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Isolation, Screening and Characterization of Bacteria Having Antibacterial Activity from Industrial Waste Effluent

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Abstract

Background: The aim of the present study was to isolate and screen antibiotic producing bacteria from potential industrial effluents samples of industrial areas mainly Abdullah Tannery Pvt. Ltd (Kanpur), Superhouse Leathers Ltd. (Unnao), UPDPL (Lucknow) and MSF Grow (Kanpur), Uttar Pradesh, India.

Methods: Thirty five soil samples were collected from waste effluent sites, serially diluted and spread on nutrient agar, modified agar-streak and agar-plug methods were used for the primary screening. Cross streak method was used to check antagonistic activity of isolated bacteria against test organisms. Agar well diffusion was used for antimicrobial activity of isolated bacteria against test organisms.

Results: Four isolates (TAAAP010, TAAAP012, TAAAP020 and TAAAP033) have been found to show high antagonistic activity during primary screening. Inhibition zones obtained from agar well diffusion test showed significance differences when compared with standard antibiotics tested against test organisms ($P<0.05$). Inhibition zone of isolate TAAAP010 against *Staphylococcus aureus* MTCC 2940, and *Bacillus subtilis* MTCC 441 were (16±2) mm and (37±1) mm, respectively, which shows greater antimicrobial activity compared to amoxicillin (4 mm). Isolate TAAAP012 showed (22±1) mm and (18±1) mm inhibition zones, respectively. Isolate TAAAP020 has shown inhibition zones of (16±1) mm and (17±1) mm, respectively. However, isolate TAAAP033 has shown high inhibition zones of (28±3) mm and (23±2) mm, respectively.

Conclusion: The research work concluded that the A10 and A33 isolates showed good antibacterial activity against Gram-positive and Gram-negative bacteria. The A10 and A33 isolates were confirmed as *Bacillus subtilis* and *Streptomyces griseus* by 16S rRNA studies.

Keywords: Antimicrobial metabolites, industrial effluent, antagonism, enzymes, soil

1. Introduction

Sewage and industrial wastewater contain high amounts of complex organic matter which is mainly in the form of proteins and lipids [1]. The biomass that reduces the complex content of the sewage is the activated sludge, biological flocs mainly composed of saprotrophic bacteria, and some protozoa and small metazoans [1]. Microorganisms not only take up some small molecules for intracellular metabolism but also enzymatically hydrolyze a large fraction of the organic matter through a series of hydrolytic reactions to smaller units, which can be taken up by the bacterial cell uptake system [2]. Previous research indicated that activated sludge is a good source for studying and discovering proteases which play a vital role in the extracellular catabolism of organic matter in activated sludge [3]. They have diverse functions ranging from catabolism to protein post-translational modification and regulation of biological processes. However, up to 80–90% of the microorganisms detected inactivated sludge by 16S rRNA genes based on molecular taxonomic studies cannot be cultured using standard cultivation techniques, leaving a potentially valuable resource largely unexplored [4].

Today both academic and industrial interest in soil bacteria (due to their several advantages over other microorganisms) is on the rise, in search of deriving these unique biologically active metabolites and novel commercially important products from them. Bacteria are present in diverse ecological habitats. They are considered highly valuable as they are used in fermentation processes, much as brewing, baking, cheese and butter manufacturing, chemical manufacturing such as ethanol, acetone, organic acid, enzymes, perfumes etc., microbial mining and they produce various antibiotics, vaccines, steroids as well as other therapeutically useful compounds with diverse biological activities [5]. Hence there is an immense possibility to screen effective bacterial strains from waste dump sites with valuable applications. To cope up with the demand for new organisms with properties of production of unique molecules for antibacterial properties there have been a constant effort in isolating novel bacteria from diverse environment. Therefore, the objective of the present study was to isolate and screen antibacterial metabolite producing bacteria from industrial waste effluent samples in Kanpur, Unnao and Lucknow. The outcome of this finding may be important to give direction for researchers and for future treatment of multidrug resistant human pathogens.

