Impact of Soil physicochemical properties on the diversity of Limestone-acquired Actinomycetes

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Abstract
Actinomycetes, a varied genus of filamentous bacteria, play an important role in soil ecosystems, especially in limestone-derived soil. This research looks at the effect of soil physicochemical qualities on the diversity and biochemical characteristics of actinomycetes isolated from limestone-rich settings. Soil samples were tested for pH, moisture content, organic matter and key nutrients to determine their impact on microbial diversity. Actinomycetes were isolated, identified and described by using morphological and biochemical methods preliminarily.

The data show that alkaline pH, calcium-rich composition and organic matter all have a substantial impact on actinomycete variety and metabolic capacities, especially antibiotic synthesis and enzymatic activity with intricate biosynthetic pathways. These findings emphasize the ecological importance of soil characteristics in creating microbial communities and their potential for biotechnological applications.

Keywords: Limestone bacteria, physico-chemical properties, biochemical characterization, secondary metabolites.

Introduction

Actinomycetes, a diverse group of Gram-positive organisms, are fascinating in microbiology due to their ability to synthesize valuable bioactive compounds[18]. They exhibit various morphologies including cocci, rods, mycelia and numerous forms. These organisms can adapt to harsh conditions such as desiccation, low temperature, stress and hyperosmotic stress, making them both terrestrial and aquatic dwelling[17]. The habitat range of these diversified actinomycetes is expanding, possibly due to their ability to withstand extreme conditions. Actinomycetes play a crucial role in the environment by mediating carbon and nutrient turnover, contributing to the formation of humus, a vital component of soil. Humus significantly influences the survival and efficiency of these microorganisms, impacting processes like respiration, photosynthesis and nitrogen fixation[9].

The southern parts of India, particularly the limestone quarries of the Deccan plateau are found in combination with calcium carbonate or calcium and magnesium carbonate rocks. They are Proterozoic sedimentary in nature[23]. Therefore, this unique ecological niche is considered to be the underexplored area for the isolation and studies of actinomycetes. Hence the culturing of uncultured actinomycetes from this terrestrial habitat may be possible. Pretreatment methods enrich the population density of actinobacteria, with *Streptomyces* being the dominant group[12,15].

Soil analysis is a quantitative estimation made to study the nutrients present in the soil sample which dictates microorganism's natural adaptation, interactions of biotic and abiotic factors and their microenvironment which ensures their functionality, sustenance and proliferation[14]. pH favours the longevity of the microorganisms and soil enzyme activity [10]; the type of bioactive molecules the organisms produce is mainly governed by the inorganic metals present in the soil sample, since it enhances several secondary metabolite-biosynthetic gene clusters in *Streptomyces* or even activates silent biosynthetic gene clusters[24]. Therefore, soil nutrients analysis report gives us the insights for optimization of the media and pH for the successful cultivation of rare/unculturable actinomycetes. The majority of the bacterial taxa display rather restricted growth tolerances in soil pH, therefore cultivating techniques proves to be an important role in cultivating new species[13,19].

Material and Methods

Sampling sites and Sample Collection: Soil samples were collected between the sedimentary slabs of two limestone quarries of Banaganapalli Mandal, Kurnool district, Andhra Pradesh, India [Latitude 15°23'26.53"N to Longitude 78°13'55.00"E; Limestone quarry-1(YL) and Latitude 15°23'34.62"N to Longitude 78°14'9.85"E; Limestone quarry-2 (PL)] at an altitude of 252 mean sea level. The collected sediment samples were brought to the laboratory in sterile zipper lock bags and stored at room temperature until further use[1].

Pre-treatment of the soil sample: Pre-treatment method is an approach to look for perhaps valuable actinobacterial community adaptable in different biological habitats. Pre-treatment of the soil samples promotes the proliferation of dormant forms of actinobacteria and at the same time eradicates the undesired fungi and bacterial propagules in the primary isolation plate[22].

Heat treatment: To ensure the successful isolation of only actinobacteria, heat treatment was employed as per the recommendations made by Jiang et al[7] and then modified.

According to which one gram of soil sample was weighed, sieved and ground to fine particles. A thin overlay of soil was spread uniformly on a clean Petri dish and subjected to the temperature at 60°C for 30 minutes followed by serial dilution and spread plate method on starch casein agar plates.

