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ANTIMICROBIAL ATTRIBUTES OF RARE ACTINOBACTERIA DETECTED FROM LIMESTONE QUARRIES

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ABSTRACT

Rare actinobacterial isolates belonging to the genera *Micromonospora, Nonomuraea, Kribbella, Lechevalieria, Saccharotherix* and commonly occurring *Streptomyces* were isolated from Limestone quarry - a unique and geographical area and harsh habitat. pH – 9.0, temperature – 45 °C and sodium chloride concentration – 3% were found to be optimum for the maximum growth and physiological ability of the test isolates indicating them as thermo-alkaliphilic in nature. It was found that, the test isolate DRQ 10 belonging to the genus *Streptomyces* and DRQ 72 belonging to the genus *Micromonospora* were very effective showing maximum zone of inhibition against the bacterial pathogen *B. subtilis* (37 mm and 35 mm) and fungal pathogen *Fusarium solani* (33 mm and 31 mm) respectively. The similar test isolates exhibited their higher efficacy with the MIC value of 2 µg/ml and 4 µg/ml for *B. subtilis* and 4 µg/ml and 8 µg/ml for *C. albicans*, and also showed more potency then the commercially available antibacterial drug, Ampicillin and antifungal drug, Nystatin.

KEYWORDS; Limestone Quarry, Soil sample, Rare actinobacteria, Antibacterial activity, Antifungal activity, Minimum Inhibitory Concentration

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INTRODUCTION

The Actinobacteria are Gram-positive organisms with high G+C composition. It is an important class within Bacterial domain with 56 families and over 280 genera. They can be found in a variety of habitats and are particularly abundant in soil and aquatic bodies[1]. These are filamentous, branching bacteria with a fungal type of morphology among which they were originally classified under the older name actinomycetes. *Streptomyces* is a familiar name to all microbiologists, but few will recognize actinobacteria, the group to which it belongs. Identified from 1870s onwards, these organisms confused their discoverers by sharing characteristics of both bacteria and fungi[2].

Different type of rocks like Cinder, China clay, Coquina, Blue rock, Granite, Grit stone, Gypsum a mineral, Limestone, Marble, Sandstone, and Slate occur naturally in the Deccan Plateau. Limestone is one of the chief available sedimentary rocks of the Deccan Traps and makes up about 10% of the total volume of all sedimentary rocks. Limestone is a sedimentary rock composed largely of the minerals calcite and aragonite, which are different crystal forms of calcium carbonate ($CaCO_3$)[3,4]. These are mainly being used as raw materials for the production of cement. Their excavation for the said purpose from the earth leaves behind huge quarries with typical habitat. The harsh climatic conditions in the lime stone quarries supposed to be a good niche for detection, which includes isolation and screening of potential isolates as well as novel bioactive molecules[5].

The screening of microbial natural products, especially antibiotics, continues to represent an important route to the discovery of novel bioactive molecules, for development of new therapeutic agents and for evaluation of the potential of established as well as new bacterial taxa[6]. Antibiotics have been used in many fields including agriculture, veterinary and pharmaceutical industries. Actinobacteria have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic, and several enzymes. Of these compounds, antibiotics predominate in therapeutic and commercial importance[7,8,9,10,11,12]. It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinobacteria[13]. Indeed, the *Streptomyces* species produce about 75% of commercially and medically useful antibiotics[14]. The search for novel metabolites especially from actinobacteria requires a large number of isolates in order to discover a novel compound of pharmaceutical interest[15]. The aim of this study was to isolate, screen and investigate the antimicrobial activities of the rare actinobacteria obtained from limestone quarries of Shahabad town of Gulbarga district, Karnataka, India.

MATERIALS AND METHODS

Sampling and Isolation

Soil samples were collected[16] from limestone quarries around Gulbarga, Karnataka, India. .Actinobacteria were isolated from the collected soil samples by following serial dilution plate culture technique employing starch casein agar[17] and ISP – 2, 3, 6 and 7 media[18]. The inoculated plates were incubated at 35 °C for 1 - 2 weeks. After the completion of incubation period, typical actinobacterial colonies were picked up from mixed colonies and sub-cultured on fresh medium to obtain pure cultures. The pure cultures were stored at 4 °C.

