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A Review on Isolation, Identification of Bacillus and Antimicrobial Activity Detection

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ABSTRACT

New antimicrobial compounds are continuously required to combat antibiotic-resistant bacteria and pathogenic yeast as such resistance increasingly limits the effectiveness of current antimicrobial drugs. New screening approaches, including the search for novel targets and exploration of non-conventional places as sources of producer microorganisms, are needed. There been several attempts made to search the antimicrobial substance from the soil and in search of its potential applications in biocontrol of drug-resistant pathogens. The genus Bacillus is one of the predominant bacterial genera found in soil. The antimicrobial potential of the wild-type isolate belonging to the genus Bacillus was determined by the special agar assay. Members of the genus Bacillus are known to produce a wide arsenal of antimicrobial substances. The present paper provides a general overview of antimicrobial activity of bacillus isolated from agricultural soil.

Keywords: Bacillus species; antimicrobial activity, agar-well diffusion, Cross-Streak Method.

INTRODUCTION

Antimicrobial resistance is an important concern for the public health authorities at global level. However, in developing countries like India, recent hospital and some community-based data showed increase in burden of antimicrobial resistance [1]. Due to the increasing numbers of resistant pathogenic bacteria and side effects caused by existing antibiotics, new antimicrobial compounds with effective properties are needed [2]. Antimicrobial peptides are attracting increasing interest as a potential substitute and the best alternatives for biological control [3].

An antimicrobial is an agent that kills microorganisms or stops their growth.[4] Antimicrobial medicines can be grouped according to the microorganisms they act primarily against. For example, antibiotics are used against bacteria, and antifungals are used against fungi. They can also be classified according to their function. Agents that kill microbes are microbicides, while those that merely inhibit their growth are called bacteriostatic agents. The use of antimicrobial medicines to treat infection is known as antimicrobial chemotherapy, while the use of antimicrobial medicines to prevent infection is known as antimicrobial prophylaxis [5].

Bacillus is a genus of Gram-positive, rod-shaped bacteria, a member of the phylum Firmicutes, with 266 named species. The genus *Bacillus* is a phenotypically large, heterogeneous collection of Gram-positive or Gram-variable spore-forming, aerobic or facultative anaerobic bacteria that have undergone considerable reclassification following the advances in molecular biology techniques. *Bacillus* species can be either obligate aerobes: oxygen dependent; or facultative anaerobes: having the ability to continue living in the absence of oxygen. Cultured *Bacillus* species test positive for the enzyme catalase if oxygen has been used or is present [6]. The cell wall of *Bacillus* is a structure on the outside of the cell that forms the second barrier between the bacterium and the environment, and at the same time maintains the rod shape and withstands the pressure generated by the cell's

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turgor. The cell wall is made of teichoic and teichuronic acids. The role of the cytoskeleton in shape generation and maintenance is important [7].

This study aimed to screen for antimicrobial activity of isolates from agricultural soil against some microorganisms. The soil samples for bacteria with antimicrobial production potential found in agricultural fields of outskirts of Chennai, Tamil Nadu. With a tropical climate harboring, the microbial strains that exist in this area with its diversity may provide rare and novel antimicrobe.

MATERIALS AND METHODS

Collection of soil samples

The soil was collected from agriculture lands in Chennai, Tamil Nadu, India. The samples were collected in plastic cans. The collected soil sample was kept for screening and isolation of different microorganisms with antimicrobial potential.

Isolation of microorganisms with antimicrobial potential

Ten grams of soil sample were taken and add 85 ml of distilled water in conical flask and kept for 24 hr. and serial dilutions from 10^{-1} to 10^{-6} were performed. Carefully selected serially diluted samples of 10^{-3} ,10^{-4} ,10^{-5} are transferred to sterile Petri plates using spread plate method.

Screening using Spread plate method

In petri dish, sterile unsolidified Special agar was poured and it was allowed to solidify and kept in laminar air flow. Then, pipette out 0.1 ml from the dilution series 10^{-3} onto the center of the surface of two agar plate. L-shaped glass spreader dipped into alcohol and flamed over a Bunsen burner. Samples were evenly distributed throughout the plates using the sterile glass spreader, while rotating the Petri dish underneath at the same time. Same method was repeated to 10^{-4} ,10^{-5}. Then these 6 special agar plates and a control plate were incubated at 37°C for 24 hours. After incubation, the bacterial colonies were isolated and purified from the plates.