Calcium Carbonate treatment: To enable the isolation of actinomycetes, the soil samples were subjected to calcium carbonate pre-treatment method in order to subdue undesired microbial load and obtain discrete actinobacterial colonies. This method was followed and altered as per the study suggested by Nimaichand et al[11].

This involves the drying up of soil sample in the room temperature followed by crushing and sieving and treating 1gram of the finely ground sample with 0.1 g $CaCO_3$ for 24 hours at room temperature. This inhibits the bacterial count. After preliminary incubation, treated soil sample was dispersed in 99 ml of sterile distilled water and shaken continuously at room temperature using an orbital shaker at 150 rpm for 30 minutes. The Erleynmer flasks containing stock were kept to still and 1 ml of the top most suspension was collected for serial dilution.

Determination of Soil Physicochemical Properties: The collected soil samples were weighed approximately 500 grams and sieved for analysing various abiotic and macro-elemental parameters that include soil pH, electrical conductivity (EC), organic carbon (OC) by volumetric method[26], available sodium, phosphorous, potassium and elements such as nitrogen by Kjeldahl method, carbon, hydrogen and sulphur[6].

Determination of Trace Elements using ICPMS (Inductively Coupled Plasma Mass Spectrometry): The ICP converts elemental atoms into ions, which are detected using a mass spectrometer. Sample was digested with suprapure hydrogen peroxide and nitric acid, then filtered and fed into an ICPMS to determine heavy metal concentration. The digestion was carried out in triplicates and the concentration of heavy metals was calculated using the mass to charge ratio[2].

Isolation of Actinobacteria: The pre-treated soil suspension was subjected for the process of isolation; 1 ml of the stock solution was transferred to 9 ml sterile test tube containing water and mixed vigorously to reach the dilution factor of 10^{-1}. Successive dilutions were carried out from 10^{-2} to 10^{-6}.

After serial dilution, a 0.1ml aliquot sample of each dilution from 10^{-2} to 10^{-6} was spread evenly on the starch casein agar medium at slightly alkaline pH of 7.8 in accordance to the standard solid spread plate technique. The plates were incubated at 30°C for nearly 7 to 14 days until the appearance of actinomycete colonies. After incubation, individual colonies were maintained on the same starch casein agar slants and maintained at 4°C for further use. The cultures were studied for their morphological and biochemical characteristics[18].

Characterization of Isolates: The pure colonies were observed; their morphological characteristics and pigmentation were recorded. Discrete actinomycetes isolates were identified based on morphological, cultural and biochemical characteristics.

Morphological Characterization: To examine their morphological characteristics using the macroscopic approach, the chosen isolates were streaked onto a Starch Casein Agar medium. During the preliminary phase, the colonies, spore and substrate morphology, aerial hyphae, branching and diffusible pigment formation on the plates was studied. To examine the Gram's property of the discrete actinobacterial colonies, Gram staining was performed[20]. Later, the organism was microscopically examined at 10x and 40x magnification and then under 100x with oil immersion objective[4].

Biochemical characterization: Biochemical characterization of actinobacterial isolates is done to study their physiology. Actinomycetes utilize a wide variety of organic substrates specially the simple sugars which are readily utilized without any fermentation resulting the media turning alkaline[21]. The biochemical tests performed throughout this study were IMViC tests, catalase activity, H_2S production, citrate utilization, gelatin liquefaction and carbohydrate utilization.

Results and Discussion

The current study expounds the correlation between soil physicochemical properties and rare, entrapped and uncultured actinomycetes in exploring novel bioactive molecules, as the growth cycle of actinomycetes is similar to that of many other microorganisms in nutrient deficit habitats. The main objective of the study is to evaluate the relationship between the distribution of actinomycetes across varied ecological niches and the impact of vegetation, soil chemical characteristics and effective pretreatment method.

Sampling sites and sample collection: A total of 18 distinct soil samples, collected from sedimentary rock formations at limestone quarry - 1 and limestone quarry - 2, comprising of eight and ten samples respectively, were placed in sterile zipperlock bags and transported to the laboratory where they were stored at room temperature until further use.

Pretreatment of the soil sample: The results of this study offer valuable insights into the impact of pretreatment on the suppression of microbial communities, excluding actinomycetes. Table 1 demonstrates that the treatment with calcium carbonate resulted in a higher number of isolates and effectively reduced the bacterial count on the primary isolation plate. The heat treatment method proved to be effective in eliminating unwanted microorganisms;

however, there were a few limestone-derived actinomycetes present.