Identification and Characterization

Actinobacterial colonies were identified and characterized morphologically, biochemically and physiologically following the standard features described in International Streptomyces Project (ISP) [18] and Bergey's Manual of Systematic Bacteriology[19, 20]. Cultural characteristics of pure isolates in various media were recorded after incubation

for 7 to 14 days at 35 °C. Morphological observations were made with a light microscope (Nikon, SE) by using the method of Shirling and Gottlieb[18]. Essential biochemical reactions were carried out following the standard procedures of Gottlieb[21]. Carbon and nitrogen utilization was determined on plates containing ISP basal medium 9 as described by Gottlieb[21].

Antimicrobial Activity

Antimicrobial activities were examined *in vitro* against standard strains of bacterial and fungal pathogens that includes, *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 29998), *Klebsialla pneumonia* (ATCC 15380), *Pseudomonas aeruginosa* (ATCC 25619), *Salmonella typhi* (ATCC 6539),*Staphylococcus aureus* (ATCC 6538), *Aspergillus fumigatus* (ATCC 46645), *Aspergillus niger* (ATCC 16404), *Fusarium solani* (ATCC 36031), *Candida albicans* (ATCC 90028), *Cryptococcus gatti* (ATCC 32609) and *Mucor indicus* (ATCC 4855) obtained from the culture depository unit, Department of Microbiology, Gulbarga University, Gulbarga. Determination of antimicrobial activity of pure actinobacterial cultures was performed by well diffusion method[22]. The test organisms were grown on Muller Hinton medium (bacteria) and Sabouraud Dextrose agar (fungi and yeast).

Wells were bored and the culture filtrate of the selected isolates of actinobacteria were dropped in the wells and kept for incubation at 37 °C for bacterial and 28 °C for fungal strains[23]. The antimicrobial activity was determined by measuring the size of the inhibition zone. The activity from 7 to 15 mm inhibition zone was recorded as low, 16 to 24 mm as medium and more than 25 mm as high activity[24].

Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MIC) of the crude extracts of all the test isolates were evaluated against the previously described bacterial and fungal pathogens by serial dilution technique according to standard methods of Noble and Sykes [25]. Dilutions of the antibiotic were made in nutrient medium containing phenol red indicator. The trays were incubated at 37 °C for 24 hours. The MIC (µg/ml) was taken as the smallest concentration of antibiotic at which the indicator remained red and yellow color indicated acid production caused by growth of the organism. Antibiotic activity against the fungal cultures was determined in tubes by serial dilution method. Tubes containing fungal and yeast pathogens were incubated at 28 °C for 1, 2 or 5 days. The MIC (µg/ml) was taken as the smallest concentration of antibiotic preventing growth of the test organism in the presence of a standard Ampicillin for bacterial and Nystatin for fungal pathogens[26].

RESULTS

Sixty three isolates of actinobacteria were obtained from about 35 soil samples collected from different sampling sites of limestone quarries of Shahabad in Gulbarga. Some among the isolates had dry powdery appearance and some have smooth appearance (Figure 1).

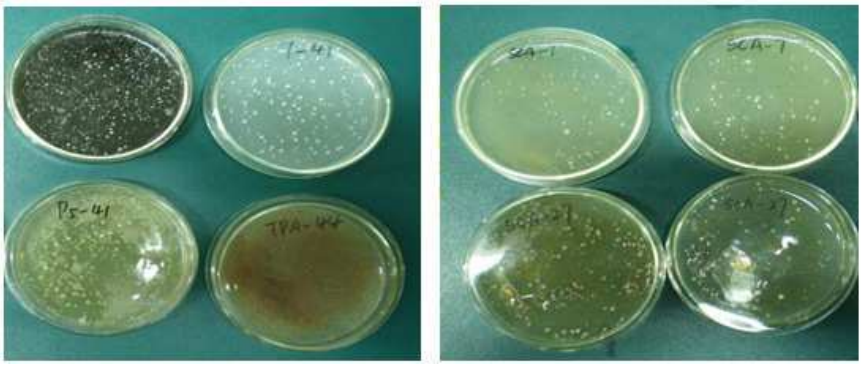


Figure 1
Actinobacteria on different ISP media

A total of six isolates (DRQ - 10, 72, 76, 133, 150 and 201) were identified as members of different genera of actinobacteria namely, *Streptomyces*, *Micromonospora*, *Nonomuraea*, *Kribella, Lechevalieria,* and *Saccharotherix* (Figure 2).

