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Characterization and Identification of *Pantoea calida* from Contaminated Soil and Its Biocontrol by *Streptomyces coeruleorubidus*

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ABSTRACT

The genus Pantoea is Gram-negative bacteria, distributed in contaminated soil but the increased numbers of Pantoea species were isolated from human bodies, indicating that these species are opportunistic pathogen and cause nosocomial infections. This research aimed to isolate, characterize, and biocontrol of Pantoea species, isolated and collected from soil. During searching for phosphate-degrading bacteria, isolate MO11 was obtained from soil samples and grow well on a medium containing tricalcium phosphate. It was identified according to morphology, physiology, and molecular methods. The antimicrobial susceptibility patterns to antibiotics were determined and the antibiotic resistance pattern reflects its clinical importance. It showed high resistance to Amoxicillin, Amikacin, and Gentamicin while it was sensitive to Imepinem and Meropenem. The isolate was identified as Pantoea calida with 97% and as P. agglomerans with 95% similarity level. Therefore, it is the first time to isolate this bacterium from the soil in Saudi Arabia. Streptomyces coeruleorubidus MM5 was previously isolated from contaminated soil and it was identified using molecular methods. It showed good inhibitory activity against Pantoea calida using the cross streak method, Agar plug diffusion method, and Agar well diffusion methods. Further, the active agent was extracted by ethyl acetate solvent from the extracellular filtrate and it showed excellent antibacterial activity against Pantoea cells which showed resistance to the routinely used antibiotics. In conclusion, although multidrug-resistant Pantoea cells pose a serious threat worldwide and the appropriate antibiotic must be prescribed after determining the sensitivity test, Streptomyces species have played a critical role as biocontrol agents.

Keywords: *Pantoea, Identification, Antibiotic sensitivity, Streptomyces, Gram negative*

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INTRODUCTION

The genus *Pantoea* is ubiquitous, it is well known for more than ten years as the genus belonged to Enterobacteriaceae and it resembles species of the genera *Erwinia* and *Enterobacter* (Akshita *et al*., 2020; Mirza *et al*., 2020; Suhartati *et al*., 2021). *Pantoea* is facultative anaerobic Gram-negative bacilli with peritrichous flagella (AbdAlhussen & Darweesh, 2016). The cultural and biochemical characters and phylogenetic approach are still mainly used to identify various *Pantoea* spp. Some species of the genus *Pantoea* have beneficial effects as a producer of antibiotic like pantocin A and B while others infect some plants, but the diversity of *Pantoea* strains and their possible association with hosts and disease are still to be demonstrated (Cruz *et al*., 2007; Walterson & Stavrinides, 2015). Its role in phosphorus solubilization which is an important macronutrient, needed for all plant growth is well known. Thus, the solubilization of organic and inorganic insoluble soil phosphate by the eco-friendly bacterium is necessary for agricultural uses (Hegyi *et al*., 2021; Madrid-Delgado *et al*., 2021; Zhang *et al*., 2022).

Pantoea species have been isolated from soil, water, eucalyptus leaves (as epiphytes or endophytes bacteria) and plant seeds and fruits, and, four *Pantoea* species were isolated and showing symptoms of blight and die-back on the plant shoot while *Pantoea allii* was recovered from onion leaf and seed (De Maayer *et al*., 2014; Sheibani-Tezerji *et al*., 2015; Achbani *et al*., 2016; Ma *et al*., 2016; Palmer *et al*., 2016). Other members of the genus *Pantoea* have been isolated from animals and human wounds and may be opportunistic pathogens. Some species were isolated from human and animal gastrointestinal tracts, feces, dairy products, human blood, and urine. Also, three new *Pantoea* species were isolated from infant formula. They cause some human infections like septic arthritis and osteomyelitis. They can live as opportunistic pathogens in the immunocompromised human, causing a wound, blood, and urinary tract infections (Brady *et al*., 2010). The species of the genus *Pantoea* included *P. agglomerans, P. ananatis*, *P. citrea*, *P. dispersa*, *P. punctata*, *P. stewartii*, and *P. terrea were isolated and identified* (Schmid *et al*., 2003). In recent years, a variety of human diseases due to *Pantoea* species are increased and nosocomial infections by this organism were reported in different hospital intensive care units, burn, hemodialysis, and oncology unit. *Pantoea* spp. was isolated during an outbreak in a Malaysian neonatal intensive care unit in 2006 (Van Rostenberghe *et al*., 2006; Liberto *et al*., 2009). In India, Tiwari and Beriha (2015) isolated *P. agglomerans* from neonatal sepsis and it is difficult to treat this bacterium. In Iraq, different antibiotics resistance *Pantoea* spp were isolated from different clinical and environmental sources (AbdAlhussen & Darweesh, 2016).