In accordance with the present findings, this study can be compared with the work of Nimaichand et al[11], who documented the isolation of the novel actinomycete *Streptomyces manipurensis* from the limestone deposits of Manipur, India, employing the calcium carbonate pre-treatment method, as the biotope of limestone deposits falls within the classification of calcareous soil or alkaline soil forms. Consequently, this elucidates the rationale behind the efficacy of calcium carbonate treatment in the isolation of actinomycetes derived from limestone.

Soil Analysis Report

Macronutrient analysis of soil: Macronutrients are the organic substances also existing as cations governing activity, functionality and stability of molecules and cell structures, often involved with the numerous biological activities, including protein synthesis and energy conservation. The findings of the result revealed distinct properties of soil, primarily, the pH of the soil inclined towards alkaline while limestone quarry-2 (PL) is greatly alkaline (Table 2).

The study findings presented a difference in soil micronutrients across two limestone quarries. The limestone quarry-1 (YL) exhibited the highest available nitrogen, phosphorous, carbon, sulphur and carbon/nitrogen ratio while limestone quarry-2 (PL) showed highest values in pH (alkaline), electric conductivity, potassium, sodium and hydrogen as illustrated in table 2. The electrical conductivity is low in both the soil samples which indicates non - saline soil. The organic carbon in both the samples is in an ideal range.

Nitrogen availability is high in both the samples which may aid the survival ability of the actinobacteria. Total phosphorous available in the soil samples is moderate, YL sample shows higher phosphorus values than PL sample. On contrary, sample PL reported high values of potassium and sodium than sample YL. The C/N ratio is high in both the samples which infers about the slow organic matter decomposition, perhaps tells us about the dormant state of the viable cells in its microenvironment.

El Karkouri et al[5] conducted an investigation into the physical and chemical parameters, encompassing pH, electric conductivity and salinity, derived from the salterns. The research findings indicated that both soil types exhibited alkaline characteristics and that Cfu/ml is directly correlated with the physico-chemical properties of the soil. The pH of the soil was significant to know in order to adjust the growth medium accordingly for the cultivation of lime-stone derived actinobacteria.

Nimaichand et al[11] reported isolation of lime stone adapted actinobacteria on Starch Nitrate Agar medium at a pH adjusted at 8.5. The electrical conductivity and sodium availability made the study to rule out the optimum requirement of sodium chloride, the ratio of C/N revealed the microbial activity and nutrient cycling since the values of organic carbon were high in both the samples, ensuring fertility, structure and moisture retention. The percentage of carbon, nitrogen, sulphur, phosphorous and potassium supported the microbial cells for their physiology and metabolism.

Table 1
Effectiveness of Pre-treatment method for the isolation of limestone-derived actinomycetes.

S.N.	Pre-treatment method	No. of isolates obtained		Media
		YL	PL	
1.	Heat treatment method.	03	07	SCA
2.	Calcium-carbonate treatment method.	09	17	SCA

YL=Limestone quarry-1; PL=Limestone quarry-2 and SCA=Starch Casein Agar

Table 2
Macronutrient analysis of the soil sample from limestone quarries.

S.N.	Sample	pH	EC (dSm^{-1})	OC (%)	Nitrogen (kg/ha)	P_2O_5 (kg/ha)	K_20 (kg/ha)	Na (mg/100g)	N (%)	C (%)	H (%)	S (%)	C/N ratio
1	YL	7.95	0.24	1.14	568.58	29.61	189.39	0.14	0.06	3.09	0.625	0.012	51.884
2	PL	8.26	0.31	1.02	508.73	27.33	264.88	0.39	0.05	2.12	0.668	0.007	40.201
3	ICAR Norms of Healthy Soil	6.0 – 7.5	< 1.0	0.75 – 1.5	> 280	22-56	108 – 280	< 0.3	~0.1 – 0.3	~1.5 – 3	Not Significant	0.01 – 0.05	10-12

YL=Limestone quarry-1; PL=Limestone quarry-2; EC=Electric conductivity; OC=Organic carbon; P_2O_5=Phosphorus; K_2O=Potassium; Na=Sodium; N=Nitrogen C=Carbon; H=Hydrogen and S=Sulphur

Elemental Analysis report: The precise relevance of micro-elements existing in the soil, inhabiting actinomycetes is less understood. Actinomycetes encounter nutrients in the soil in their existence which can switch on cryptic gene clusters through the expression of cofactors required to induce the unique bio-chemical pathways for the synthesis of secondary metabolites. It also serves as an elicitor for the actinomycetes to adapt and to evolve. Table 3 illustrates the element finding from the soil sample of different limestone quarries.