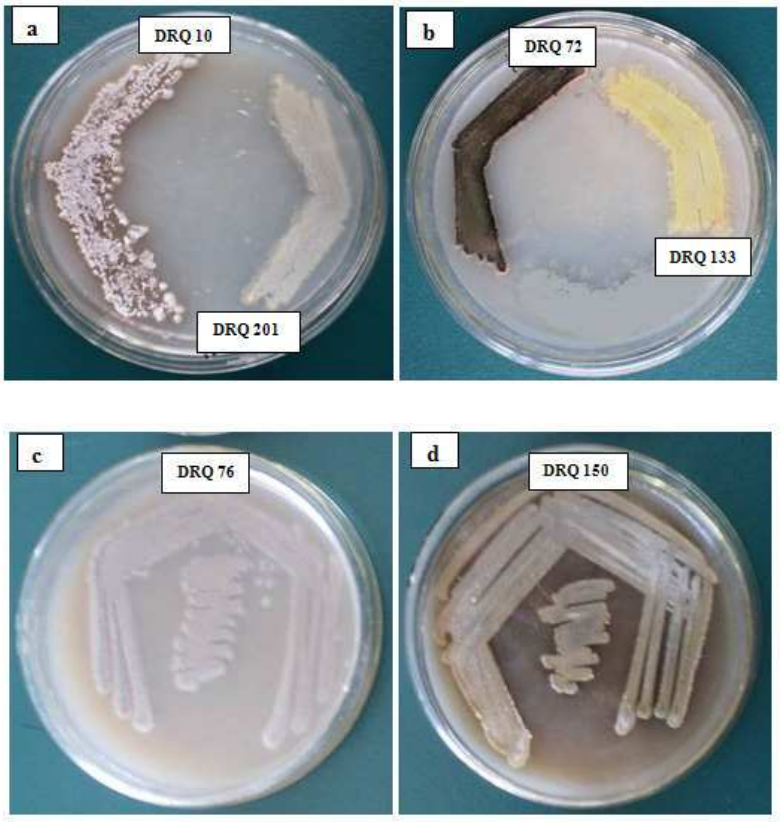


Figure 2
Selected Isolates of actinobacteria grown on Oatmeal agar (ISP3 medium)

a: DRQ 10: Streptomyces sp. & DRQ 201: Saccharotherix sp.
b: DRQ 72: Micromonospora sp. & DRQ 133: Kribbella sp.
c: DRQ 76: Nonomuraea sp.
d: DRQ 150: Lechevalieria sp.

The color of the substrate and aerial mycelia were varied. DRQ 10 and DRQ 72 produced diffusible pigments on different ISP media. Diffused brownish pigment was produced on peptone-yeast extract agar (ISP 6) and tyrosine agar (ISP 7) by DRQ 10 and DRQ 72 (Table 1).

Table 1
Colony characters of the selected isolates of actinobacteria

Isolate	Aerial mycelium	Substrate mycelium	Microscopic features	Soluble pigment
DRQ 10	Greyish White	Greyish Brown	Gram positive Flexuous Smooth	Greyish Brown
DRQ 72	Not Determined	Light Greyish Black	Gram positive Straight Rough	Brownish
DRQ 76	Light Pink	Reddish Pink	Gram positive Hooked/Curled Smooth	No Pigmentation
DRQ 133	Light Yellow	Yellow	Gram positive Elongated Rods Smooth	No Pigmentation
DRQ 150	White/ Light Yellow	Yellowish Orange	Gram positive Elongated Rods Smooth	No Pigmentation
DRQ 201	Yellowish White	Beige/Olive	Gram positive Rod shaped Smooth	Greyish Brown

DRQ 10: Streptomyces sp., DRQ 72: Micromonospora sp., DRQ 76: Nonomuraea sp., DRQ 133: Kribbella sp., DRQ 150: Lechevalieria sp., DRQ 201: Saccharotherix sp.

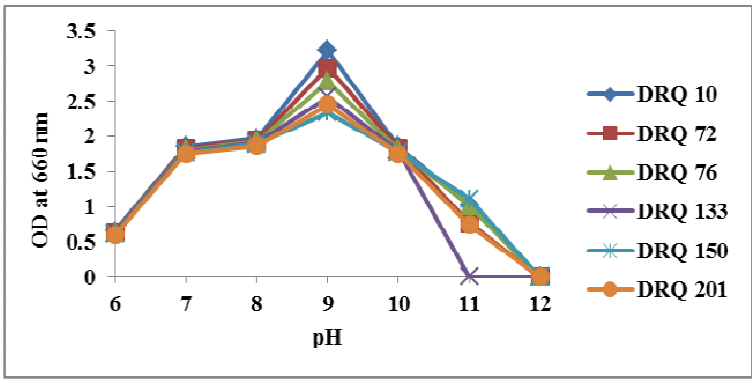
The important biochemical tests of the confirmed isolates of actinobacteria are presented in Table 2.