Screening of bacillus isolates in Special agar media

Morphologically distinct bacterial were found and isolated in new special agar medium in petri dish. There were 2 types of morphologically different bacteria were found. Two petri dish was taken and each plate one type of bacteria is inoculated from the culture medium using streak plate method.

Screening of bacillus isolates using Streak plate

Preparing Special agar medium for 50ml. The necessary ingredients added to distilled water are 0.5g of Peptone, 0.5g of Mannitol, 0.1g of phenol red, 0.5g of NaCl and 1g of Agar powder. After autoclaving the medium is poured into two perfidies and kept it in laminar air flow for setting. Yellow and red colonies were taken and using cotton swab or loop a quick swap is taken. Lightly drag the cotton swab or loop across the agar surface in a zigzag pattern. Turn the plate 90 degrees and drag the loop through the area you have just streaked two to three times and

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continue to drag the loop in a "zig-zag" formation in the remaining half of the plate without touching that area again. Repeat these steps again untill the remaining area of the plate is filled with zig-zag formation. Avoid to touch any of the areas you previously streaked. Then 2 agar plates were incubated at 37°C for 24 hours. After incubation, the bacterial colonies were isolated and purified from the plates. The well grown bacterial cultures stored at 4°C.

Antimicrobial Activity Detection

Two different assays, the cross-streak method and the agar-well diffusion methods were used to detect antimicrobial activity. All experiments performed in duplicate. The cross-streak method was performed using MHA (12) agar plates. The agar-well diffusion method was performed using MHA (2) agar plates.

Preparation of MHA medium

Preparing MHA medium for 300ml. For that 150ml of distilled water is taken in a conical flask and- the necessary ingredients are added, 6.3g of Mueller Hinton broth and 8.4g of Agar Agar powder. Mix the ingredients. Add 150ml of distilled water to the flask and close the opening and kept it in autoclave and wait for it to sterilize. After autoclaving the medium is poured into 12 petri dish and kept it in laminar air flow for setting.

The Cross-Streak Method

Cross-streak technique, is a pre-screening method used to determine antimicrobial activity. Cross - streak technique antimicrobial agents used to give quick results. Cross-streak techniques is easy technique that allows for relatively rapid screening of cultures in research for the discovery of new antibiotics. However, the biggest disadvantage of the Cross-streak test is the difficulty in obtain quantitative data. Because the edges of the inhibition zone are usually very fuzzy and unclear. This technique is both a fast and inexpensive method. Multiple isolates or isolates in their studies is a very convenient test method for determining the most active isolates. In this way, cost and duration of multi-isolates it is greatly decreasing.

Two strains of Bacillus were investigated. Originally, the strains were isolated from agricultural soil. The antimicrobial activity was investigated against 12 indicators of pathogenic bacteria and fungi (Table 01).

Table 01: Pathogenic bacteria and fungi that are used as indicators

Indicators	**Growth/maintain media**	**Growth temperature (°C)**
Pseudomonas aeruginosa (PA)	Mueller Hinton Agar	37
Staphylococcus aureus (SA)	Mueller Hinton Agar	37

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Staphylococcus aureus II *(SA-II)*	Mueller Hinton Agar	37
Vancomycin-resistant Enterococcus faecium (VREF)	Mueller Hinton Agar	37
Escherichia coli	Mueller Hinton Agar	37
Candida albicans (CA)	Mueller Hinton Agar	37
Escherichia coli II (EC-II)	Mueller Hinton Agar	37
FRCA 1	Mueller Hinton Agar	37
FRCA 2	Mueller Hinton Agar	37
FRCA 3	Mueller Hinton Agar	37
Enterococcus faecalis (EF)	Mueller Hinton Agar	37
Methicillin resistant staphylococcus aureus (MRSA)	Mueller Hinton Agar	37

The cross-streak method was performed using MHA (12) agar plates on which selected isolates were inoculated as 5-7cm long lines, 0.6cm in width, and incubated at 37°C for 3 days in 5%CO2 atmosphere. The plates were then cross-streaked with microbial inoculate strains, incubated aerobically at 37°C for 24 h. The plates were examined to inhibit growth of microbial inoculum strains around the streak line of selected isolates.

