The Pharma Innovation Journal 2018; 7(11): 287-291

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(11): 287-291

www.thepharmajournal.com
Received: 04-09-2018
Accepted: 08-10-2018

VVSR Krishna Kishore
Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India

M Vijayalakshmi
Professor, Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India

Correspondence
VVSR Krishna Kishore
Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur, Andhra Pradesh, India

Isolation, morphological and biochemical characterization of antibiotic producing bacteria from various sewage sites of Visakhapatnam, Andhra Pradesh

VVSR Krishna Kishore and M Vijayalakshmi

Abstract
The emergence of antibiotic resistance among pathogens and need for better, broad spectrum antibiotics is always in high demand. Hence isolation of antibiotic producing bacterial strains from sewage was carried out in the laboratory. In the present study, antibiotic producing bacteria were isolated from various sewage sample collected from Visakhapatnam. After primary screening, three bacterial strains (AntC1, AntC3 and AntC8) were isolated, which showed antimicrobial activity against some common bacteria namely, *E. coli*, and *S. aureus*. The isolated strains showed prominent zones of inhibition against *E. coli* and *S. aureus*. To identify the isolated strains, Microscopic examination and biochemical tests were performed and it was found that the strains were related to be Bacillus species with some differences in cultural characteristics. Identification of those strains by 16s rRNA gene sequencing will be useful in improving these strains for antibiotic production.

Keywords: Antibiotic producing strains, sewage effluents

Introduction
Antibiotics were one of the highly imperative economically exploited secondary metabolites produced by the bacteria and employed in an extensive range. Though many antibiotics were discovered so far, endeavors to find new antibiotic is still proceed. Bacteria are rich in different environments like air, water and soil, they are using in the production of various antibiotics, vaccines, steroids as well as other therapeutically useful compounds as they are proved to be having high biological activities [1]. Sewage water or industrial waste water is rich pathogenic micro organisms and as well as it contains antibiotic resistance microorganisms. The microbial biomass that reduces the complex content of the sewage is the activated sludge, biological flocs mainly composed of saprophytic bacteria, and some protozoa and small metazoans [2]. Currently industrial interest in soil bacteria is highly increasing as they are having several advantages compare to other microorganisms, in searching for the novel antibiotics and commercially important products from them. From the last 55 years more than 12,000 antibiotics have been discovered [3, 4]. The predominant soil bacteria were the bacillus species because of their resistant endospore formation and vital antibiotics production like bacitracin etc. were persistently found inhibiting the various microbial growths [5]. Along the various sources of antibiotics, sewage is the most significant target for scientists in the innovation of novel antibiotics. According to Sunil kumar and Dheerendra Kumar (2017), sewage microbes have persistently been determined for their useful biological active metabolites such as antibiotics at present. Subsequently there is an enormous possibility to screen viable bacterial strains from waste dump localities with important applications. To meet the demand for production of novel antibiotics there have been a steady efforts are carried out by the researchers in isolating novel bacteria from diverse environment. Accordingly, the goal of the present investigation was undertaken to isolate the novel antibiotic producing bacteria from sewage samples collected from industrial effluent dumping sites in vizag city. These sewage samples were chosen for analysis because of the likely hood of isolating novel antibiotics producing bacteria from this area is elevated. The result of this finding might be vital in searching for the isolation of novel antibiotic producers even from waste effluents (Sewage) and for future treatment of multidrug resistance pathogens.

Material Methods

Sample collection: Sewage water from exclusive areas have been collected for this study specifically from three different locations like domestic sewage, industrial waste effluent and hospital dumping sites located around Visakhapatnam Fig: 1, (Table 1) in a sterile glass bottles with a sterile spatula and were transported to the laboratory and stored at 4 ^{0}C throughout the project period.