Table 2
Biochemical properties of the selected isolates of actinobacteria

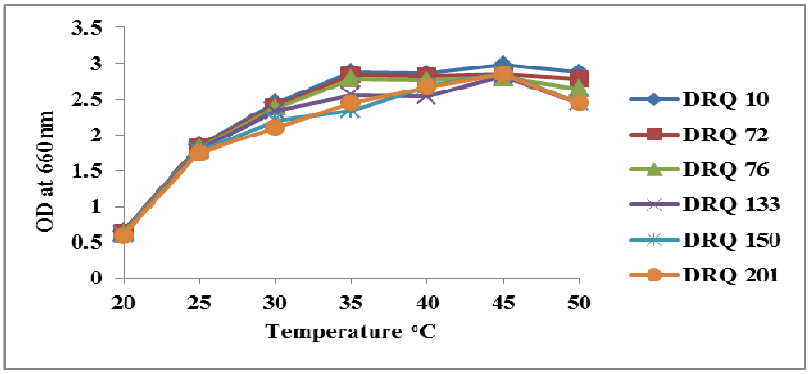
Properties	Reduction of	Hydrolysis of			Production of	
Isolates	Nitrate	Starch	Casein	Gelatin	H_2S	Catalase
DRQ 10	+	+	+	+	-	+
DRQ 72	+	+	+	-	+	-
DRQ 76	+	+	+	+	-	-
DRQ 133	-	+	+	-	+	+
DRQ 150	+	+	+	-	-	+
DRQ 201	+	-	+	+	-	+

DRQ 10: Streptomyces sp., DRQ 72: Micromonospora sp., DRQ 76: Nonomuraea sp., DRQ 133: Kribbella sp., DRQ 150: Lechevalieria sp., DRQ 201: Saccharotherix sp.
+: positive, -: negative

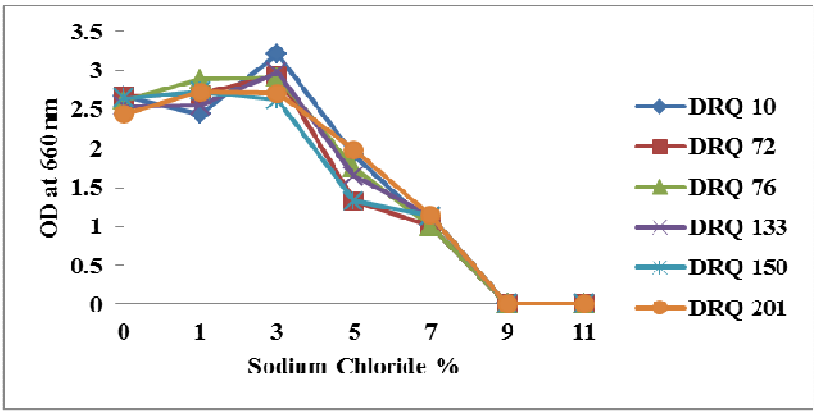
Effect of pH, temperature and salt concentration on the growth and physiological ability of the selected isolates were assessed at different range. All the six isolates grew optimally in alkaline pH - 9.0 (Graph 1), temperature - 45 °C (Graph 2), and at sodium chloride concentration of 3.0 % (Graph 3).