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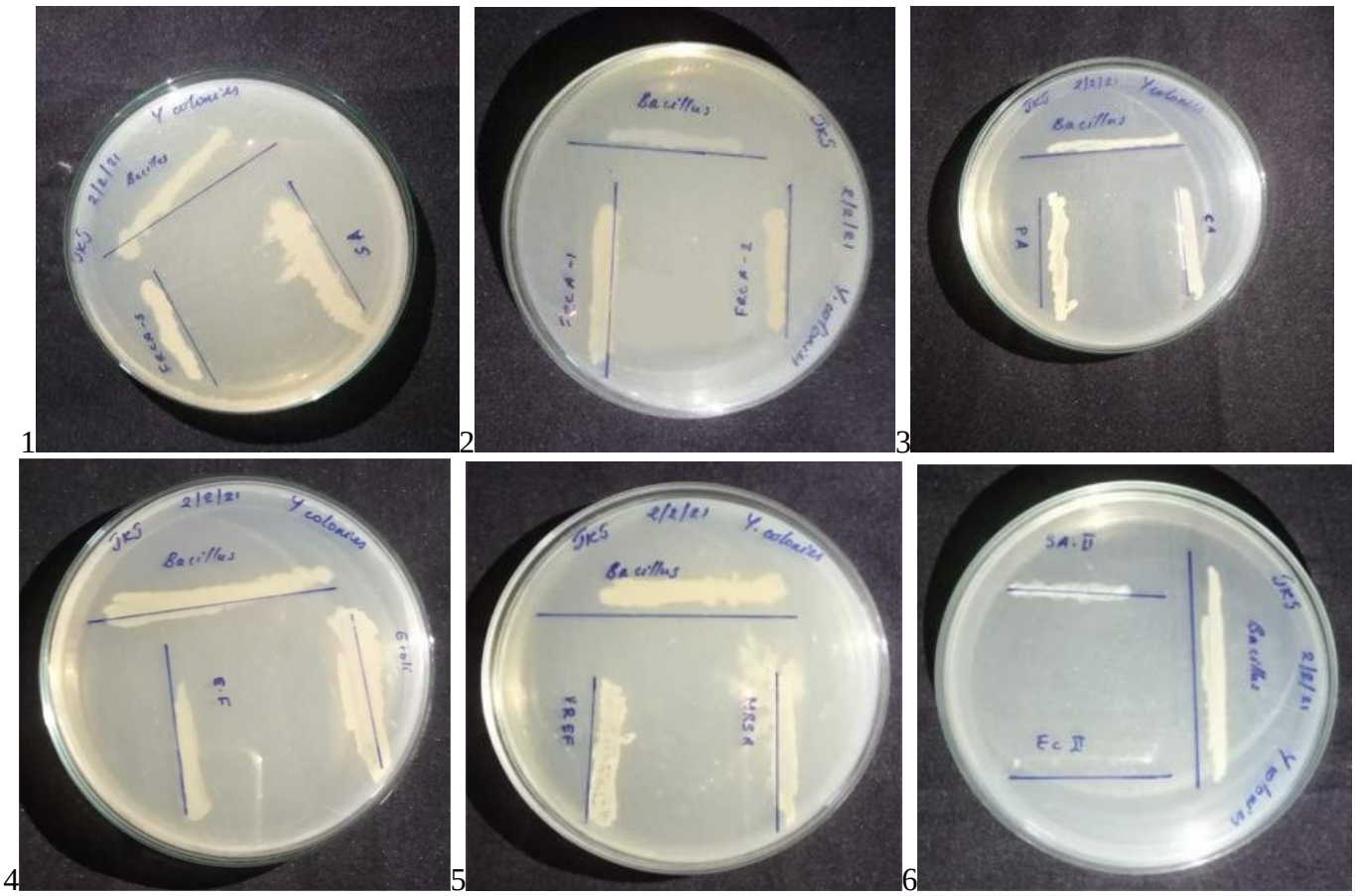

Fig. 1 Yellow colonies – (1) SA & FRCA 3, (2) FRCA 1 & FRCA 2, (3) CA & PA, (4) EF & E coli, (5) VREF & MRSA, (6) SA II & EC II