2. Materials and Methods

2.1 Study area and period

The study area was located at Abdullah Tannery Pvt. Ltd (Kanpur) 26°25'57.5"N 80°23'57.2"E, Superhouse Leathers Ltd. (Unnao) 26°32'30.7"N 80°29'58.9"E , UPDPL (Lucknow) 26°46'11.8"N 80°52'30.8"E and MSF Grow (Kanpur) 26°28'59.0"N 80°20'34.6"E. The study was carried out from September 2014 to August 2016. The details of the samples are given in Table 1.

2.2 Sampling and isolation of bacteria

Samples were collected from industrial waste effluent (Abdullah Tannery Pvt. Ltd (Kanpur), Superhouse Leathers Ltd. (Unnao), UPDPL (Lucknow) and MSF Grow (Kanpur), Uttar Pradesh, India) waste effluent disposal areas. During the study, 35 waste effluent samples were collected aseptically from 4 sites at different depth (5, 8 and 11 cm) of the disposal using standard methods[6]. The collected samples were transferred to research laboratory of microbiology, Department of Biotechnology, RR Institute of Modern Technology, Lucknow and Faculty of Biotechnology, Institute of Biosciences and Technology, Shri Ramswaroop Memorial

University, Barabanki where the entire research work was carried out. The waste effluent samples were sieved through 250 µm pore size sieve. From each sample, 1 ml of effluent sample was then added in different test tubes containing 9 mL physiological saline (NaCl, 8.5 g/L) and shaken well using vortex mixer. These test tubes were considered as stock cultures for different effluent sample sites.

From the stock cultures, a volume of 1 mL was transferred aseptically and added to a test tube containing 9 mL of sterile physiological saline and mixed well. From this test tube, 1 mL of aliquot was again transferred and mixed with another 9 mL of sterile physiological saline to make 10^{-2} dilution factor. Similarly, dilutions up to 10^{-5} were made using serial dilution technique for all waste effluent samples. A volume of 1 mL of suspension from 10^{-4} and 10^{-5} serially diluted tubes were taken and spread evenly with sterile L-shaped glass rod over the surface of sterile nutrient agar plates aseptically using spread plating technique. The plates were incubated aerobically at 37°C for overnight [1 , 7]. The identified colonies were purified by repeated streak plate method [6, 7].

2.3 Microbiological and biochemical characteristic of isolated bacteria

Identification of the isolated bacterial species were done by performing biochemical tests and comparing the results with standard description given in Bergey's Manual of Systematic Bacteriology [8]. Morphological determination was done based on Gram straining results. And further identification was done by biochemical characterization of the isolates. The biochemical tests performed were Catalase Test, Indole Test, Methyl Red Test, VP Test, Urease Test, Starch Hydrolysis Test and Sugar Fermentation Test [2, 9].

2.4 Antagonism of bacterial strains

Each strain was cultured in their respective broth and incubated at 37°C for 24-48 hrs. These isolates were tested for their antagonistic activity by the cross-streaking method at room temperature and at 37° C. The cross-streaking method was performed as described by Gillers & Govan et al. (1969). The strain to be tested was inoculated as a 1.5-cm-wide streak (instead of 1 cm) diametrically across duplicate nutrient agar plates. The plates were incubated overnight at either room temperature or 37° C. A wider streak of the original inoculum was used because the inhibitory zones produced were larger and clearer. The plates were incubated at room temperature or 37° C overnight, and inhibition was recorded where the indicator strains crossed the original inoculum. This procedure was followed until each of the strains had been tested against each other.

2.5 Molecular Characterization using 16S rRNA sequencing

The isolates show good zone of inhibition at minimum concentration was selected for 16S rRNA sequencing studies.