Graph 1
Effect of pH on the growth of isolates of actinobacteria

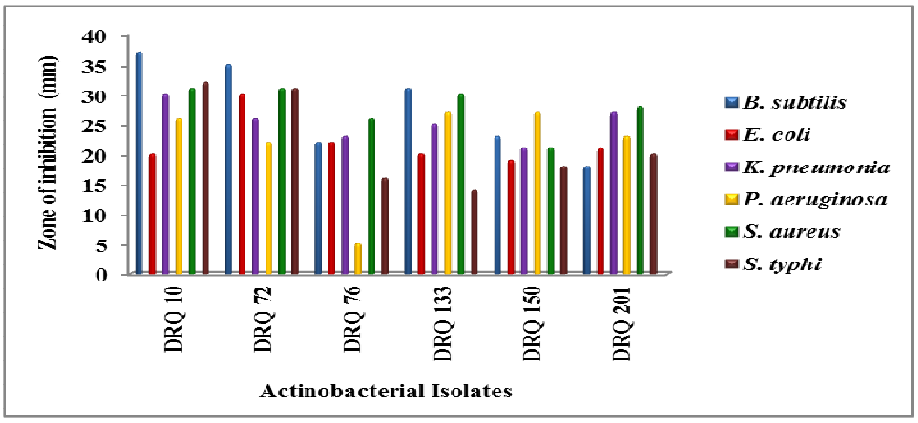


Graph 2
Effect of temperature on the growth of isolates of actinobacteria

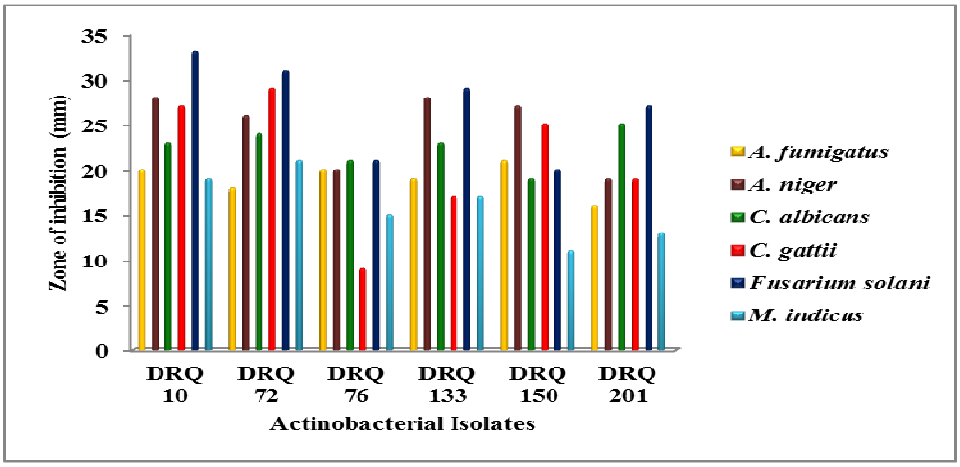


Graph 3
Effect of salt concentration on the growth of isolates of actinobacteria

The antimicrobial activity of the test isolates were found to be varied. Six out of sixty three actinobacterial isolates have shown very potent *in vitro* antimicrobial activity against the standard strains of the pathogenic microorganisms. The results of the antibacterial activity and antifungal activity of active isolates are presented in Graph 4 and 5, respectively.