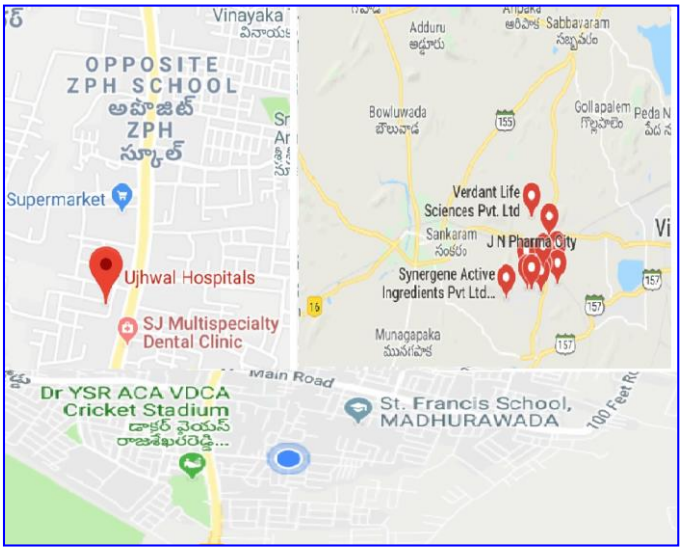

Fig 1

Table 1: Sample location

S. No	Sample Code	Sample Collection Point	Area
1	PCSS-I	JN Pharma City(Parawada)	vizag
2	HSS-II	ZPH School raod (Madhurawada)	vizag
3	DSS-III	PM Palem	vizag

PCSS: pharmaceutical industry sewage sample; HSS: Hospital sewage sample; DSS: domestic sewage sample

Sample preparation: A sample preparation was done by adding 1 g of the sample in 10 ml of distilled water in a previously labeled sterile test tube for each soil sample and is vortexes. 1 ml from this the first test tube transferred to the second test tube and is vortexes and from second to third up to 10^{-7}. Like this each sample was serially diluted under sterile conditions.

Isolation of bacteria from test samples: 0.1ml of each sample from 10^{-6} and 10^{-7} were transferred on to separate sterile nutrient agar plates and distributed evenly with a sterile bent glass rod the plates were incubated at 37 ^{0}C for 24 hours in an incubator [2, 6].

Sub culturing: Bacterial colonies with clear margins were picked Fig: 2 and purification was accompanied by continues streaking of the isolated colonies on fresh nutrient agar plates with a sterile loop using streak plate method and plates were incubated at 37 ^{0}C for 24 hours [6, 7].

Test Micro organism: Gram positive and three gram negative organisms were used as test organisms in this study. Two test organisms *E. coli* -5346 and staphylococcus aureus-4841 were collected from NCIM culture collection center. Staphylococcus aureus is a gram positive bacterium while Escherichia coli are gram negative bacteria. Cultures were maintained on nutrient agar slants.

Screening for antimicrobial property: The antimicrobial property of purified strains namely AntC1, AntC3 and AntC8 was determined by well diffusion method [8]. For the screening of antimicrobial activity of test samples, test cultures were inoculated on to the surface of Mueller Hinton agar (MHA) medium by using spread plate method. Sterile cut tips were used to prepare well at the centre on the agar plates. Each well was filled with test supernatants. Control was prepared with test organism only (without supernatants). And plates were incubated at 37 ^{0}C for 24 hours. Zone of inhibition around each well was measured after appropriate incubation period Fig: 3.

Supernatant preparation

For this initially purified strains were inoculated into a sterile nutrient broth in sterile conical flask of 100ml capacity and were kept on orbital shaker cum incubator for 24 hours at 37 ^{0}C after incubation culture suspensions were centrifuged at 6000rpm for 10 minutes and collected supernatants in sterile eppendorf tubes and were labeled as sample-I and sample-II.

Identification Methods

Identification was done by following biochemical tests and the results were compared with standard description as in Bergey's Manual of Systematic Bacteriology [9].

Microscopic examination: Gram staining technique was performed for AntC1, AntC3 and Antc8 to determine the morphological characteristics. Gram staining technique was performed according to the method instructed in Remel, Lenexa, KS gram staining kit.