During the past decade, increasing antibiotic resistance to currently used drugs cause high morbidity and mortality

throughout the world, and novel alternative antibiotics with the heterocyclic structure are needed. Actinomycetes, the filamentous Gram-positive, aerobic spore-forming bacteria had DNA rich in GC content (60-80 %) and is the most attractive bacteria with abilities to produce unique or novel bioactive compounds, beneficial to humans against some pathogens like multidrug-resistant bacteria, *Serratia marcescens*, *Staph aureus* and MRSA (Khamna *et al*., 2010; Genilloud, 2017; Kemung *et al*., 2018; Takahashi & Nakashima, 2018). The soil had microbial diversity of Actinomycetes which form extensive branching substrate and aerial mycelia and play significant geochemical roles in soil. They are highly valued for their capacity to form biological secondary metabolites with antimicrobial activities and today, most of the used antibiotics are Actinomycetes natural secondary products (Barton, 2006). On another hand, unexplored soil areas have a significant potential for exploring new bacterial isolates that present under increased competition in the environment like limited nutrient content and producing antimicrobial substances against each other. Some *Streptomyces* isolates produce many secondary metabolites with excellent inhibitory activities against different human microbial pathogens (Sadoway *et al*., 2013; Al-Ansari *et al*., 2019). Production of bioactive agents or antibiotics by actinobacteria with antibacterial and/or antitumor by members of the genus *Streptomyces*, *Amycolatopsis, Micromonospora,* and *Nocardia* were documented (Nakaew *et al.,* 2009; Arifuzzaman *et al*., 2010; Rangseekaew & Pathomaree, 2019). Out of 22,000 bioactive secondary metabolites, 16,000 compounds were selected as bioactive antibiotic activities and about 45% of these compounds were from Actinomycetes, and more than 8000 useful bioactive compounds were from the genus S*treptomycetes* (Berdy, 2005; Khamna *et al*., 2010). From soil, *S. coelicolor* and *S. violaceoruber* produce an important antibiotic actinorhodin with a wide range of applications in the medical sector (Palanichamy *et al.,* 2001). *Streptomyces* sp. SM01 was isolated from Indian soil and it produced a novel antibiotic picolinamycin which was effective against multi-drug resistant bacteria (Maiti *et al*., 2020). The present study was focused on the isolation and identification of *Pantoea* species from soil and its biocontrol by soil actinomycetes.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were collected in sterilized bags from different depths, 5, 15, and 20 cm, from contaminated soil, collected from Jeddah, Saudi Arabia. Soil samples were kept under 4ºC until used for bacterial isolation on nutrient agar using serial dilutions methods. The obtained bacterial colonies were selected, purified, and preserved on the same medium.

Phosphate solubilization and indole production

All the bacterial isolates were screened for phosphate solubilization on phosphate mineral agar containing g/l: 10 glucose, 5 $Ca_3(PO_4)_2$, 5 $MgCl_2.6\ H_2O$, 0.25, $MgSO_4\ 7H_2O$, 0.2 KCl, 0.1 $(NH_4)_2SO_4$ (Nautiyal, 1999) and all plates were incubated for 5 days at 30°C. The colonies with clear halo zones were considered to be phosphate-solubilizing bacteria and the mean diameter of the clear zone (mm) around the tested bacterial colony was measured (Janardan & Verma, 2012). The bacterial growth was measured and represented as +++ (high growth) and ++ (moderate growth). The positive bacteria with clear zone were selected for indole acetic acid production (IAA) in a medium supplemented with 2 mg/ml of L-tryptophan and IAA was extracted from the culture filtrate with ethyl acetate and the quantity was detected according to the method described before by measuring the $A_{530\ nm}$ and preparing a standard curve of IAA (Bano & Musarrat, 2003; Ahmed *et al.*, 2004). IAA-producing *Azotobacter* sp. was obtained from the culture collection of the faculty of Science, KAU, Jeddah, Saudi Arabia, and used as the reference strain.

