**Bulletin of Environment, Pharmacology and Life Sciences**
Bull. Env. Pharmacol. Life Sci., Vol 10 [1] December 2020 : 162-167

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.876
Universal Impact Factor 0.9804
NAAS Rating 4.95


**ORIGINAL ARTICLE** **OPEN ACCESS**

# Actinomycetes - A Promising Source of Anti Biofilm Agent

**Sreelakshmi K S[1] and Usha R[2*]**
[1]Department of Bioscience, SNIST arts and science college, Karumallor, Ernakulam, Kerala,
[2]Department of Microbiology, Karpagam Academy of Higher Education, Coimbatore, Tamilnadu.
*Email: ushaanbu09@gmail.com

**ABSTRACT**
*Medical devices coated with biofilm serves as the major source of infections. Biofilm producing microorganisms show more resistance to antibiotic drugs due to the incomplete diffusion of drugs all the way through the biofilm mats. Among the producers of bioactive compounds, actinomycetes serves as the producers of novel compound. These compounds possess antibacterial, antifungal, antiviral, activity. In this study, around 30 types of actinomycetes isolated in AIA, SCA (glycerol storage) from various regions of different soil types. Biofilm forming organisms, E.coli, Pseudomonas, and Staphylococcus from urinary catheter isolated by rolling plate method and carried out tube assay, microtitre plate method. Primary selection of most active actinomycetes were done by line streak method against the above said pathogens. Selected actinomycetes inoculated to fermentation broth for the production of bioactive metabolites. Extraction proceeded with liquid- liquid extraction using ethyl acetate. Extracts concentrated, antibacterial tests were performed against desired biofilm producers. In Well, broth dilution method, three pathogens were inhibited. Zone of inhibition was highest in Pseudomonas sp.*
***Key words:*** *Biofilm, Multi* drug *resistance, Bioactive compounds, Actinomycetes, Antibacterial.*

Received 21.10.2020 Revised 16.11.2020 Accepted 14.12.2020

## INTRODUCTION

Biofilm can be formed with the aggregation of bacteria which produces exopolysaccharides, nucleic acids, proteins [1]. Biofilm formation results in health care infections causing death, especially those associated to the implant of medical devices. The bacteria form Biofilm initially by the conditioning of the surfce, followed by the irreparable attatchment [2]**.** Biofilm forming bacteria are more resistant to antibiotics. Targeting on the locations of Biofilm related infections, transplant units, neonatal intensive care unit, burn units, dialysis unit, oncology units were mostly affected. Huge amounts of antibiotic drugs used, resulted in the development of multidrug resistance in Biofilm formers. Multi drug resistance can be developed in bacteria by the accretion of multiple genes, on resistance (R) plasmid and also by the expression of genes that codes for multidrug efflux pump for wide range of drugs [3].
As the multidrug resistance persists, many antimicrobial agents are getting reduced its effectiveness. As they also targets some non pathogens, it is very important to develop more environment friendly agents. Actinomycetes, soil dwelling microorganisms can contest with the existing problem [4,5]. Actinomycetes are gram-positive, filamentous, aerobic, non sporing organisms which provide a novel source for the production of many potential bioactive compounds. These organisms are unique enough to classify them as individual group [4]. As the bioactive compounds posses antibacterial, antifungal, antiviral, anti proliferative and anti-inflammatory properties, it can be targetted against biofilm forming microorganisms especially formed on medical devices [6].

## MATERIAL AND METHODS

### Collection of Soil and processing of sample for isolation of Actinomycetes

The sample collection was done in such a way that from 5cm top portions of different niche habitats of Ernakulum and Thrissur district, Kerala. They were collected and transferred to sterile polythene bag and properly labeled the date of collection. Then the soil samples were mixed carefully and passed through a 2 mm sieve to remove gravel and debris **[**7**]**. Fifty samples were collected within a period of 4 months.

Pretreatment of samples were done by drying in hot air oven and stored at 4°C until it is plated. This was done mainly to avoid bacterial and fungal contamination.