Procedure: According to Remel, Lenexa, KS gram staining kit

- Thin smear was prepared on a glass slide.
- Slide was allowed to air-dry completely.
- Smear was fixed on the slide by passing 3 or 4 times through the flame of a Bunsen burner.
- Gram Crystal Violet was applied for 1 minute to the smear.
- Slide was washed under running tap water and overlayed with Gram Iodine mordant for 1 minute.
- Flooded with Gram Decolorizer until the solvent flows colorless from the slide (10-30 seconds).
- Slide was washed under running tap water and treated with Gram Safranin for 30 seconds.
- Finally the slide was washed with water and allowed to dry.

Biochemical test: The Following biochemical tests were performed for the biochemical characterization of AntC1, ActC3 and AntC8; Catalase Test, Indole Test, Methyl Red Test, VP Test, Urease Test, Starch Hydrolysis Test and Sugar Fermentation Test [10, 11].

Results and Discussion

A total of 30 bacterial strains with clear marginal zones were isolated in 10^{-6} dilution plate from the industrial waste effluents Fig: 2 where as no marginal zone colonies were isolated from domestic and hospital sewage. From which 15 bacterial cultures with clear marginal zones were selected and were screened for antibiotic production by crowded plate technique with two test organisms namely *Staphylococcus aureus* and *E. coli*. Out of 15 there were 9 cultures showed antimicrobial property.

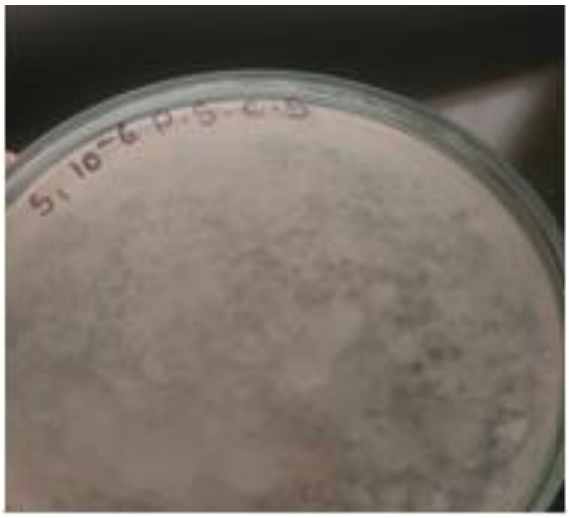

Fig 2

9(AntC1-AntC9) cultures were finally screened for their antimicrobial property (Table 2) by agar well diffusion method. The antimicrobial property was studied on muller hinton agar medium [12].

Table 2: Screening for Sensitivity of test bacterial strains by Agar well diffusion method

Sample no	Test Bacterial samples	Gram positive Bacteria Staphylococcus aureus	Gram negative bacteria *E. Coli*
1	AntC1	+++	-
2	AntC2	-	-
3	AntC3	-	+++
4	AntC4	-	-
5	AntC5	-	-
6	AntC6	-	-
7	AntC7	-	-
8	AntC8	+++	+++
9	AntC9	+	-

+++ High zone of Inhibition, ++ Median zone of Inhibition, + Less zone of Inhibition, - No Inhibition.

Only three were selected because of showing clear antimicrobial property against Staphylococcus aureus and *E. coli* Fig: 3 and these three colonies (AntC1, AntC3 and AntC8) were identified by morphological characteristics, microscopic examination and biochemical tests (Table 3, 4 and 5). The bacterial isolates were identified as gram positive rod shaped bacterial strains. These microbial strains were sub cultured on agar slants and stored for further identification by 16s rRNA sequencing at 4 ^{0}C. Antimicrobial compounds are the most vital bioactive substances for the treatment of infectious diseases. Be that as it may, now, in view of the crises of multi- drug resistance pathogens, there are fundamental difficulties for viable treatment of those infectious diseases. Hence, because of the risk of high recurrence of multi drug resistance pathogens on the earth, there has been expanding enthusiasm for seeking powerful anti-infection agents from soil microorganisms in various environmental regions [13].