The findings indicate that the soils of YL contain elevated levels of calcium and cobalt whereas silver is present in minimal quantities, followed by bismuth. In contrast, the soils of PL demonstrate higher concentrations of copper, magnesium, sodium, zinc and arsenic, with cobalt being the least abundant element, with respect to the soils of YL (Table 3). Furthermore, Gitari et al[6] emphasized the significance of micro-elemental analysis across diverse land systems prior to the isolation of actinomycetes. The study concluded that abiotic factors such as nutrient availability, pH, temperature and light exposure are crucial in influencing pigment production by actinomycetes.

This study investigates the relationship between soil physico-chemical properties and the emergence of rare and uncultured actinomycetes, aiming to uncover novel bioactive molecules, given that the growth cycle of actinomycetes parallels that of various other microorganisms in nutrient-deficient habitats[25]. The majority of studies on soils have concentrated on how macronutrients (such as potassium, phosphorus and nitrogen) and soil pH affect the composition of soil microbial communities.

Table 3
Elemental analysis of the soil sample from limestone quarries. YL=Limestone quarry-1 and PL=Limestone quarry-2

S.N.	Elements	mg/kg	
		YL	PL
01	Bismuth	1.18	1.25
02	Calcium	36443.07	27022.12
03	Cobalt	13.14	10.40
04	Copper	43.96	82.91
05	Iron	1716.45	1839.63
06	Magnesium	1908.57	3182.53
07	Manganese	5855.31	5879.16
08	Sodium	792.06	950.76
09	Silver	0.51	1.86
10	Zinc	406.83	820.37
11	Arsenic	8.87	12.74
12	Nickel	35.67	38.64

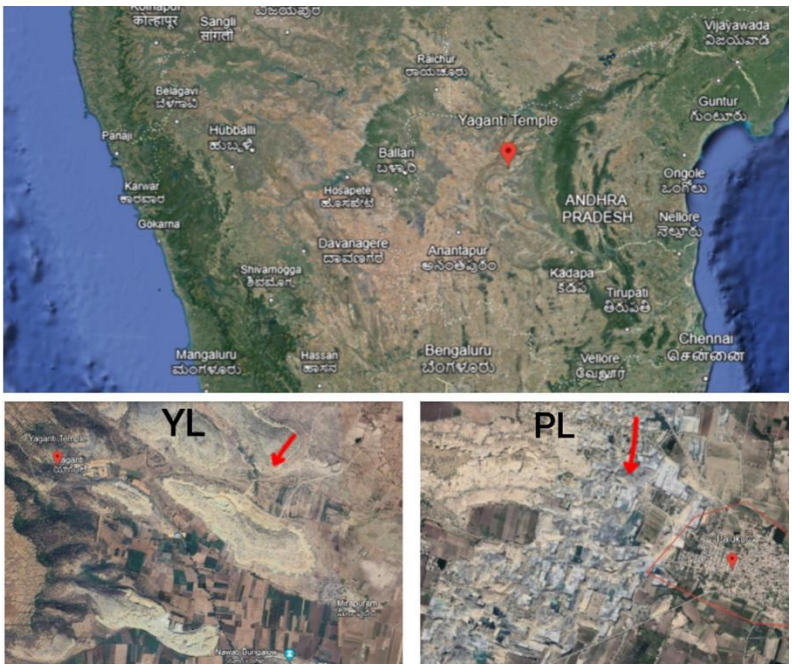

Fig. 1: Geological map depicting the locations of limestone quarries sampling sites of southern India. YL=Limestone quarry-1 and PL=Limestone quarry-2

