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ORIGINAL ARTICLE **OPEN ACCESS**

Isolation and identification of Rhizospheric Actinomycetes with potential application for bio control of Black spot of *Ricinus communis* L.

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ABSTRACT
Bacterial pathogens pose substantial threats to agricultural systems, while drug-resistant bacteria are critical concerns for public health. Consequently, there is a pressing need to discover novel antimicrobial compounds from alternative sources. Considering these challenges, our study aimed to investigate the antibacterial properties of Streptomyces sp. BNPA72 against Black spot disease, which is caused by Xanthomonas sp. in Ricinus communis L. In our study, we isolated 76 strains of Actinomycetes from the rhizospheric region of the castor plant in Gujarat. Subsequently, we conducted a secondary screening, which revealed that 3 of these strains exhibited antagonistic activity against Xanthomonas sp. Among these, one Actinomycete strain, designated as BNPA72, displayed notable antibacterial properties. Morphological, biochemical, and molecular analyses confirmed that BNPA72 belongs to the Streptomyces genus. Subsequent chemical exploration of the Ethyl acetate extract resulted in the isolation and purification of an antibacterial compound. To determine its structure, FTIR spectroscopic analysis was employed. Secondary metabolite identification was performed using the TLC-Bioautography method targeting Xanthomonas sp. The outcomes of this analysis revealed the existence of antibacterial compounds, further substantiated by the formation of a distinct clear zone in the disc diffusion test. To the best of our knowledge, our study represents the initial demonstration of BNPA72 as antibacterial effectiveness against Xanthomonas sp. The results of our investigation support the potential application of the compound produced by Streptomyces sp. BNPA72 as an antimicrobial agent for combating Black spot disease in Ricinus communis L.
Keywords*: Actinomycete, Xanthomonas sp., Ricinus communis L., Bioautography,*

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INTRODUCTION

Actinomycetes are a group of Gram-positive filamentous bacteria known for their distinctive appearance resembling rays (actini) and fungi-like (mycetes) characteristics. These microorganisms are widely distributed in various natural environments, such as soil and water sources. They possess unique morphological features and have a high guanine plus cytosine (G+C) content in their DNA. Actinomycetes are highly valuable for their role in producing antibiotics. They have contributed to the production of a range of antibiotics, including tetracycline, macrolide, chloramphenicol, nucleosides, and polyenes. Notable antibiotics like erythromycin, gentamycin, rifamycin, and streptomycin have been derived from Actinomycetes found in soil [1]. The presence and availability of antibiotic-producing Actinomycetes can vary based on soil characteristics and cultivation conditions. While Actinomycetes are globally distributed, only a limited number of them have been systematically screened for antibiotic production. Therefore, there is a significant potential to discover diverse Actinomycetes with antibiotic-producing capabilities in water sources, given the similarities between soil and water as natural habit [2]. Metabolomics is a scientific approach that involves the comprehensive analysis of biologically active substances in microorganisms, biological fluids, plants, and food samples, with the aim of both identifying and quantifying these compounds [3]. Metabolomics is a scientific discipline dedicated to the identification and quantification of small-molecule metabolites within the metabolome of biological samples. This field employs high-throughput techniques to gather data on all the metabolites present in cells, organs, or microorganisms. These metabolites encompass a wide range of compounds, such as peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, pharmaceuticals, food additives, phytochemicals, and