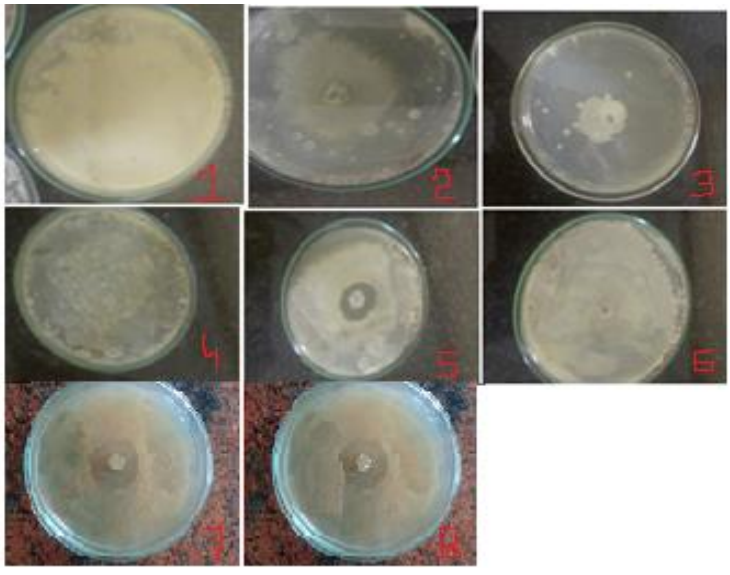

Fig 3

1. Control with test microorganism *E. coli*; 2. Supernatant of AntC1 with *E. coli* test organism; 3. Supernatant of AntC3 with *E. coli* test organism; 4. Control with test microorganism Staphylococcus aureus; 5. Supernatant of AntC1 with Staphylococcus aureus test organism; 6. Supernatant of AntC3 with Staphylococcus aureus test organism 7. Supernatant of AntC8 with Staphylococcus aureus; 8. Supernatant of AntC8 with *E. coli*.

Table 3: Morphological identification

Colony morphology on Nutrient Agar plate	AntC1	AntC3	AntC8
Shape	Circular	Circular	Circular
Color	White	White	Creamy
Profile	Flat	Flat	Flat
Surface nature	Smooth	Smooth	Irregular
Margins	Undulate	Smooth	Irregular

Table 4: Microscopical examination

S. No	Bacterial strains	Gram staining	Shape
1.	AntC1	Gram positive	Rod
2.	AntC3	Gram positive	Rod
3.	AntC8	Gram positive	Rod

Table 5: Biochemical examination

Test	Reaction with AntC1	Reaction with AntC3	Reaction with AntC8
Catalase	+ve	+ve	+ve
Indole	-ve	+ve	-ve
Methyl Red	-ve	-ve	-ve
VP	+ve	+ve	+ve
Urease	+ve	-ve	-ve
Starch Hydrolysis	+ve	+ve	+ve
Sugar Fermentation	+ve	+ve	+ve

In the present investigation, the chosen waste effluents samples were taken from various regions like domestic, hospital and industrial areas for isolation of microscopic organisms showing anti microbial properties. AntC1 indicated antimicrobial property on Staphylococcus aureus just yet not on *E. coli*, AntiC3 demonstrated its antimicrobial property on *E. coli* however not on Staphylococcus aureus as opposed to this AntC8 have Showed its antimicrobial property on both test cultures (Staphylococcus aureus and *E. coli*). The effective segregation of microbes from ecological examples requires a better understanding of the potential waste example regions and natural elements influencing their development. Recent investigations demonstrated that determination of various potential regions, for example, sewage effluents were a critical action for segregation of various sorts of powerful antibiotic producing soil microorganisms [14].

The present investigation of essential screening utilizing Agar well techniques demonstrated that, three (10%) out of 30 bacterial strains indicated potential antimicrobial action against two test microscopic organisms. This outcome (10%) is lower than 21.88%, 59.09% and 13.3%, from past reports [13, 15-16].