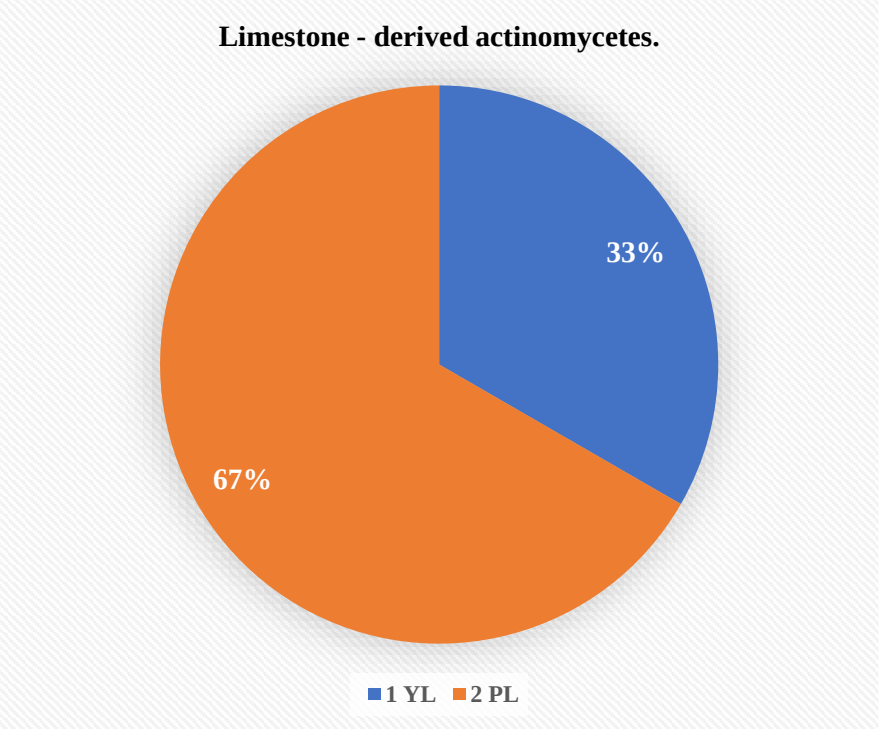

Fig. 2: Comparison of results between YL=Limestone quarry-1 and PL=Limestone quarry-2 concerning the isolates obtained

On the other hand, little is known about how soil metal micronutrients such as Fe, Cu, Mn and Zn support vital biological processes and how they affect the activity and composition of soil microorganisms. Despite having concentrations as low as milligrams per kilogram or less, micronutrients are essential to microorganisms and are crucial for redox homeostasis and cell proliferation. In typical biochemical processes, such as the synthesis of proteins and biomolecules, they function as enzyme cofactors and participate in the formation of enzyme-substrate complexes. This suggests that micronutrients, which were previously overlooked, may have a major impact on the microbial communities[13].

Isolation of Actinomycetes: On observing all the necessary factors required for the cultivation of actinomycetes from the underexplored ecological niche, a total of 37 different isolates were obtained from the soil samples of limestone quarries. Limestone quarry-1 and quarry-2 yielded 12 isolates and 25 isolates respectively (Fig. 2). There was a somewhat favorable and significant correlation between the actinomycetes population and soil pH, since soil pH is directly proportional to that of amount of sodium, calcium and magnesium present.

This research examines the findings of Nimaichand et al[11] who successfully isolated *Streptomyces manipurensis* from limestone deposits in Manipur, India, employing a calcium carbonate pretreatment method. The biotope of limestone deposits is categorized under calcareous or alkaline soil types, underscoring the effectiveness of calcium carbonate treatment in isolating actinomycetes sourced from limestone. Similarly, Li et al[8] reported a novel *Streptomyces canchipurensis* from the same limestone deposits of Manipur. El Karkouri et al[5] studied abiotic factors and physico-chemical parameters of soil, encompassing pH, electric conductivity and salinity, sourced from salterns.

Nimaichand et al[11] documented the isolation of limestone-adapted actinobacteria using starch nitrate agar medium, with the pH meticulously adjusted to 8.5. One of the most widely reported pharmaceutical applications of actinobacterial isolates producing antimicrobial agents was proven to be novel by Quadri and Agsar[15] where six novel genera belonged to the lime stone origin of the deccan traps of India which showed antagonistic activity against many microorganisms. Nimaichand et al[11] isolated a novel actinomycete, *Streptomyces manipurensis* from a limestone quarry of Hundung, Manipur, India.

Characterization of actinobacterial isolates

Morphological characterization of actinomycetes isolates: A total of 37 isolates of limestone origin, were analyzed for macroscopic characteristics on Starch Casein Agar (SCA) and Tryptone Glucose Beef Extract Agar (TGB). All isolates exhibited Gram-positive characteristics. Observations were conducted on colony morphology, the color of aerial and substrate mycelium and the production of diffusible pigments. The actinomycetes isolates exhibited varied morphology and color of the isolates, that ranged from dark grey, light grey, white, light brown and creamish white. The diffused pigments in the starch casein agar media observed were light brown, dark brown, pale pink and yellow.