toxins. Notably, metabolites are categorized into two main types: primary and secondary metabolites [4][5]. Throughout the life cycle and energy utilization of microorganisms, primary metabolites are regularly synthesized. Primary metabolites, including glucose, proteins, amino acids, and fatty acids, are vital for cell growth, development, and reproduction. On the other hand, secondary metabolites are derived from primary metabolites under particular conditions, and they serve a significant function in defense against pathogens [6]. Low-molecular-weight metabolites constitute a valuable class of compounds with diverse applications, including pharmaceuticals (e.g., antibiotics and anti-tumor agents), agrochemicals (e.g., pesticides), biofuels (e.g., oleoresin), and food additives (e.g., essential oils). Some secondary metabolites synthesized by fungi and bacteria have proven effective as antibacterial agents, like the phenazine produced by *Streptomyces kebangsaanensis* [4]. Gas chromatography-mass spectrometry (GC-MS) is a valuable technology for separating the different components present in secondary metabolites. It has found applications in various fields, including the study of lipids, drug metabolites, environmental pollutants, and forensic science. One notable advantage of GC-MS is its ability to identify microbial species based on the time it takes for compounds to pass through the instrument and the pattern of fragmentation produced when they are ionized using electron voltages of around -70 eV. These fragmentation patterns are consistent and not influenced by the specific instrument used, allowing for the creation of databases and data sharing among researchers. Additionally, GC-MS facilitates the quantitative identification of the substances being analyzed [7]. A pure culture of non- Actinomycetes bacteria has been found to possess inhibitory properties against Actinomycetes. As a result, six distinct methods have been devised for the selective isolation of soil Actinomycetes. These methods are as follows: nutritional selection: This approach involves using growth media with specific nutrients that Actinomycetes preferentially consume. Selective inhibition: In this method, the growth inhibitors such as antifungal drugs and antibiotics are added to the media to prevent the growth of non- Actinomycetes bacteria. Physical or chemical sample pre-treatments: Techniques are employed to treat soil samples physically or chemically to limit the presence of non-Actinomycetes bacteria. Enrichment approaches: Nutritional media can be supplemented with additional nutrients that promote the growth of Actinomycetes while inhibiting the growth of other microorganisms. Membrane filter method: This method doesn't require pre-treatment, specific media, or antibiotics. It utilizes a membrane filter to selectively capture Actinomycetes. Integrated method: This comprehensive approach involves the combination of various procedures to ensure the selective isolation of soil Actinomycetes [8]. Recent research on Actinomycetes in the rhizospheric soil of plants has highlighted the potential of plant-associated Actinomycetes as biocontrol agents in agriculture. These Actinomycetes show promise in stimulating plant growth and protecting plants from phytopathogens. While most of these beneficial microorganisms have been found in the rhizospheric soils of vegetables and fruit trees, Actinomycetes associated with medicinal plants represent a unique and promising resource. Actinomycetes linked to medicinal plants are of particular interest for two main reasons. Firstly, they may serve as valuable sources of bioactive natural compounds. Secondly, these Actinomycetes could possess biological activities and produce bioactive substances similar to those present in the medicinal plants themselves. For instance, *Citrullus colocynthis*, known for its therapeutic properties, including antimicrobial and antitumor effects, is a focus of this study. Based on this hypothesis, our current research aims to isolate Actinomycetes strains from the rhizosphere of Citrullus colocynthis. We are investigating their antifungal capabilities and identifying the antimicrobial molecules they produce. This work seeks to better understand the potential benefits of *Citrullus colocynthis*-associated Actinomycetes, offering insights into their role in both agriculture and medicine [9].

MATERIAL AND METHODS

Isolation of Actinomycetes: Soil samples were collected from various locations in Mehsana District, Gujarat, India, including Kherva, Visnagar, Patan, and Mansa. Sterile new zip lock bags and a sterile spatula were used for sample collection. These samples were transported to the laboratory for the purpose of isolating Actinomycetes[10]. The process of enriching and isolating soil Actinomycetes was carried out as follows: One gram of sediment soil was placed into 100ml of starch casein broth, which was supplemented with Calcium carbonate (0.02g), Ferrous sulfate (0.01g), Magnesium sulfate (0.05g), Potassium nitrate (2g), Potassium phosphate (2g), Sodium chloride (2g), Soluble starch (10g), and Casein (0.3g). The mixture was then incubated in a rotary shaker at 36°C for a duration of 7 days. After the incubation period, streaking was performed on Starch casein agar plates to isolate individual colonies. Selected colonies from these plates were subcultured and maintained at 4°C for future use[11]. Isolation of plant pathogens: To isolate plant pathogens from Ricinus plant leaves exhibiting symptoms of bacterial black spot, aseptic techniques were employed. Infected leaves were collected and subsequently crush thoroughly using a sterilized mortar and pestle. The crush leaf material was then transferred from Nutrient broth to Nutrient agar plates

for culturing. These agar plates were subsequently incubated at a temperature of 37°C for a duration of 24 to 48 hours, as previously documented in references [12]. For further research purposes, bacterial cultures were transferred into sterile Nutrient agar slants. These slants were maintained under frozen conditions to preserve the cultures for subsequent investigation, as described in reference[13]. Screening of Actinomycetes: The isolation process encompasses a biphasis screening approach, encompassing both primary and secondary evolutions. In the primary screening phase, the antibacterial potency of the isolated Actinomycetes was determined by utilizing the cross-streak technique, as alaborated in reference [11] and secondary screening by using agar well diffusion method [14]. Molecular identification of BNPA72: In phylogenetic analysis, we pick up to 10 similar sequences from different groups among the top 1000 matches in a database. We use a tool called MUSCLE to align these sequences. We create a phylogenetic tree to show how species are related in terms of evolution. Each branch in the tree represents a group of species we're comparing. The starting point of a branch (node) marks where species diverged from a common ancestor. The tree's root represents the oldest ancestor for all species. Phylogenetic trees can be rooted (with a known ancestor) or unrooted (without one). We focus on branch lengths to gauge evolutionary distance—longer branches mean more genetic differences. The horizontal lines (branches) display how different lineages evolved, with more changes happening in longer branches. Numbers at nodes are bootstrap percentages, indicating how often branches had the same arrangement during analysis. A higher percentage means more confidence in that branch's placement. In short, phylogenetic analysis helps us understand species relationships through a tree, where branch lengths and bootstrap values are key, and longer branches mean more genetic change over time[15][16].