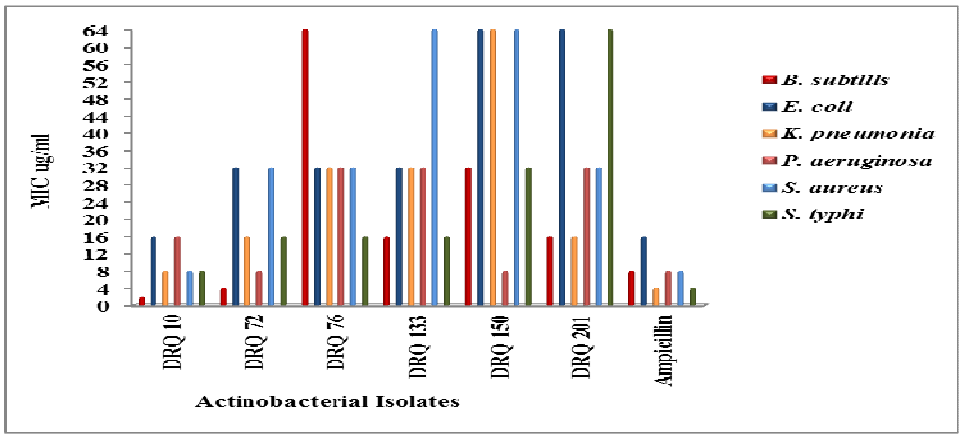


Graph 4
Antibacterial potentials of selected isolates of the actinobacteria



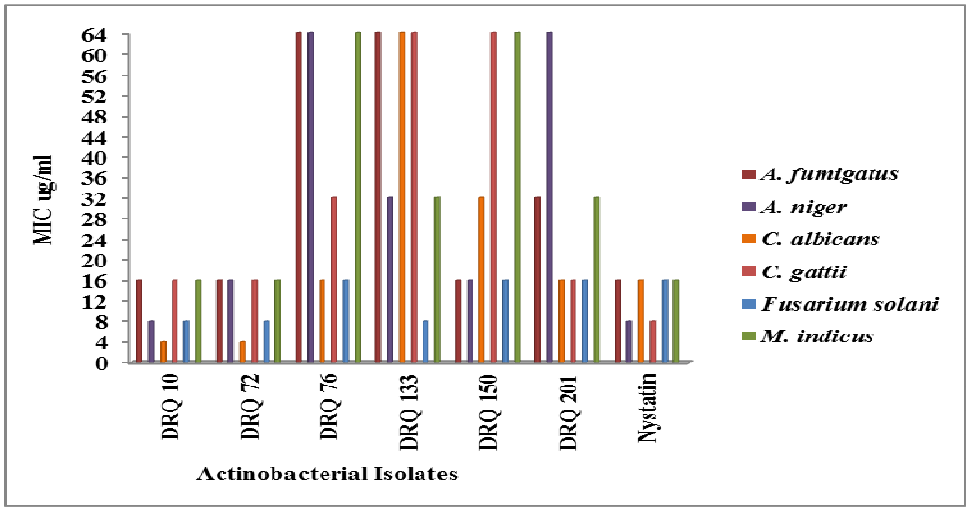
Graph 5
Antifungal potential of selected isolates of the actinobacteria

The minimum inhibitory concentrations of the culture extracts are shown in the Graph 6 for bacterial pathogens and Graph 7 for fungal pathogens.



Graph 6

MIC of extracts obtained from test isolates of actinobacteria against bacterial pathogens



Graph 7

MIC of extracts obtained from test isolates of actinobacteria against fungal pathogens

DISCUSSION

Actinobacteria are diverse and ubiquitous in nature and because of their diversity they are the most explored group among microorganisms, not only for their bioactivities but for their importance in medical and biotechnological applications. The most commonly occurring actinobacteria is Streptomyces, which is being extensively studied for all its capacities (physical, chemical, genetics etc) but emphasis is now shifting towards the rare occurring actinobacteria for different/ novel bioactivities. In the present investigation, an attempt was made to explore the potentials of rare actinobacteria specifically for their antagonism towards bacterial and fungal pathogens. A total of sixty three isolates obtained from the limestone quarry soil samples were subjected for antimicrobial potentials. Although there are several reports of rare actinobacteria isolated from different habitats like forests, mountains[27], desert[28], fresh water sites[29], marine system[30] and alkali/soda lakes[31], it is the first report on

detection of rare actinobacteria and their potentials from the limestone quarry soils.

The discovery of new classes of antibiotics is necessary due to the increased incidence of multi drug resistance among pathogenic microorganisms to drugs that are currently in clinical use[32]. There are of course many reports of antimicrobial activities of actinobacteria and rare actinobacteria, but, the observations recorded in the present investigation are highly diverse. DRQ 10 and DRQ 72 test isolates have shown the maximum antimicrobial activity against *B. subtilis* (37 mm and 35 mm) and also against *Fusarium solani* (33 mm and 31 mm), when compared to any other recorded literature[33]. However, the antimicrobial activity of DRQ 10 and DRQ 72 test isolates against *S. typhi* (32 mm and 31 mm) and *S. aureus* (31 mm and 31 mm), and also against *C. albicans* (23 mm and 24 mm) and *Fusarium solani* (33 mm and 31 mm), were similar to the observations made by Zakir Sultan *et al*[34]. This may be due to the biogeographical attributes and habitat conditions from where the isolates have been obtained. The more diverse is the habitat, the more chance of getting potential isolates and bioactive molecules. Furthermore, different soils have different types of microorganisms which produce different type of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including actinobacteria. However, adaptation to the harsh geographical conditions prevalent in limestone quarries might have in turn lead the actinobacteria to produce their own potential secondary metabolites which are more effective than those produced by other group of microorganisms.