The used actinomycete isolate

The isolate *Streptomyces coeruleorubidus* MM5 was previously isolated from the industrial zone, Jeddah (Aburas *et al*., 2021) and kindly provided from the culture collection of the faculty of Science, KAU, Jeddah, Saudi Arabia. It was grown on a Starch Nitrate agar medium (Shirling & Gottlieb, 1966), examined under light and scanning microscopes at the KHMR center, KAU, and preserved on the same medium.

Preparation of the bacterial suspension

The bacterial suspension was prepared in a sterile Falcon tube containing 3 ml of sterile nutrient broth medium, these tubes were incubated at 30˚C for 24 hrs and growth was measured at $OD_{\ 520\ nm}$ using Spectrophotometer and it was adjusted to 0.5.

Screening of Actinomycetes for antimicrobial activity

The Actinomycete isolate, *Streptomyces coeruleorubidus* MM5 was screened for inhibitory substances production against the tested bacterium MO11 using different methods. Primary screening using Cross streak method is applied by inoculating a single streak of the selected isolate on the center of the Mueller Hinton agar plate and incubating for 5 days at 30ºC, then the plates are seeded with the isolate MM11 in a perpendicular arrangement and the plates were re-incubated again for 24 hrs (Oskay, 2009). Also, the Agar plug diffusion method was used to determine the inhibitory activity of the selected actinomycete against the isolate MO11. By sterile cork borer, a disc of 8 mm diameter of bacterial growth was put on the center of the Mueller Hinton agar plate inoculated with the tested pathogen MO11. The antimicrobial activity of the bacterial growth disc was detected by the inhibition zone (mm). Also, the selected actinomycete, *S. coeruleorubidus* MM5 was grown on Starch Nitrate agar medium at 30˚C for 5 days at 80 rpm and the culture supernatant was filtrated sterilized through bacterial filter 0.22 µm and the filtrate was extracted with ethyl acetate (1:1V/V). The extract was dried and dissolved in DMSO. Then, 100 µl of the extract was used to fill each agar well with dimmer 6 mm on Muller Hinton agar plates or blood agar inculcated by the tested bacterium, and the plates were incubated at 30˚C for 24 hrs. The mean diameter of the produced inhibition zone was determined (Magaldi *et al.,* 2004; Valgas *et al*., 2007).

Characterization of the selected bacterium

The morphological characterization and biochemical tests were carried out for the most active bacterial isolate MO11. The selected isolate was examined on Nutrient, MacConkey, and blood agar plates after incubation at 30˚C under aerobic conditions for 2 days. Cells were examined under a light microscope using an oil immersion lens after Gram staining. Catalase test, oxidase test, starch hydrolysis, coagulate test,

blood hemolytic, and antibiotic sensitivity were also recorded. VITEK-2 system at KAUH was used for bacterial identification. Antibiotic susceptibility testing was carried out on Mueller-Hinton agar by "The Kirby-Bauer disc diffusion method. The fresh bacterial suspension was prepared with OD 0.5 and inoculated on Mueller-Hinton plates and antibiotic discs were applied o the agar surface. All the inoculated plates were incubated at 30 ◦ C for 24 hrs, and the susceptibility of the tested isolate to each antimicrobial agent was recorded.

Molecular identification of selected isolate

The selected isolate MO11 was grown on Nutrient agar medium for 2 days at 30˚C, then, DNA was extracted, purified, and amplified using twp primers. After PCR, the sequences of the 16S rRNA were determined at Macrogen. The information on the used primer was summarized in **Table 1**.

Table 1. Primer used for molecular identification of the isolate MO11

The information on the used Primers	
PCR (primer name and sequence)	Sequence (primer name and sequence)
27F 5' (AGA GTT TGA TCM TGG CTC AG) 3	785F 5' (GGA TTA GAT ACC CTG GTA) 3'
1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3'	907R 5' (CCG TCA ATT CMT TTR AGT TT) 3'

Statistical analysis

Statistical Package for Social Science (SPSS for windows, version 17) was used for comparing the data, a mean value of three reading ± SD was used and a t-test was used to detect any significant difference between the sample and control. Significant results were obtained at $P<0.05$.