Quadri et al[16] documented the cultural characteristics of *Nonomuraea indica*, a novel actinomycete isolated from a limestone open pit mine. The research indicated that the aerial mycelium exhibited a light pink hue whereas the substrate mycelium displayed extensive branching without any pigment diffusion.

Biochemical characteristics: From the findings of biochemical attributes of the isolates, it can be concluded that the physiology of these actinomycetes is unique from one another, also none of the isolate was positive for gas production in the carbohydrate fermentation tests. From the findings, most of the isolates showed negative results for the biochemical tests performed except for few nominal positive results for citrate utilization, nitrate reduction and catalase test. Therefore, provided the results and with reference to Bergey's manual, the genus identification perhaps can be made.

Das et al[3] highlighted the importance of the physiological properties which further substantiate the genus and to a certain degree, the species, predicated on the utilization of particular sugars and amino acids.

Conclusion

The aim of the present study was to examine the suitable pre-treatment method to tap the hibernating/dormant actinobacteria. The choice of pretreatment method substantiated with that of the properties of soil at micro level, revealing it to be an organic rich soil with pH slightly alkaline, indirectly agreeing the point of actinobacterial sustenance and exclusive isolation of its cells. The soils of YL contain elevated levels of calcium and cobalt, while silver is present in minimal quantities, followed by bismuth.

In contrast, the soils of PL demonstrate higher concentrations of copper, magnesium, sodium, zinc and arsenic, with cobalt being the least abundant element. Gitari et al[6] emphasized the significance of micro-elemental analysis across diverse land systems prior to the isolation of actinomycetes, concluding that abiotic factors such as nutrient availability, pH, temperature and light exposure are crucial in influencing pigment production by actinomycetes.

In order to cultivate rare soil actinomycetes, this study significantly affected the soil's physicochemical qualities and shed light on how to set up physiological conditions, determine the trace element requirements (in the form of inorganic salts) and choose appropriate ISP media. In addition, the correct pretreatment technique and media conditioning for the growth of fastidious actinobacteria had a substantial impact on the approach to isolation procedures used to cultivate the propagules from suspensions of the samples in the lab. While the heat treatment method was a shortcoming, the calcium carbonate pretreatment method helped to eliminate a number of fast-growing and spreading bacteria. Actinomycetes between the slabs of sedimentary rocks may provide a peculiar population of actinomycetes. The characterization of the discrete colonies was the pre-requisite for their identification.

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References

1. Aini N.N. and Sulistyani N., Isolation of Actinomycetes from Sugarcane (*Saccharum officinarum*) Rhizosphere and the Ability to Produce Antibiotic, *Advances in Health Sciences Research*, **18**, 11-16 **(2019)**

2. Avula B., Wang Y.H., Smillie T.J., Duzgoren-Aydin N.S. and Khan I.A., Quantitative Determination of Multiple Elements in Botanicals and Dietary Supplements Using ICP-MS, *J. Agric. Food Chem*., **58**, 8887–8894 **(2010)**

3. Das M., Agsar D. and Shivaveerakumar S., Production of L-glutaminase by *Streptomyces rochei* Detected from the Lime Stone Quarries of Deccan Trap, *J. Pure Appl. Microbiol*., **7(3)**, 2251-2260 **(2013)**

4. Davies J.A., Anderson G.K., Beveridge T.J. and Clark H.C., Chemical mechanism of the Gram stain and synthesis of a new electron-opaque marker for electron microscopy which replaces the iodine mordant of the stain, *J. Bacteriol*., **156**, 837–845 **(1983)**

5. El Karkouri A., Assou S.A. and El Hassouni M., Isolation and screening of actinomycetes producing antimicrobial substances from an extreme Moroccan biotope, *Pan. Afr. Med. J*., **33**, 329 **(2019)**

6. Gitari J.M., Muraya M.M., Onyango B.O. and Maingi J.M., The Influence of Soil's Physicochemical Properties and Land Use Systems on the Abundance of Actinomycetes Populations, *South Asian J. Res. Microbiol.*, **16**, 20–37 **(2023)**

7. Jiang Y., Li Q., Chen X. and Jiang C., Isolation and Cultivation Methods of Actinobacteria, In Actinobacteria - Basics and Biotechnological Applications **(2016)**