The Minimum Inhibitory Concentration (MIC) is very important and critical in determining the concentration of a biomolecule at which a pathogen can be totally inhibited. It also exhibits the specificity of concentration to a particular pathogen[35]. It helps in choosing the right concentration against a pathogen for treatment. The experimental results of the test isolates DRQ 10 and DRQ 72 has shown a promising level of inhibition to the bacterial pathogens *B. subtilis* (2 µg/ml and 4 µg/ml), and in case of fungal pathogens *C. albicans* (4 µg/ml and 4 µg/ml). The isolates DRQ 10 and DRQ 72 were very effective in comparison to previously reported data[34, 36]. It is noteworthy that, the MIC values of the test isolates DRQ 10 and DRQ 72 have shown lower concentrations for the complete inhibition of the pathogens when compared to the standard and commercially available antibiotics like Ampicillin and Nystatin which were tested along with the pathogens in the investigation.

The isolates of rare actinobacteria obtained from the samples of limestone quarry appear to be very prominent with potential antimicrobial features. However, the active biomolecules required to be studied further for their potential prospectives.

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REFERENCES

1. Goodfellow, M. and K.E. Simpson. Ecology of Streptomycetes. Front. Applied Microbiol., 2: 97-125, (1987)
2. Hopwood D. A, An introduction to the actinobacteria. Microbiology Today. 60-62, May 2007, (2007)
3. Dunham, R. J., Classification of carbonate rocks according to depositional textures, in

Ham W.E. (ed.), Classification of carbonate rocks*:* Am. Assoc. Petroleum Geologists Mem, 1: 108-121, (1962)

4. Folk, R.L., Petrology of Sedimentary Rocks. Hemphill Publishing, Austin, Texas, (1974)
5. Syed Raziuddin Quadri and Dayanand Agsar "Detection of melanin producing thermo-alkaliphilic *Streptomyces* from limestone quarries of the Deccan traps" World Journal of Science and Technology. 2, 2. (*In Press*) (2012)
6. Kurtboke, D.I. and H.G. Wildman,. Accessing Australian biodiversity towards an improved detection of actinomycetes-an activity report. Actinomycetes, Vol. 9, (1998)
7. Waksman, S.A.,. The Actinomycetes, Classification, Identification and Description of Genera and Species. Baltimore: The Williams and Wilkins Company. Vol. 2, P.P. 61-292, (1961)
8. Hopwood, D.A.,. Streptomyces in Nature and Medicine. Oxford University Press, New York, USA. (2007)
9. Lachevalier, H.A.,.. The Actinomycetes III, A Practical Guide to Generic Identification of Actinomycetes. Bergey's Manual of Systematic Bacteriology. Williams & Wilkins Company, Baltimore. 2344-2347. (1989)
10. Williams, S.T., M. Goodfellow and G. Alderson,. Genus Streptomyces Waksman and Henrici 1943. In: Bergey's Manual of Systematic Bacteriology, Holt, J.G., S.T. Williams and S.T. Williams (Eds.). Williams and Wilkins Company, Baltimore, USA., pp: 2452-2492. (1989)
11. Ouhdouch, Y., M. Barakate and C. Finance,.. Actinomycetes of Moroccan habitats: isolation and screening for antifungal activities. European Journal of Soil Biology, 37: 69-74. (2001)
12. Saadoun, I. and R. Gharaibeh,. The Streptomyces flora of Badia region of Jordan and its potential as a source of antibiotics active against antibiotic- resistant bacteria. J. Arid Environ, 53: 365 - 371. (2003)
13. Takizawa, M., R.R. Colwell and R.T. Hill,. Isolation and diversity of action. Environ. Microbiol., 59: 997-1002. (1993)
14. Miyadoh, S.,. Research on antibiotic screening in Japan over the last decade: a producing microorganism approach. Actinomycetologica, 7: 100-106. (1993)
15. Oskay, M., U.A. Tamer and C. Azer,.. Antibacterial activity of some Actinomycetes isolated from farming soils of Turkey. Afri. J. Biotechnol, 3 (9): 441-446. (2004)
16. Grant, W.D., W.E. Mwatha and B.E. Jones,. Alkaliphiles: Ecology, diversity and application. FEMS Microbiol. Lett., 75: 255-270, (1990)
17. Cochrane V. W.,. Physiology of Actinomycetes, Annu.Rev. Microbiol*,* 15: 1-24, (1961)
18. Shirling, E.B. and D. Gottlieb,.. Methods for characterization of Streptomyces species. International Journal of Systematic Bacteriology, 16: 313-340, (1966)
19. Bergey, D.H.,. Bergey's Manual of Systematic Bacteriology. Vol. 4. Williams and Wilkins Company, Baltimore, USA:. (1989)
20. Locci, R.,. Streptomyces and Related Genera. In: Bergey's Manual of Systematic Bacteriology, Williams, S.T., M.E. Sharpe and J.G. Holt (Eds.). Williams and Wilkins Co., Baltimore, Maryland, USA., pp: 2451-2508, (1989)
21. Gottlieb D.,. An evaluation of criteria and procedures used in the description and characterization of *Streptomyces*:A cooperative study, Appl.Microbiol*,* 9: 55 – 65, (1961)
22. Egorov, N.,. Microorganisms -antagonists and biological methods for evaluation of antibiotic activity. Moskva: Vissha shkola, 200. (1995)
23. Singh, L.S., B. Indra and T.C. Bora,. Actinomycetes of Loktak Habitat: Isolation and Screening for Antimicrobial Activities, Biotechnology, 5 (2): 217-221, (2006)
24. Denitsa Nedialkova and Mariana Naidenova,. Screening The Antimicrobial Activity Of Actinomycetes Strains Isolated