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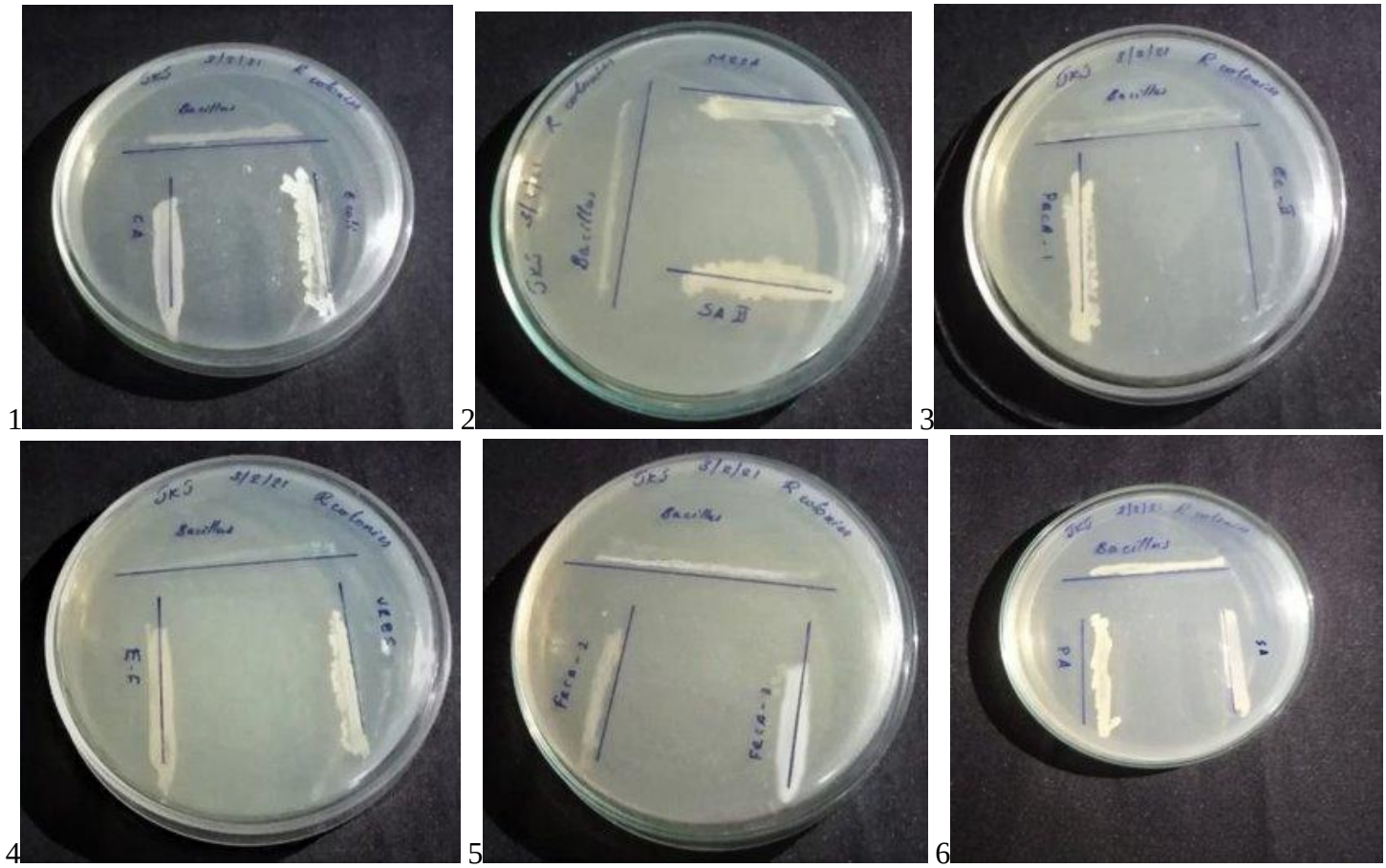

Fig. 2 Red colonies- (1) CA & E coli, (2) MRSA & SA II, (3) EC II & FRCA 1, (4) E. F & VREF, (5) FRCA 2 & FRCA 3, (6) PA & SA

Preparation of culture broth

Preparing culture broth for 1000ml(1L). For that 500ml of distilled water is taken in a conical flask and- the necessary ingredients are added, 10g of Peptone, 10g of Mannitol, 10g of NaCl. Mix the ingredients. Add 500ml of distilled water to the flask and close the opening and kept it in autoclave and wait for it to sterilize. After autoclaving the broth is poured into 2 conical flask and 1 test tube. The amount contains in one conical flask about 50oml and other with 490 ml of broth and the remaining 10ml on test tube which is used as control in later test. The test tube is stored in 4°C for later. The bacillus colonies are introduced in both conical flasks, red and yellow respectively. The flasks are kept in Orbital Shaker at room temperature for 48hr. After the bacteria are cultured, they broth were poured into falcon tubes and then centrifuged at 7000 rpm for 15min. The supernatant is collected and tubes were discarded.

