. strains namely K2NRBAY005 and K2NRBAW008 showing species similarity with *Streptomyces variabilis* and *Streptomyces albofaciens*, respectively were obtained from the non-rhizosphere region of Fore dune of CSD. These strains were screened for antimicrobial activity, hydrolytic enzyme production, and plant growth-promoting attributes.

Streptomyces sp. K2NRBAY005, in the present study, showed similarity with *Streptomyces variabilis* exhibited 15 and 20 mm zone of inhibition towards *S. aureus* and *P. vulgaris*, respectively. *Streptomyces variabilis* is known to produce variapeptin, citropeptin, and ammosamide D bioactive compounds **[25]**. *Streptomyces variabilis* PO-178 isolated from soil of Western Ghat Agumbe, Karnataka, India, exhibited antibacterial activity against *S. aureus* and *Pseudomonas aeruginosa* **[26]**. So far there are no reports of *S. variabilis* exhibiting antibacterial activity against *P. vulgaris*.

Streptomyces sp. K2NRBAW008 isolated in the current study showed the most species similarity with *Streptomyces albofaciens*. This strain exhibited the highest zone of clearance witha 27 mm zone diameter against *P. vulgaris*. Whereas, it exhibited an 11 mm zone of clearance against *S. aureus*. *S. albofaciens* is a well know producer of commercially available antibiotics oxytetracycline, spiramycin, albopeptin A, albopeptin B, and alpomycin (Uniprot, LPSN bacterio.net). *Streptomyces albofaciens* has been reported from the marine sediment of Chennai coast overlooking the Bay of Bengal which gave antimicrobial activity against *S. aureus* and *Enterococcus faecalis*, giving 19 and 21 mm zone of clearance, respectively **[27 & 28]**. So far there are no reports of *S. albofaciens* showing antibacterial activity against *P. vulgaris*. *S. afghaniensis* VPTS3-1 isolated from the east coast of India, showed antibacterial activity against *P. vulgaris* with a 16 mm zone of clearance **[29]**. *Streptomyces* spp. RM17 and RM42 isolated from CSD zone from Ernakulam to Kannur (Kerala West Coast of India) showed activity against *S. aureus* with 18 and 17 mm zone diameter, respectively **[30]**. Various strains of *Streptomyces* sp. isolated from CSD zone of coastlines of Goa and Maharashtra, showed activity against *S. aureus* with the zone of clearance ranging from 12 to 21 mm **[31]**. *Streptomyces* sp. isolated from CSD of Point Calimere, East Coast of India produced antimicrobial compounds active against *Bacillus subtilis* and *Escherichia coli* **[32]**.

According to an NNISS report (2004), *Staphylococcus aureus* is one of the most infectious pathogens and globally causes health-care-associated infections such as skin infections, soft tissue infections, and endovascular infections, septic arthritis, pneumonia, and osteomyelitis. *P. vulgaris* shows resistance towards antibiotics due to the presence of plasmid, making the infection difficult to cure. In the present study, two strains obtained from CSD have shown promising antibacterial activity against these pathogens. These strains have great potential as antibiotic producers to treat diseases caused by these pathogens.

These strains were checked for enzyme production and plant growth promotion ability. *Streptomyces* sp. K2NRBAY005 produced the maximum number of hydrolytic enzymes such as amylase, protease, lipase, and cellulase with 18 mm, 4 mm, 9 mm, and 15 mm zone of hydrolysis, respectively. Whereas, *Streptomyces* sp.K2NRBAW008 produces an amylase enzyme with a 10 mm zone of hydrolysis. Thus, these could have a wide potential in the food, detergent, and textile industries. Sangeetha *et al*. (2012) isolated and screened Actinobacteria from the rhizosphere of CSD of Chennai (India), for the production of lytic enzymes **[33]**. Several strains of *Streptomyces variabilis* are reported to have various other properties including antioxidant activity, saccharification, production of bioethanol, L-glutaminase, and lipase **[34-37]**.

The environmental conditions of CSD are harsh and stressed making life forms including plants difficult to grow. It is known that microorganisms inhabiting CSD are helping the plants in their growth by providing various factors. In the current study, *Streptomyces* sp. K2NRBAY005 and *Streptomyces* sp. K2NRBAW008 were screened for PGP factors such as the production of IAA, siderophores, ammonia, EPS, and solubilization of inorganic calcium phosphate. Both these strains showed positive results for all these tests. *Streptomyces* sp. K2NRBAY005 and *Streptomyces* sp. K2NRBAW008 from the current study demonstrated promising results in PGP and thus could be utilized as bio-fertilizers. *S. diastaticus* obtained from vermicompost was able to promote plant growth **[38]**. *S. variabillis* showed solubilization of inorganic phosphate **[39]**. *S. wadayamensis* isolated from plant tissue of *Citrus reticulata* showed antagonistic property towards plant pathogens *Xylella fastidiosa* **[40]**. *S. pseudovenezuelae* isolated from the rhizosphere of *Ebenus sibthorpii*

showed an antagonistic effect towards plant pathogens *Rhizocto niasolani* **[41]**. *S. rochei* isolated from decomposed cow dung can alleviate stress in chickpea, caused by salt **[42]**. *S. coelicolor* produces siderophores **[43]**. *Streptomyces* sp. K2NRBAY005 in the present study showed the capability of producing enzyme lipase and protease. These enzymes produced by bacteria provide them with an added advantage since these enzymes are capable of degrading the cell wall components of those pathogens which are made up of lipoproteins **[44]**. Having able to produce such enzymes could give the strain additional benefits in eliminating the pathogen. There are commercially available *Streptomyces* bio-fertilizers based on *S. lydicus* WYEC 108, *S. griseoviridis* and *S. avermitilis* which are majorly used for inhibiting soil-borne plant pathogens such as *Phytophthora* spp., *Pythium* sp., *Rhizoctonia* sp., *Sclerotinia* sp. and *Fusarium* sp. **[6]**.

CONCLUSION

The present study establishes *Streptomyces* strains inhabiting CSD to have promising potential in the medical field as a producer of antibacterial agents, agricultural field as bio-fertilizers, and producer of commercially important hydrolytic enzymes. The current study proposes the CSD as a resourceful source for obtaining potential actinobacterial isolates which could have multiple prospective.

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