FTIR analysis of BNPA72:

The FP's particular functional groups were examined using FT-IR spectroscopy, utilizing a Nicolet iS10 FT-IR spectrophotometer from Thermo Scientific in the USA. To do this, a straightforward KBr pellet method was employed, and the spectrophotometer captured spectra of the pellet sample while comparing it to a reference KBr pellet. The spectral range covered 400 to 4000 cm−1, with a resolution of 4 cm−1[17].

Extraction of Bioactive Compound

The production of bioactive compounds was achieved through submerged fermentation, as described in reference [18]. Actinomycete isolates were inoculated into 50 mL of SCA broth within a sterile 250 mL conical flask. These flasks were then incubated at 32°C for a duration of 7 days, with continuous rotation at 150 rpm, maintaining aseptic conditions throughout [19]. Following the fermentation period, the culture medium underwent centrifugation at 10,000 rpm for 10 minutes to separate and eliminate cells and debris. The resulting supernatant, referred to as the fermented broth, was obtained. To extract the bioactive compounds, an equal volume of Ethyl acetate was added to the fermented broths. The mixtures were vigorously shaken using a rotary shaker. Subsequently, the solvent phase was isolated and allowed to evaporate naturally at room temperature. This process yielded completely dried residues, which were then re-dissolved in dimethyl sulfoxide (DMSO) and subjected to lyophilization, thereby preparing them for further research and analysis[18][20].

Bioautography: Thin layer chromatography (TLC) was utilized with a solvent system consisting of a mixture of Ethyl acetate and Chloroform in a ratio of 9:1 (v/v). This method aimed to analyze the presence of antimicrobial compounds within a diethyl ether (Et2O) extract. The results were visualized by examining the chromatogram under UV light. To determine the quantity of antimicrobial compounds present, TLC strips were aseptically placed onto the surface of nutrient agar (NA) plates that had previously been inoculated with a test culture of Xanthomonas. These TLC strips were then allowed to incubate at 4°C for one hour, allowing the active metabolites to diffuse into the agar medium. Subsequently, the plates were incubated for a period of 24 to 48 hours at 37°C for bacterial growth. The presence of inhibition zones on the agar plates indicated the presence and number of active compounds within the solvent extract. These inhibition zones serve as an indicator of the antimicrobial activity of the compounds present in the extract [21].

Purification of antimicrobial compound from Streptomyces sp. BNPA72: The active compound was purified and characterized through a series of scientific procedures. To purify antimicrobial compounds from a 50 mg sample extracted with diethyl ether, silica gel chromatography was employed. A column measuring 35 × 1.0 cm was packed with silica gel with a particle size of 60–120 mesh, and chloroform was used as the initial solvent. The elution process involved a stepwise gradient of solvents, with the following chloroform:ethyl acetate ratios: 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 (v/v), followed by 100% ethyl acetate (200 ml each). The elution was carried out at a flow rate of 2 ml/min, resulting in the collection of 88 fractions, each with a volume of 25 ml. These fractions were then concentrated. To assess the antimicrobial activity of the collected fractions against Xanthomonas bacteria, the standard Kirby-Bauer disc diffusion method was employed. Nutrient agar plates were inoculated with

100 µl of the test bacterium, with an optical density equivalent to the Mac Farland standard of 0.5. Subsequently, 6 mm diameter discs loaded with the residue obtained from the concentrated fractions were placed on the agar plates. These plates were allowed to incubate at their respective temperatures, and the resulting zones of inhibition were measured [22].