The Agar-Well Diffusion method

Using this method, microbial extracts can be screened for antimicrobial activity against pathogenic microbial species. The Muller-Hinton agar plates (2) were prepared for the antibacterial activity. About 0.1 mL of the fresh 18hr old broth culture was spread on the respective media. EC2 (E.coli) is introduced to agar plates using the

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spread plate technique, wells of 6 mm in diameter were made at the center of the plate by using sterile cork borer. The wells were open with the help of sterile forceps. Then 50µL, 100 µL of yellow colonies stock solution (supernatant) was added by using micropipette in two well and control broth in third well. Same is done with red colonies stock solution. The extract was allowed to diffuse; hence the prepared plates were left at room temperature. The antimicrobial extract solution gradually diffuses through the agar medium and inhibits the growth of the bacterial species tested.

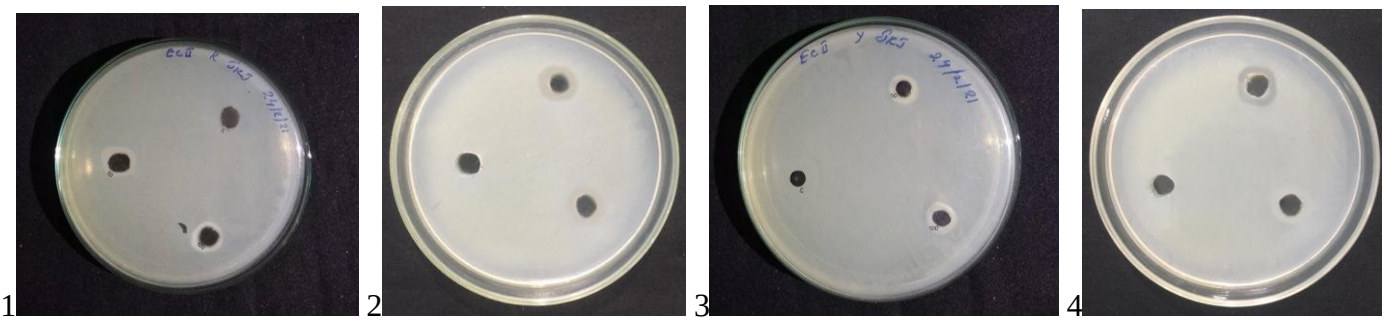

Fig. 3 (1) Red colonies (close), (2) Red colonies (open), (3) yellow colonies (close), (4) yellow colonies (open)

Results

Isolation of microorganisms with antimicrobial potential

Strain was initially identified on the basis of its morphological and biochemical characteristics and according to the results it belongs to the genus Bacillus. In the course of screening for novel substances with antimicrobial potential from the agricultural fields of outskirts of Chennai, Tamil Nadu, two bacterial colonies (yellow and red) were isolated using special agar medium and screened using Special agar medium. The isolated cultures were sub-cultured on Special agar medium and preserved in a refrigerator at 4°C.

Isolation and Identification of bacillus

Special medium is used to cultivate microorganisms to isolate bacillus genus bacteria. This medium is called Special Agar Medium and is a medium used only for growing bacillus colonies. This medium allows only bacillus genus bacteria to grow and others are unsuitable to grow or destroyed. The bacterial isolates present in the agricultural soil were isolated by Serial dilution technique. Serially diluted samples of 10^{-3} ,10^{-4} ,10^{-5} are screened using spread plate method. Since, 10^{-3} have more colonies compared to others it is selected for further proceedings. Both yellow and red colonies are screened individually. Morphologically distinct bacterial were found and isolated in new special agar medium. There were 2 morphologically different types of bacteria were found. Two petri dish was taken and each plate one type of bacteria is inoculated from the culture medium using streak plate method. The well grown bacterial cultures stored at 4°C. Morphological studies showed that the isolates are Gram-positive, sporulating, rod shaped bacteria.

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Preliminary screening for antimicrobial activity

The isolated bacterial strains from agricultural soil were screened for antimicrobial activity by *Cross-streak technique*, method under conditions which shown as optimal in previous experiments. Fig.1 shows the potential antibacterial activity of yellow colonies of bacillus spp against Escherichia coli II (EC-II). Red colonies of bacillus spp showed potential antibacterial activity against Escherichia coli II (EC-II), but no antifungal activity. Fig. 2 shows antibacterial activity of red colonies of bacillus spp against Escherichia coli II (EC-II). *The Agar-Well Diffusion*, when incubated, the antimicrobial extract solution gradually diffuses through the agar medium and inhibits the growth of the bacterial species tested. Finally, the inhibition zone can be observed. This proves that bacillus ssp have antimicrobial activity against Escherichia coli.