2.6 Isolation of genomic DNA from bacteria

Bacterial genomic DNA was isolated using the genomic DNA isolation Kit, as per the kit instruction below procedure followed. An isolated bacterial colony was picked and suspend in 1 ml of sterile water in a microfuge tube. It was centrifuged for 1 minute at 10,000-12,000 rpm to remove the supernatant. Add 200 µl of to the pellet and incubated at 56°C for 15 minutes. Vortexed at high speed for 10 seconds and the tube were placed in a 100°C in heat block or boiling water bath for 8 minutes. The content vortexes at high speed for 10 seconds and then spinned at 10000 – 12000 rpm for 2 minutes. In result 20 µl of the supernatant was used per 50 µl polymerase chain reactions.

2.7 PCR amplification

The A-10 and A-33 organisms DNA was amplified by PCR using purified genomic DNA as a template. 16S rRNA universal primers gene fragment was amplified using Thermal Cycler. 1 µL of template DNA was added in 20 µL of PCR reaction solution. 27F-AGAGTTTGATCMTGGCTCAG, 1492R-TACGGYTACCTTGTTACGACTT primers were used as primer, and then PCR reaction was performed with below conditions: initial denaturation 94°C for 2 minutes and then 35 amplification cycles at 94°C for 45 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, a final

extension at 72°C for 10 minutes. DNA fragments were amplified to about 400 bp.

2.8 Sequencing and bioinformatics protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis. The sequencing and phylogenetic analysis was performed by Cytogene R & D Pvt. Ltd., Lucknow.

2.9 Data analysis

The collected and recorded data were analyzed using SPSS 16 Version software. The different inhibition zone measurements in triplicate were compared by performing One-way ANOVA ranked with Duncan's multiple range tests with descriptive analysis type on different isolates against different test pathogens. All statistical results with $P<0.05$ were considered to be statistically significant.

3. Results

3.1 Sampling and isolation of bacteria

From a total of 35 waste effluent samples, 30 different bacterial isolates were obtained at different depth of the waste effluents. Of these 30 isolates, only 4 (13.3%) showed antibacterial activity hence these four samples were processed for further analysis (Table 1).

3.2 Screening of isolated bacteria for their antimicrobial activities

Primary screening

As the results of primary screening, four (13.3%) bacterial isolates showed antimicrobial activity against one or more test bacteria. The detailed antibacterial activity of TAAAP010, TAAAP012, TAAAP020 and TAAAP033 has been explained in Table 3.

3.3 Biochemical Characteristics of the isolates from the waste

The biochemical properties of those four bacterial strains marked as TAAAP010, TAAAP012, TAAAP020 and TAAAP033 has been shown in Table 2.

3.4 Antagonism assay

Eight test isolates were used for antagonistic assay. These test organisms includes (*S. aureus*, and *B. subtilis*). Four isolates TAAAP010, TAAAP012, TAAAP020 and TAAAP033 were tested against these two test organisms using cross-streaking method to determine the antagonism among the bacterial strains. An evaluation of the antagonism possessed by the bacterial isolates is described (Table 3). TAAAP010 and (TAAAP033) referred as A10 and A33 isolates showed maximum antagonistic activity against the both two test organism while A12 (TAAAP012) showed against one test organism i.e., *B. subtilis* whereas A20 (TAAAP020) against only one test organism *S. aureus.*

3.5 Polymerase chain reaction and 16S rRNA sequencing

TAAAP010 and (TAAAP033) referred as A10 and A33 isolates having good inhibitory activity on test microorganisms were selected for 16S rRNA sequencing studies. According to the 16S rRNA sequence studies, A10 and A33 isolates were found to be *Bacillus subtilis* and *Streptomyces griseus*, respectively. Antibacterial compound may be bactericidal or bacteriostatic in nature. Since, industrial waste effluent harbors a wide range of microflora; there is a possibility of finding our new species and strains. The phylogenetic tree analysis of the two samples is shown in figure 1 and 2.