RESULTS:

Isolation of Bacteria:

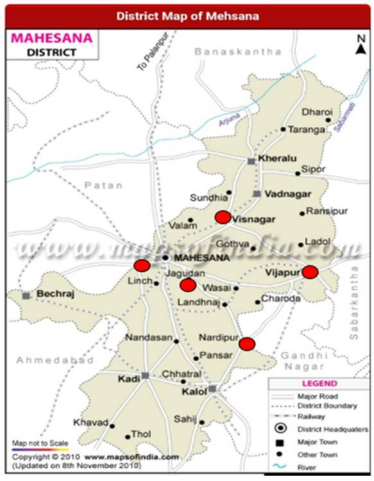

Figure 1 Sample collection site

Table 1 List of the isolated strains of Actinomycetes from Mehsana District

Sr. No	Location site	Designation of strain
1	Gozariya	BNPA1-BNPA15
2	Kansa	BNPA16-BNPA27
3	Panchot	BNPA28-BNPA43
4	Kherva	BNPA44-BNPA55
5	Langhnag	BNPA56-BNPA66
6	Ranasan	BNPA67-BNPA76

To combat Bacterial Blight disease in Ricinus plant, a survey was conducted to identify the specific pathogens present in the leaves of these plants. The bacteria were isolated from crushed leaf samples and cultured on nutrient agar. These bacterial isolates exhibited a characteristic appearance, appearing as shiny yellow and having a mucoid texture. Upon further analysis, it was determined that these bacteria were Gram-negative and displayed morphological and biochemical traits consistent with the Xanthomonas species. Isolation of Actinomycetes: A total of 76 Actinomycetes were successfully isolated from various locations within Mehsana district, specifically from the rhizospheric soil associated with Ricinus plants as show in (Table 1). These Actinomycetes were meticulously sub-cultured to obtain pure cultures and were subsequently maintained as slant cultures for future experimentation. Among the various types of growth media tested, it was found that Starch Casein Agar proved to be the most effective medium for the isolation of Actinomycetes in this study. Notably, a majority of the isolated Actinomycetes exhibited a gray-colored mycelial growth pattern, which was the predominant characteristic among the obtained isolates. Primary screening of Actinomycetes against plant pathogens: In the primary screening of Actinomycetes against plant pathogens, we employed the cross-streak method on Mueller-Hinton agar to assess the antibacterial activity of isolated Actinomycetes strains. First, Actinomycetes cultures were prepared, and an inoculum was streaked onto Mueller-Hinton agar plates to shield it from potentially harmful pathogenic bacteria. Subsequently, all the plates were incubated at a temperature of 37°C for a duration of 24 to 48 hours following the inoculation of the bacteria. We observed antagonistic interactions by examining the inhibition zones caused by the plant pathogen on the Mueller-Hinton agar medium [29]. Secondary screening of isolates: Certain isolates demonstrated significant effectiveness against plant pathogens. To further assess the antibacterial activity of these Actinomycetes, we employed the Agar well diffusion method. The Actinomycetes isolates were cultivated in Erlenmeyer flasks containing Starch casein broth and incubated at 32°C while agitating at 150 rotations per minute (rpm) for a duration of 6 to 7 days. Subsequently, the

Actinomycetes cultures underwent lateral centrifugation at 10,000 revolutions per minute (rpm) for a period of 30 minutes [33].

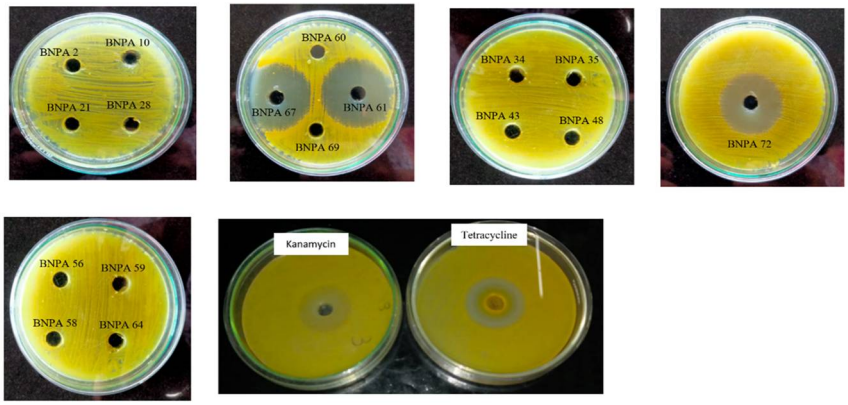

Figure 2 Secondary screening of isolates:

Table 2 Zone measurement of selected isolates:

Sr.no	Name of Isolates	Zone of inhibition (mm ± S.D)
1	BNPA61	39.82 ± 0.05
2	BNPA67	37.05 ± 0.06
3	BNPA72	29.05 ± 0.06
4	Kanamycin	22.22 ± 0.05
5	Tetracycline	26.02 ± 0.05

Biochemical Characterization of potent Actinomycetes: Biochemical characterizations of the selected isolate, BNPA72, are provided in Table 4. It was observed that this isolate exhibited positive reactions for various biochemical tests, including citrate utilization, oxidase activity, starch hydrolysis, and lipid hydrolysis. Furthermore, BNPA72 displayed the capability to utilize glucose, galactose, fructose, and sucrose as sole carbon sources, indicating its metabolic versatility in utilizing these sugars for growth and energy production.