RESULTS AND DISCUSSION

A bacterium was isolated from soil samples, collected from the contaminated area. Out of 20 bacterial isolates obtained on Nutrient agar, 7 isolates were phosphate-solubilizing bacteria and they had different abilities to produce IAA **(Table 2)**. The isolate MO11 was the most active isolate, thus it was selected for more detailed studies.

Under light microscopic, the selected isolate MO11 appeared as Gram-negative motile bacilli **(Figure 1a)**. It grows well on different agar media, Nutrient agar **(Figure 1b)**, MacConkey agar, and blood agar plates. It appeared as punctuate circle smooth colonies, middle in size, while on blood agar it produce smooth non-hemolytic colonies. It had positive results for catalase, indole, and citrate production and fermentation of Raffinose. It showed negative results to produce H_2S and other biochemical reactions had variable results **(Table 3)**. The isolate MO11 was resistant to Amoxicillin, Amikacin, and Gentamicin while it was sensitive to Imepinem and Meropenem **(Figure 1c)**. The isolate MO11 was identified by an automated VITEK-2 compact system using GN-ID cards as *Pantoea* spp. A phylogenic analysis was performed to identify the selected isolate MO11using a partial sequence of the 16S rRNA which showed a high level of sequence similarity with member genus *Pantoea* with a homology level of 97% to *P. calida* as in the phylogenic analysis **(Figure 2)**.

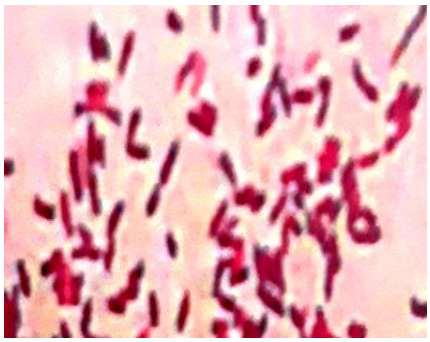
Table 2. The growth of the obtained bacterial isolates on nutrient agar, Gram strain, production of IAA, and phosphate solubilization

Isolate	Soil depth (cm)	Shape	Color	Gram stain	IAA (mg/l)	Phosphate solubilization	
						Growth	Halo Zone Diameter (mm)
MM1	5 cm	Monococcus	White	G +ve	1.19±0.12*	++	14±1.9*
MM4	5 cm	Bacilli	Yellow	G -ve	ND	++	12±1.3*
MM9	1 5 cm	Bacilli	White	G +	1.45±0.14*	++	18±2.1*
MM11	15 cm	Bacilli	White	G- ve	0.49±0.49*	+++	34±2.0
MM15	20 cm	diplococci	White	G -ve	1.69±0.11*	++	22±2.8*
MM16	5 cm	Bacilli	Yellow	G-ve	2.89±0.22	++	21±1.0*
MM20	5 cm	Bacilli	Gray	G-ve	1.90±0.09*	++	20±1.9*
Control (Azotobacter sp.)		Cocci	Black	G-ve	3.11	++	30±5.0

+++: high growth, ++: moderate growth, IAA: Indole acetic acid, G+ve: gram-positive, G-ve: gram-negative, ND: Not detected, *: Significant differences compared to control.

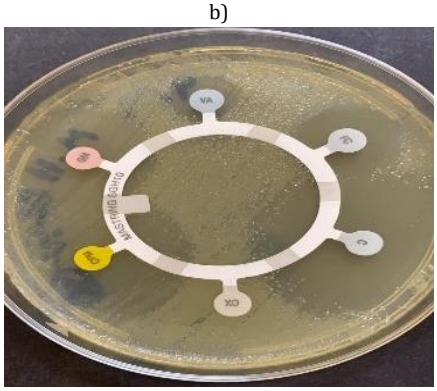
Table 3. Biochemical test for identification of *Pantoea* sp.MO11

Test	Result	Test	Result
Shape	Small rod	Citrate	+
Catalase	+	VP	+
Oxidase	-	Gas	+
Gelatinase	-	H_2S	-
Hemolysis	γ	Urease	+
Motility	+	Indole	+
Fermentation of Raffinose	+	MR	-



a)

b)



c)

Figure 1. The isolate MO11, a) After Gram stain, b) grown on nutrient agar, c) resistance of the isolate to some antibiotics.