Table 1: Study area for sample collection with their grid reference

S. N.	Sample code	Sample collection point	Location	Grid Reference
1	TAAAP010	Abdullah Tannery Pvt. Ltd	Kanpur	26º26'1.968"N, 80 º 24'8.5284"E
2	TAAAP012	Superhouse Leathers Ltd.	Unnao	26 º 26'6.6696"N, 80 º 24'38.9664"E
3	TAAAP020	UPDPL	Lucknow	26 º 45'58.968"N, 80 º 52'38.6364"E
4	TAAAP033	MSF Grow	Kanpur	26 º 28'57.3492"N, 80 º 20'43.2816"E

Table 2: Biochemical characteristics of isolated bacteria

Biochemical Test	Isolates			
	TAAAP010	**TAAAP012**	**TAAAP020**	**TAAAP033**
Catalase Test	+ve	+ve	-ve	+ve
Indole Test	+ve	+ve	-ve	-ve
Methyl Red Test	-ve	+ve	-ve	+ve
VP Test	-ve	-ve	+ve	-ve
Urease Test	-ve	+ve	-ve	+ve
Starch Hydrolysis Test	-ve	+ve	+ve	-ve
Sugar Fermentation Test	Glucose +ve Lactose -ve Maltose -ve	Glucose +ve Mannitol +ve Sucrose +ve	Sucrose +ve Glucose +ve Maltose +ve	Glucose +ve Sucrose +ve Maltose +ve

'+' = Positive; '–'= Negative

Table 3: Comparison of the antagonistic activity of isolates against test organisms during primary screening

Isolate	***Zone of Inhibition (mm)***	
	S. aureus	*B. subtilis*
TAAAP010	15± 1	25± 2
TAAAP012	0	31± 1
TAAAP020	24± 3	0
TAAAP033	19± 2	35± 4

Values are means ±SD.