Conclusion

Streptomyces griseus, is one of the outstanding anti-microbial maker present in soil which delivers the bactericidal compound Streptomycin which battles a few pathogenic microscopic organisms like Mycobacterium tuberculosis [17]. They have been generally used over the past decades to battle a few bacterial infections. In any case, one noteworthy issue that we confront today is the developing microbial resistance towards the effectively accessible anti-microbial agents [18]. Hence, there is continues need to find out alternatives and discover novel antibiotics to fight against these resistant pathogens [19]. There are numerous other potential antibiotic producing microorganisms present in the earth particularly in waste effluents and high number of microorganisms that still can't seem to found or used. Since extensive variety of micro-flora is available in nature, there is a possibility of discovering new species and strains that have this property. Therefore, this was the principle point of the investigation to isolate and screen an antibiotic producing microorganism from the sewage or waste effluents of different sources. From the outcome of this study, the colonies that showed zones of inhibition were taken and sub-cultured over and over to acquire a pure culture. This was on account of the strain that showed a zone of inhibition due to the compound that repressed the development of the other microorganism in the crowded plate. This colony was then subject to morphological, microscopical and biochemical tests to determine the genus of the bacteria and identified that all three would be related to Bacillus Species. The pure culture was then screened for antibiotic property against two bacterial cultures. Further purification, structural elucidation and characterization are recommended to know the quality, novelty and commercial value of these antibiotics.

Acknowledgements

The Authors are exceedingly valued the Pious Science lab Staff Vishakhapatnam and Department of Microbiology Achrya Nagarju University Nambur for giving their proceeds with help in successful fruition of this research work.

References

1. Demain AL. Small bugs, big business: the economic power of the microbe. Biotechnol Adv. 2000; 18(6):499-514 [PMID: 14538099].
2. Berdy J. Bioactive microbial metabolites. J Antibiot (Tokyo). 2005; 58(1):1-26 [PMID: 15813176 DOI: 10.1038/ja.2005.1]
3. Goldstein DA, Tinland B, Gilbertson LA, *et al*. Human safety and genetically modified plants: A review of antibiotic resistance markers and future transformation selection technologies. J Appl Microbiol. 2005; 99:7-23.
4. Badosa E, Moreno C, Montesinos E. Lack of detection of ampicillin resistance gene transfer from Bt176 transgenic corn to culturable bacteria under field conditions. FEMS Microbiol Ecol. 2004; 48:169-78.
5. Cafini F, del Campo R, Alou L, Sevillano D, Morosini MI, Baquero F, *et al*. J Antimicrob Chemotherapy. 2006; 57(2):224-229.
6. Reddy NG, Ramakrishna DPN, RG 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):1-14.
7. 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].
8. Perez C, Paul M, Bazerque P. Antibiotic assay by agar well diffusion method. Acta. Bio Med Exp. 1990;

15:113-117.
9. 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].
10. Czaplewski L, Bax R, Clokie M, Dawson M, Fairhead H, Fischetti VA *et al*. 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]
11. Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R *et al*. 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].
12. NCCLS-National Committee for Clinical Laboratory Standatrds, Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard. National Committee for Clinical Laboratory, Wayne, PA, 1999.
13. Abo-Shadi M, Sidkey NM, AM AM. Antimicrobial agent producing microbes from some soils' rhizosphere. J Am Sci. 2010. 6(10):915-925.
14. Kannan RR, SG V. Molecular characterization of antagonistic streptomyces isolated from a mangrove swamp. Asian J Biotechnol. 2011; 3(3):237-243.
15. 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. 17:242-249.
16. Dheerendra Kumar, *et al*., Isolation, Screening and Characterization of Bacteria Having Antibacterial Activity from Industrial Waste Effluent Am. J. Biomed. Sci. 2018, 10(1):9-17; doi: 10.5099/aj180100009 © 2018 by NWPII.
17. Thakur D, Yadav A, Gogoi BK, Bora TC. Isolation and screening of Streptomyces in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites, Journal de Mycologie Médicale. 2007; 17:242-249.
18. Eddie C. The soil resistome: The anthropogenic, the native, and the unknown, Soil Biology & Biochemistry. 2013; 63:18-23.
19. Nithya, Pandian. Isolation of heterotrophic bacteria from Palk Bay sediments showing heavy metal tolerance and antibiotic production, Microbiological research. 2010; 165:578-593.