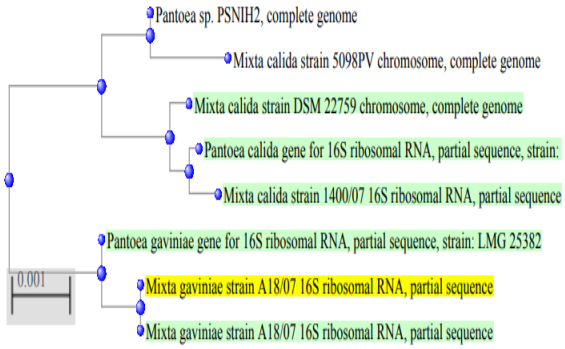
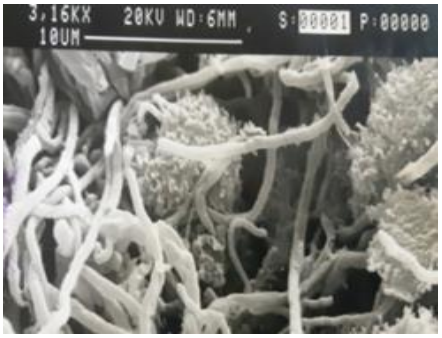
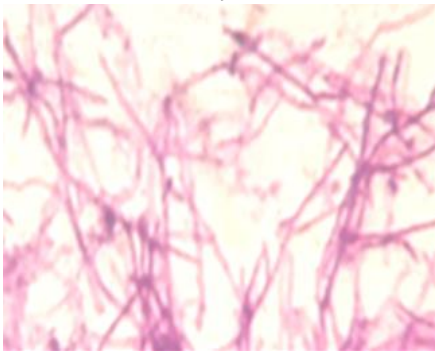


Figure 2. The phylogenic tree of the isolate MO11 and the most related isolates

a)

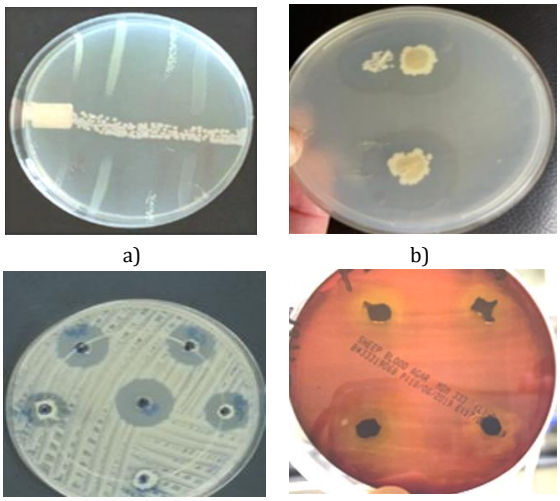


b)



c)

Figure 3. Streptomyces coeruleorubidus MM5, grow on starch nitrate agar (a) for 7 days, under a scanning electron microscope (b) and after Gram stain (c).



a) b) c) d)

Figure 4. The inhibitory activity of Streptomyces coeruleorubidus MM5 against Pantoea calida MO11 using the three tested techniques, cross streak method (a), Agar plug diffusion method (b), and Agar well diffusion methods on Muller Hinton (c) and blood agar (d).

Isolate *Streptomyces coeruleorubidus* MM5 was grown on starch nitrate agar for 5 days and examined under a light and scanning microscope. *Streptomyces coeruleorubidus* MM5 has a dark yellow color with aerial and substrate well-developed mycelia **(Figure 3)**. It showed good inhibitory activity against *Pantoea calida* MO11 using the three tested techniques, cross streak method Agar plug diffusion method, and Agar well diffusion

method **(Figure 4)**. It is noticed that the ethyl acetate extracts of the isolate *S. coeruleorubidus* MM5 gave a good inhibition zone using agar well diffusion methods either on Muller Hinton or blood agar.