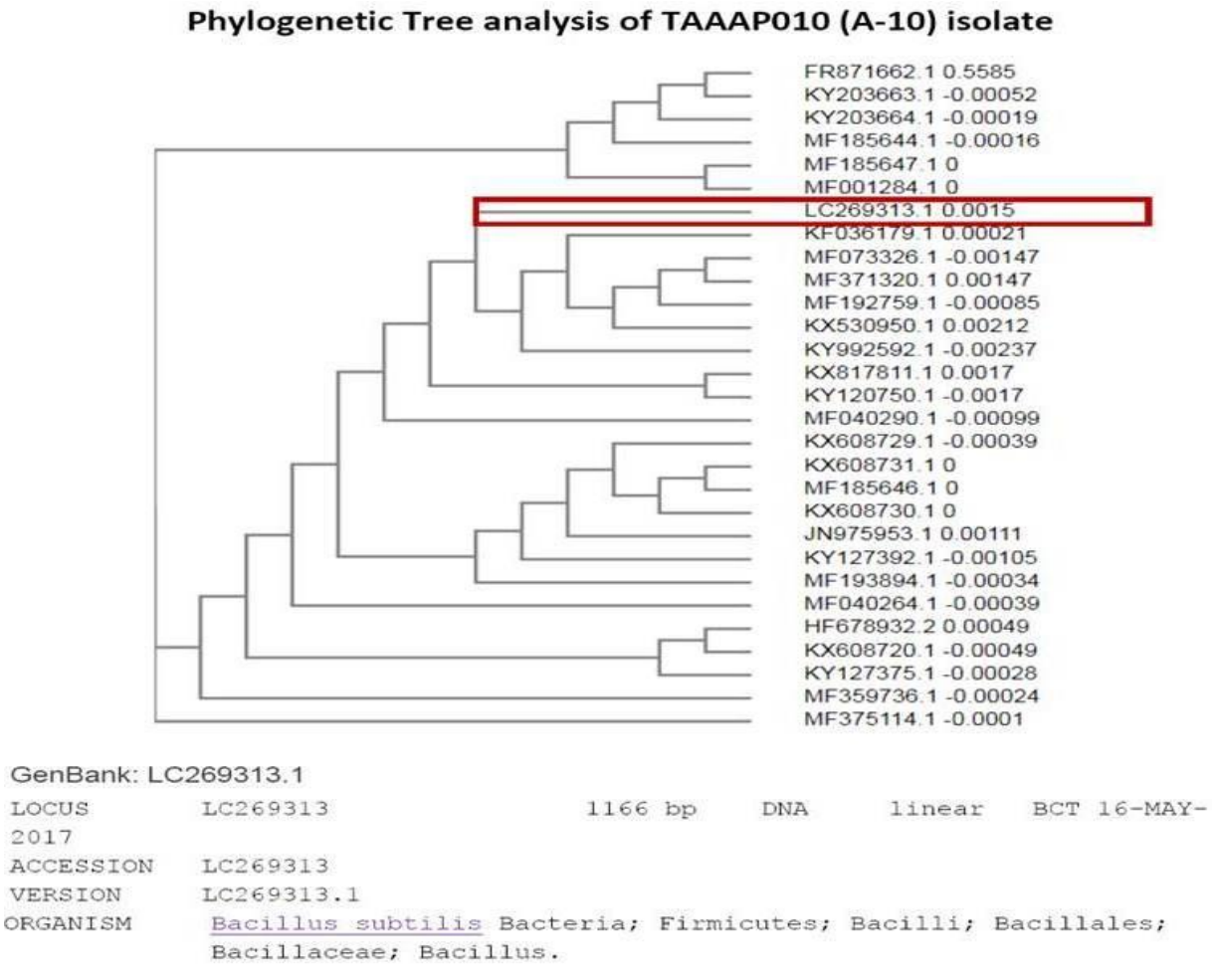

Figure 1. Phylogenetic tree analysis of A-10 isolate

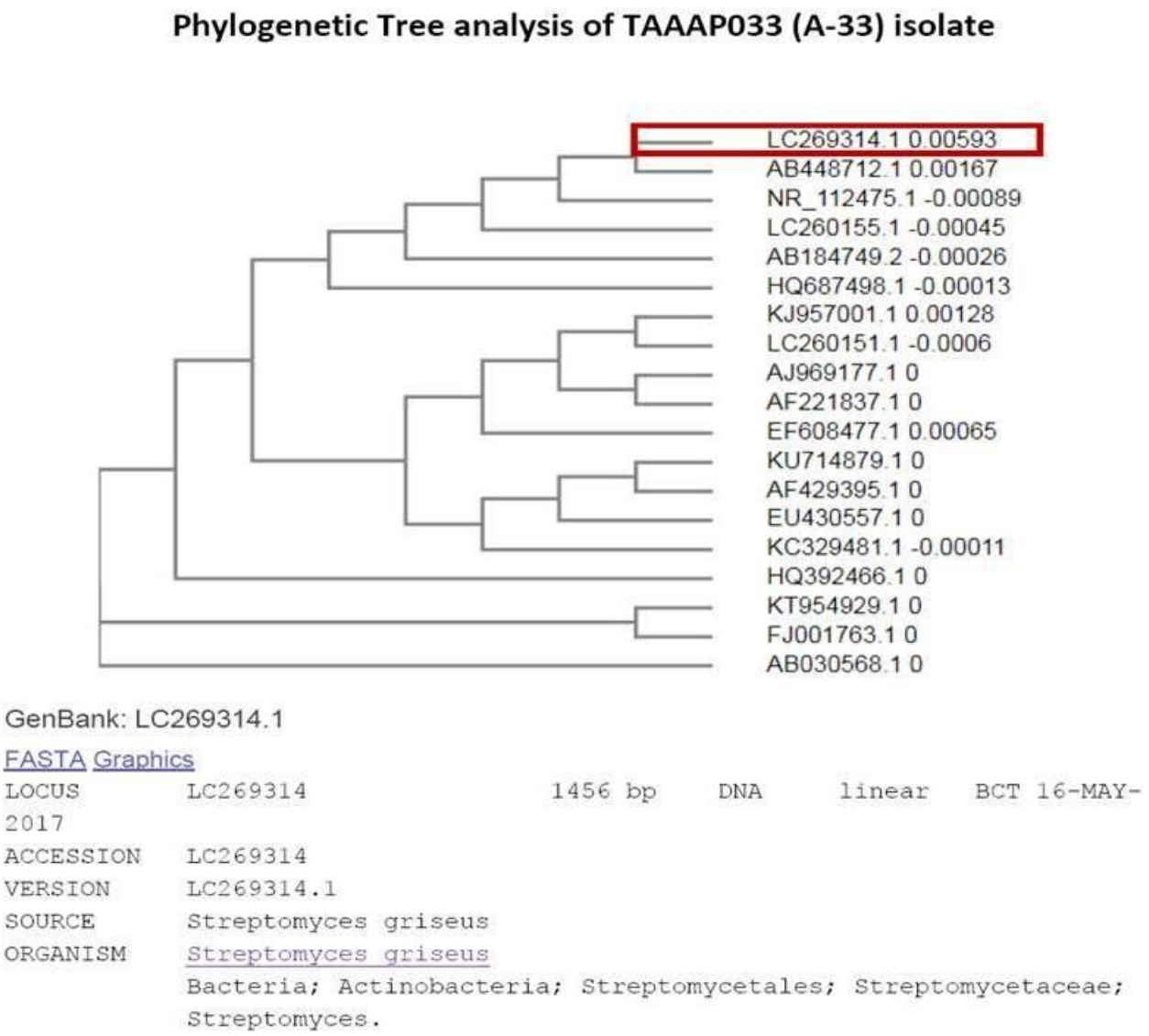

Figure 2. Phylogenetic tree analysis of A-33 isolate.

4. Discussion

Antibiotics are the most important bioactive compounds for the treatment of infectious diseases. But now, because of the emergence of multi-drug resistant pathogens, there are basic challenges for effective treatment of infectious diseases. Thus, due to the burden for high frequency of multidrug resistant pathogens in the world, there has been increasing interest for searching effective antibiotics from soil microorganisms in diversified ecological niches [10].

In the present study, the randomly selected waste effluents samples were taken from industrial areas for isolation of bacteria. The successful isolation of bacteria from environmental samples requires an understanding of the potential waste sample areas and environmental factors affecting their growth. Previous studies showed that selection of different potential areas such as waste effluents samples were an important activity for isolation of different types of potent antibiotic producing soil microorganisms [11].

The present study of primary screening using single streak methods indicated that, four (13.3%) out of 35 bacterial isolates showed potential antimicrobial activity against one or more test bacteria. This result (13.3%) is lower than 21.88% and 59.09% from previous reports [10, 12].