Table 2 Biochemical Characterization of BNPA72

Sr. No.	Characteristics	BNPA72
1	Reduction of nitrate	+
2	Hydrolysis of Urea	-
3	Productionof Ammonia	-
4	H2S Production	-
5	Oxidase	+
6	IMVIC test	-
7	Casein Hydrolysis	+
8	Starch Hydrolysis	+
9	Sucrose	++
10	Fructose	+++
11	Galactose	+
12	Glucose	+++
13	Lactose	-
14	Maltose	-

Molecular identification of BNPA72:

Table 3 Genetic identification

Sr. No.	Isolate	Identified as a	Accession No.
1	BNPA72	*Streptomyces rochei*	MN114054
2	BNPP6	*Xanthomonas sp.*	OR030421

FTIR analysis of BNPA72: The FTIR spectrum of the ethyl acetate extract derived from *Streptomyces rochei* is illustrated in Figure 23. This spectrum provides valuable information about the active components in the extract by identifying characteristic peaks associated with specific functional groups. The data regarding the peak values and the likely functional groups present in the extract are summarized in Table 6. The FTIR analysis revealed distinct bands at specific wavenumbers, which correspond to particular functional groups within the extract. Notably, there were peaks observed at 3466 cm^-1, indicating the presence of hydroxyl (O-H) groups. Additionally, a peak at 1737 cm^-1 suggested the presence of carbonyl (C=O) groups, while another peak at 1232 cm^-1 indicated the presence of amino (C-N) groups within the active components of the extract. These findings contribute to our understanding of the chemical composition of the *Streptomyces rochei* extract and its potential biological activities.

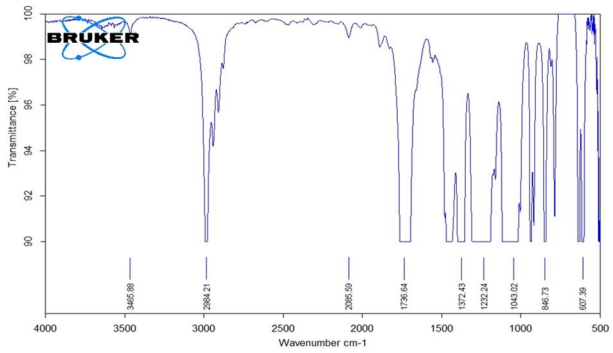

Figure 3 FTIR analysis of Ethyl acetate extract of BNPA72

Table 4 FTIR analysis

Number of Compound	Peak value	Bond
1	3466 cm^-1	(O-H) groups
2	1737 cm^-1	(C=O) groups
3	1232 cm^-1	(C-N) groups

Extraction, separation and bioautography of antimicrobial compound: Diethyl ether was used to extract antimicrobial metabolites from the culture supernatant because it provided the best recovery of metabolites in terms of inhibition zone. Diethyl ether (Et2O) extract showed pronounced activity against Xanthomonas. Thin-layer chromatography of Et2O extract followed by bioautography against Xanthomonas revealed the presence of one active compound with an Rf value of 0.9.

Purification of antimicrobial compound from Streptomyces sp. BNPA72:

For the purification of the antimicrobial compound, Et2O extract was subjected to silica gel column chromatography. Antimicrobial activity against Xanthomonas was found in eight fractions (32–40 fractions) eluted with chloroform: ethyl acetate (60:40, v/v). The purified compound was named BNPA72.

CONCLUSION

The Actinomycetes members displayed significant diversity, as determined by a comprehensive analysis of their morphology and biochemical profiles, as well as the range of compounds they synthesized. We successfully isolated various strains of Streptomyces sp. from different geographical locations within the Mehsana District of Gujarat, India. These strains demonstrated the potential to produce a wide variety of antibacterial compounds, warranting further investigation due to the notable antimicrobial activity observed in our study. Our research team, working in partnership with our colleagues, is presently conducting both controlled laboratory experiments (pot study) and field trials to assess the impact of the bioactive compounds identified in our study on *Ricinus communis* L. We anticipate that comprehensive

information about the molecular properties of these Actinomycetes will be presented in forthcoming publications.

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