During the screening of soil bacteria for their ability for phosphate solubilization, many isolates showed excellent activity and they also produce IAA in a liquid medium. Isolate MO11 was the most active isolate and it was identified using morphological and physiological methods (Grimont & Grimont, 2005; De Maayer *et al*., 2014). After molecular studies, it was identified as a bacterial pathogen of the genus *Pantoea* using 16S rDNA sequencing which is mainly used as a most powerful technique for bacterial identification (Yokota, 1997; Xu *et al*., 2021). The 16S rDNA sequence was determined and the phylogenic tree was obtained. Many authors reported that *Pantoea* species can produce IAA and auxin-related compounds which affect the root system structure and shape (Duca *et al*., 2014; Spaepen, 2015; Stringlis *et al*., 2018). *Pantoea* has pigmented a rod-shaped, non-spore-forming, Gram-negative bacterium, belonging to the *Enterobacterales* order, mainly isolated from soil, urine or associated with plants (Walterson & Stavrinides, 2015; Zhang *et al*., 2015) and may be used as a growth-promoting bacteria or as biocontrol agents (Dutkiewicz *et al*., 2016; Luziatelli *et al*., 2020a,b). It processes an excellent ability to remove heavy metals. In contrast, some species of the genus *Pantoea* is the obligate infectious agent in humans and can cause opportunistic wound infections or act as hospital-acquired pathogens, mostly in immunocompromised individuals. Septic arthritis, endophthalmitis, periostitis, endocarditis, and osteomyelitis or synovitis in addition to epidemics of nosocomial septicemia were recorded as a frequent clinical result of external infection with *P. agglomerans* (Dutkiewicz *et al*., 2016). They added that exposure of hospitalized patient or immunodeficient individuals to medical equipment or fluids contaminated with this bacterium cause serious infection and many fatal cases were recorded in several countries. This bacterium infects cultivated plants, cotton, onion, rice, maize, and sorghum, causing serious diseases or acting as a tumourigenic agent, inducing gall formation on some plant roots. Moreover, it was resistant to most used antibiotics and the resistance increased with time. They are implicated in nosocomial outbreaks of neonates and immunocompromised patients (Habsah *et al*., 2005). The prevalence and antibiotic susceptibility of *Pantoea* species during 12 years was recorded by Gajdács, (2019) who studied 70 isolates of *Pantoea* spp. and the highest resistance was to amoxicillin/clavulanic acid and ampicillin, thus the development of multidrug-resistant (MDR) Gram-negative bacteria is a serious concern, and the development of MDR *Pantoea* spp. may be expected in the future.

The search for novel antibiotics and other bioactive microbial metabolites from soil actinomycetes especially Streptomycetes which for a long time have been recognized as high producers of useful bioactive compounds with many applications is increased. Therefore, it is important to fight against new and emerging pathogens by application of new antimicrobial agents from actinomycetes which form unique natural agents and bioactive metabolites with great interest. *Streptomyces* species is still a promising and fruitful source to obtain new antibiotics (Berdy, 2005). *S. coeruleorubidus* have a good ability to produce natural bioactive antimicrobial material which was extracted and showed inhibition activity against *Pantoea, thus it* could be very useful for pharmaceutical and agricultural uses.

The used *S. coeruleorubidus* generally showed good growth and inhibited the growth of *P. calida* using the cross streak method which is one of the best techniques to detect the inhibitor activity of any isolates. This technique is a primary screening method and required a short incubation time to get the result. The isolate showed good inhibitory activity in primary screening, it was subjected to secondary screening using the agar plug method which is often used to highlight the antagonism between microorganisms). *S. coeruleorubidus* showed high antagonistic activity against *P. calida* using the plug diffusion method. Moreover, the Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants and microbial extract (Magaldi *et al.,* 2004; Valgas *et al*., 2007). Ethyl acetate was standardized as the best solvent to extract secondary materials. Similarly, the crude extract of *Streptomyces* sp. showed high activity against Gram-positive and negative bacteria in addition to MRSA strains. These organisms may produce a high molecular weight glycopeptide with novel anti-MRSA activity (Reddy *et al*., 2011; Park *et al*., 2014; Tan *et al*., 2015).

CONCLUSION

Screening of various actinomycetes for the production of the new drug is a continuous process that should be effective against present-day's antibiotic-resistant pathogenic bacteria. Actinomycetes have been proven as important sources of various useful secondary metabolites. Also, a recent investigation indicated the potential of marine *Streptomyces* sp. as a sustainable and useful source of novel bioactive compounds. Hence, the findings of this study revealed that *Streptomyces* sp. with antibiotic substances production capability was an important application. Also, *Streptomyces* species from unexplored regions are likely to yield novel antibacterial agents. *Streptomyces* sp. could be a promising microorganism for the development of a novel antibacterial drug against a wide range of pathogenic bacteria.

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