Observation of clear inhibition zones around the wells on the inoculated plates is an indication of antimicrobial activities of antibiotics extracted from actinomycetes against test organisms. Gurung et al. [13] reported 0-18 mm inhibition zone of crude extracts against selected test organisms. From the present study, a range of recorded inhibition zone of bacterial isolates against test organisms were 0-20 mm which were higher than the result reported by Gurung et al [13].

The previous study indicated that, the inhibition zone of bacterial isolates against S. aureus ranged from 0-24 mm [14]. In this study, inhibition zone of from four bacterial isolates against *S. aureus* and *B. subtilis* ranged from 0-35 mm which was found to be good when compared to Yucel and Yemac's results [14]. The results of the present study were interesting and encouraging because the bacterial isolates may have promising antibiotics for treatment of *S. aureus* and *B. subtilis*. According to the present result, amoxicillin had (22 ±2) mm and (18±1) mm inhibition zone against *S. aureus* and *B. subtilis* respectively, which had lower inhibition zone when compared to bacterial isolates. But, it is quite possible that the crude extracts may show good inhibition zones like after purification. Therefore, further purification process is significant to get pure antibiotic substance for the application of treatment of different pathogenic microorganisms. In conclusion the A10 and A33 isolates showed good antibacterial activity against Gram-positive and Gram-negative bacteria. The A10 and A33 isolates were confirmed as *Bacillus subtilis* and *Streptomyces griseus* by 16S rRNA studies.