CONCLUSION

Soil is a prosperous source of microorganism that produces a wide range of antibiotics including peptide antibiotics [8]. The development of antibiotics resistance and lesser safety margins provoked scientists to search for antimicrobial agents with modifed properties and maximum activity. Bacillus species have been considered as extremely useful microorganisms for producing antimicrobial agents [9]. Bacillus species are aerobic or facultative anaerobic, sporulating, rod-shaped, Gram-positive bacteria [10]. Some species may turn Gram-negative with age (Baron, 1996). The Bacillus species are known for the synthesis of secondary metabolites with remarkable diversity both in structure and function [11]. The Bacillus species are most popular for producing peptide antibiotic compounds such as polymyxin, colistin [12]. In this study we have also identified a Bacillus strain with strong antimicrobial activity.

The identified B. subtilis shows potent antibacterial activity. The most sensitive strain to its antibacterial activity was *Escherichia coli* II (EC-II), against whom *B. subtilis* forms a 10 mm inhibition zone. And other *Bacillus sp*. isolate against *Escherichia coli II* (EC-II) and determined a 08 mm inhibition zone. In our study, *B. subtilis* showed no antifungal activity, but antimicrobial studies of Moshafi al. 2015 determined its ability to inhibit *C. albicans*, *A. flavus* and *A.niger* as well [13].

his study shows a critical literature review and from the study, it is recommended further research should be carried out using other agricultural products or from other sources like water, plants, enteric tract of insects and mammals, etc.; for the isolation and antimicrobial activity. In addition, testing for the activity of these strains over a broader range of pathogenic microorganisms, including resistance testing and possible clinical application, are projected for future research. While there is so much study on the isolation of Bacillus Subtillis commonly found in soil, there is limited literature on other related bacteria such as Bacillus amyloliquefaciens etc.

CONFLICTS OF INTEREST

The authors have no relevant financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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REFERENCES

1 Ganesh Kumar Saya,Belgode N Harish, Sujatha Sistla Jawaharlal Institute of Postgraduate Medical Education & Research; Journal of Natural Science Biology and Medicine 4(2):286-291

2 Devasahayam, G., W. M. Scheld, P.S. Hoffman (2010). Newer antibacterial drugs for a new century. Expert. Opin. In. Establish. Drugs. 19:215–234.

3 Papagianni, M. (2003.) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnol. Adv. 21: 465–499

4 "Antimicrobial". Merriam-Webster Online Dictionary. Archived from the original on 24 April 2009. Retrieved 2009-05-02.

5 Weichenthal, B. A; Russell, H. G (1970): "Beef cattle feeding suggestions : nutrient requirements, balancing rations, protein supplements, suggested rations" Urbana, IL : University of Illinois at Urbana-Champaign , College of Agriculture, Cooperative Extension Service

6 Turnbull PCB (1996). "Bacillus". In Baron S; et al. (eds.). Bacillus. In: Barron's Medical Microbiology (4th ed.). Univ of Texas Medical Branch. ISBN 978-0-9631172-1-2.

7 https://www.ncbi.nlm.nih.gov/books/NBK7699/

8 Awais, M., A. Pervez, S. Qayyum, M. Saleem (2008). Effects of glucose, incubation period and pH on the production of peptide antibiotics by Bacillus pumilus. Afr. J. Microbiol. Res. 2: 114-119

9 Amin, A., A. M. Khan, M. Ehsanullah, U. Haroon, F. M. S. Azam, A. Hameed (2012). Production of peptide antibiotics by Bacillus sp. GU 057 indigenously isolated from saline soil. Braz. J. Microbiol. 43(4): 1340-1346.

10 Graumann, P. (2007). Bacillus: Cellular and Molecular Biology. 1st ed. Caister Academic Press, Ali Janabi et al.

11 SiloSuh, L. A., B. J. Lethbridge, S. J. Raffel, H. He, J. Clardy, J. Handelsman (1994). Biological activities of two fungistatic antibiotics produced by Bacillus cereus UW85. Appl. Environ. Microbiol. 60(6): 2023-2030

12 Katz, E., A. L. Demain (1997). The peptide antibiotics of Ba-cillus, chemistry, biogenesis, and possible functions. Bacteriol. Rev. 41: 449-474

13 Moshafi, H. M., H. Forootanfar, A. Ameri, M. Shakibaie, G. Dehghan-Noudeh, M. Razavi (2011). Antimicroabial ac-tivity of Bacillus sp. strain FAS 1 isolated from soil. Pak. J. Pharm. Sci. 24(3): 269-275.

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