8. Li W.J., Nimaichand S., Jiang Z., Liu M.J., Khieu T.N., Kim C.J., Hozzein W.N., Park D.J., Wadaan M.A.M. and Ningthoujam D.S., *Streptomyces Canchipurensis* sp. Nov., Isolated from a Limestone Habitat, *Antonie van Leeuwenhoek*, **106(6)**, 1119–1126 **(2014)**

9. Meenakshi S., Hiremath J., Meenakshi M.H. and Shivaveerakumar S., Actinomycetes: Isolation, Cultivation and its Active Biomolecules, *J. Pure Appl. Microbiol.*, **18**, 118–143 **(2024)**

10. Ndabankulu K., Egbewale S.O., Tsvuura Z. and Magadlela A., Soil Microbes and Associated Extracellular Enzymes Largely

Impact Nutrient Bioavailability in Acidic and Nutrient Poor Grassland Ecosystem Soils, *Sci Rep*, **12(1)**, 12601 **(2022)**

11. Nimaichand S. et al, *Streptomyces manipurensis* sp. nov., a novel actinomycete isolated from a limestone deposit site in Manipur, India, *Antonie Van Leeuwenhoek*, **102**, 133–139 **(2012)**

12. Nimaichand S., Devi A.M., Tamreihao K., Ningthoujam D.S. and Li W.J., Actinobacterial diversity in limestone deposit sites in Hundung, Manipur (India) and their antimicrobial activities, *Front. Microbiol*., **6**, 413 **(2015)**

13. Peng Z. et al, The neglected role of micronutrients in redicting soil microbial structure, *NPJ Biofilms Microbi.*, **8**, 103 **(2022)**

14. Pham V.H.T. and Kim J., Cultivation of Unculturable Soil Bacteria, *Trends Biotechnol*, **30(9)**, 475–484 **(2012)**

15. Quadri S. and Agsar D., Antimicrobial attributes of rare actinobacteria detected from limestone quarries, *International Journal of Pharma and Bio Sciences (IJPBS)*, **3**, 137–147 **(2012)**

16. Quadri S.R. et al, *Nonomuraea indica* sp. nov., novel actinomycetes isolated from lime-stone open pit mine, India, *J Antibiot.*, **68**, 491–495 **(2015)**

17. Ramijan K. et al, Stress-induced formation of cell wall-deficient cells in filamentous actinomycetes, *Nat Commun.*, **9**, 5164 **(2018)**

18. Shivaveerakumar S., Madhusudhan D.N. and Dayanand A., Catalytic zone, a novel screening approach for the production of extracellular tyrosinase by *Streptomyces vinaceusdrappus* DSV5, *J. Pure. Appl. Microbiol*., **7(4)**, 2799-2808 **(2013)**

19. Shivaveerakumar S., Madhusudhan D.N., Raghavendra H. and Agsar D., Screening of Streptomyces and Process Optimization for the Production of Tyrosinase, *J Pure Appl Microbiol*., **8(4)**, 3245-3252 **(2014)**

20. Siddharth S., Vittal R.R., Wink J. and Steinert M., Diversity and Bioactive Potential of Actinobacteria from Unexplored Regions of Western Ghats, India, *Microorganisms*, **8**, 225 **(2020)**

21. Skinner C.E., Emmons C.W. and Tsuchiya H.M., Henrici's Molds, yeasts and actinomycetes: a handbook for students of bacteriology, J. Wiley & Sons, London **(1947)**

22. Subramani R. and Aalbersberg W., Culturable rare Actinomycetes: diversity, isolation and marine natural product discovery, *Appl Microbiol Biotechnol*., **97**, 9291–9321 **(2013)**

23. Sunitha V., Reddy B.M. and Reddy M.R., Mineral Resources of Cuddapah Basin **(2014)**

24. Tyc O., Song C., Dickschat J.S., Vos M. and Garbeva P., The Ecological Role of Volatile and Soluble Secondary Metabolites Produced by Soil Bacteria, *Trends Microbiol*, **25(4)**, 280–292 **(2017)**

25. Waksman S.A. and Curtis R.E., The Actinomyces of the Soil, *Soil Sci.*, **1(2)**, 99–134 **(1916)**

26. Walkley A. and Black I.A., An Examination of the Degtjareff Method for Determining Soil Organic Matter and a Proposed Modification of the Chromic Acid Titration Method, *Soil Sci.*, **37(1)**, 29-38 **(1934)**.

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