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Conflict of interest

None.

References

1 Berdy J. Bioactive microbial metabolites. ***J Antibiot (Tokyo)*** 2005; 58(1): 1-26 [PMID: 15813176 DOI: 10.1038/ja.2005.1]

2 Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA, Foster S, Gilmore BF, Hancock RE, Harper D, Henderson IR, Hilpert K, Jones BV, Kadioglu A, Knowles D, Olafsdottir S, Payne D, Projan S, Shaunak S, Silverman J, Thomas CM, Trust TJ, Warn P, Rex JH. Alternatives to antibiotics-a pipeline portfolio review. ***Lancet Infect Dis*** 2016; 16(2): 239-251 [PMID: 26795692 DOI: 10.1016/S1473-3099(15)00466-1]

3 Semple KT, Reid BJ, Fermor TR. Impact of composting strategies on the treatment of soils contaminated with organic pollutants. ***Environ Pollut*** 2001; 112(2): 269-283 [PMID: 11234545]

4 Russell AD, Furr JR. The antibacterial activity of a new chloroxylenol preparation containing ethylenediamine tetraacetic acid. ***J Appl Bacteriol*** 1977; 43(2): 253-260 [PMID: 412828]

5 Demain AL. Small bugs, big business: the economic power of the microbe. ***Biotechnol Adv*** 2000; 18(6): 499-514 [PMID: 14538099]

6 Aghamirian MR, Ghiasian SA. Isolation and characterization of medically important aerobic actinomycetes in soil of iran (2006 - 2007). ***Open Microbiol J*** 2009; 3: 53-57 [PMID: 19440253 PMCID: PMC2681176 DOI: 10.2174/1874285800903010053]

7 Reddy NG, Ramakrishna DPN, and R.G. SV., Morphological, physiological and biochemical studies of marine Streptomyces rochei (MTCC 10109) showing antagonistic activity against human pathogenic microorganisms. ***Asian J Biol Sci.*** , 2011. 4((1)): p. 1-14

8 Ruan J. [Bergey's Manual of Systematic Bacteriology (second edition) Volume 5 and the study of Actinomycetes systematic in China]. Wei Sheng Wu Xue Bao 2013; 53(6): 521-530 [PMID: 24028053]

9 Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A, Tripathi CK. Isolation, Screening, and Identification of Novel Isolates of Actinomycetes from India for Antimicrobial

Applications. ***Front Microbiol*** 2016; 7: 1921 [PMID: 27999566 PMCID: PMC5138215 DOI: 10.3389/fmicb.2016.01921]

10 Abo-Shadi M, Sidkey NM, and A.-M. AM, Antimicrobial agent producing microbes from some soils' rhizosphere. ***J Am Sci***, 2010. 6(10): p. 915-925.

11 Kannan RR and V. SG., Molecular characterization of antagonistic streptomyces isolated from a mangrove swamp. ***Asian J Biotechnol***, 2011. 3(3): p. 237-243.

12 Thakur D, et al., Isolation and screening of Streptomyces in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites. ***J Med Mycol***., 2007. 17: p. 242-249.

13 Gurung TD, et al., Isolation and characterization of antibacterial actinomycetes from soil samples of Kalapatthar, Mount Everest Region. ***Nepal J Sci Technol***, 2009. 10: p. 173-182.

14 Yucel S, Yamac M. Selection of Streptomyces isolates from Turkish karstic caves against antibiotic resistant microorganisms. ***Pak J Pharm Sci*** 2010; 23(1): 1-6 [PMID: 20067859]