From Antarctica, Journal Of Culture Collections, 4: 29-35, (2004-2005)

25. Noble, M., D. Noble and R.G.B.B. Sykes,. 7063-2 a new nitrogen containing antibiotic of the apoxydon group isolated from fermentation broth of a species of *Streptomyces*. J. Antibio., 30:415. (1977)

26. Manivasagan, P., S. Gnanam, K. Sivakumar, T. Thangaradjou, S. Vijayalakshmi and T. Balasubramanian,. Antimicrobial and Cytotoxic Activities of an Actinobacteria (Streptomyces Sp. PM-32) Isolated from an offshore Sediments of the Bay of Bengal in Tamilnadu, Advances in Biological Research, 3 (5-6): 231-236, (2009)

27. Xu, L., Q. Li and C. Jiang,. Diversity of soil actinomycetes in Yunnan, China. Appl. Environ. Microbiol. 62:244–248. (1996)

28. Hozzein, W.N., W. Rabie and M.I.A. Ali,.. Screening the Egyptian desert actinomycetes as candidates for new antimicrobial compounds and identification of a new desert Streptomyces strain. Afri. J. Biotechnol., 10: 2295-2301. (2011)

29. Sibanda, T., L.V. Mabinya, N. Mazomba, D.A. Akinpelu, K. Bernard, A.O. Olaniran and A.I. Okoh,. Antibiotic producing potentials of three freshwater actinomycetes isolated from the eastern cape province of South Africa. *Int. J. Mol. Sci.* 11: 2612-2623. (2010)

30. Asha Devi N.K., M. Jeyarani and K. Balakrishnan ,. Isolation and Identification of Marine Actinomycetes and their Potential in Antimicrobial Activity. Pakistan Journal of Biological Sciences, 9: 470-472. (2006)

31. Jones, B.E. and W.D. Grant,. Microbial diversity and ecology of the Soda Lakes of East Africa. In: Proceedings of the 8th International Symposium for Microbial Ecology, August 9-14, 1998, Halifax, Canada, pp: 681-687. (1998)

32. Burgess, J.G., E.M. Jordan, M. Bregu, A. Mearns-Spragg and K.G. Boyd,. Microbial antagonism, a neglected avenue of natural products research. J. Biotechnol., 70: 27–32. (1999)

33. Ghadin, N., N.M. Zin, V. Sabaratnam, N. Badya, D.F. Basri, H.H. Lian and N.M. Sidik,.. Isolation and Characterization of a Novel Endophytic Streptomyces SUK 06 with Antimicrobial Activity from Malaysian Plant. Asian Journal of Plant Sciences, 7: 189-194. (2008)

34. Sultan, M.Z., N.A. Khatune, Z.S. Sathi, S.A.M.D. Bhuiyan and G.M. Sadik *et al*.,.. In vitro Antibacterial Activity of an Active Metabolite Isolated from Streptomyces Species. Biotechnology, 1: 100-106. (2002)

35. Senekal, M.,. Importance of minimum inhibitory concentration (MIC) values. Continuing Med. Edu., Vol. 28. (2010)

36. Abdul Alim Al-Bari, M., M. Abu Sayeed, A. Khan , M.R. Islam , P. Khondokar , M.M.S. Rahman and M.A. Ul Islam,. In vitro antimicrobial activities and cytotoxicity of ethyl acetate extract from streptomyces maritimus. Biotechnology, 6: 81-85. (2007)