**Isolation of Actinomycetes and maintenance**

To 9 ml distilled water, 1 gram of each dried samples were mixed and serially diluted and in this way up to $10^{-4}$ dilution were prepared. Actinomycetes isolation agar (AIA) and starch-casein-nitrate-agar (SCA) medium used for each sample with using modified sea water (50ml) and autoclaved the media [8]. An aliquot of 0.1 ml of $10^{-4}$, $10^{-5}$ dilutions were taken and spread plated with L-rod over the surface of AIA and SCA supplemented with Cyclohexamide (100µg/ml) **[**9]**.** Plates incubated at 30°C . It is then observed until 7 days for the powdered colonies. Plates with 100-110 colonies were chosen for detailed analysis and suitable colonies were recultivated several times for purity. The purified colonies were streak plated and stored with the addition of glycerol (25%v/v) **[**10].

**Rolling plate method to isolate test pathogens**

Test organism was isolated by rolling plate method. The nutrient agar was prepared and the Biofilm coated catheter tube was rolled on to the surface of the medium aseptically. Plates were incubated at 37°C for one day. From colonies formed, three strains were selected for further studies **[**6]**.**

**Congo red agar method to detect biofilm**

These strains were streaked on to Congo red agar (CRA) plates. Congo red agar method is used as a qualitative test for the detection of microorganisms which forms the Biofilm. Congo red indicator (8g/l) is concentrated and solution is made with brain heart infusion agar supplemented with sucrose and autoclaved for 121°C for 15 minutes. CRA plates were inoculated with the test organisms and incubated at 37°C for 24 hours **(**6**).**

**Tube assay to detect biofilm organism**

Tube tests were done for the detection of Biofilm forming organisms. Here, in this test microorganisms inoculated to 10 ml tripticase soy broth (TSB) with 1% glucose. The inoculated TSB broth was incubated at room temperature for 24 hours. Tubes were then washed with phosphate saline broth (pH 7.3) and were then stained with crystal violet. Finally, the tubes were washed with deionized water and dried in inverted arrangement (6). The strains selected were *Escherichia coli*, *Staphylococcus* sp, and *Pseudomonas* sp.

**Characterization and Screening of potential strains of Actinomycetes**

All the isolated actinomycetes were morphologically and biochemically characterized. The colonies were observed for the texture and the colour. Gram's stain and biochemical tests were performed. Screening involves 2 methods. Primary screening was done by cross streak/perpendicular streak method **[**11]. Secondary screening was done by tube dilution and well diffusion method with the crude extract obtained by ethyl acetate (liquid-liquid extraction) **[**12]**.**

**Extraction of bioactive compound**

Production of secondary metabolite was done by submerged fermentation process. Active strains of actinomycetes were transferred to 50ml of starch casein agar media in 250 ml conical flasks and incubated at 30°C for 7 days at 150 rpm. After fermentation, media were centrifuged at 10,000 rpm for 10 min. Then the broth was added with equal amounts of ethyl acetate and solvent phase were extracted and concentrated **[**12]**.**

**Determination of anti bacterial assay**

Partially purified extracts were checked for antibacterial quality by Well diffusion and Tube dilution method [13]. Cell concentrations of test organisms under study were adjusted according to 0.5 McFarland turbidity standards and swabbed on to MHA plates by using sterile cotton swabs [6]. Six wells of 8 mm diameter were punched into the medium (four wells for 20, 40, 80 and 100µl of extract and the rest were kept for positive and negative control)**.** The plates then incubated at room temperature for 4 hours**.**

**Determination of Minimum Inhibitory Concentration (MIC)**

MIC of potential extracts of isolates against test organisms determined by broth dilution method **[**13]. Microtitre method also performed **[**6]**.**

## RESULTS AND DISCUSSION

An attempt has been made to isolate actinomycetes for the novel compounds which inhibited the biofilm forming microorgansms. Total 30 types of actinomycetes were isolated from different regions (Fig 1). All the selected potential strains were gram positive (14). In the primary selection, cross streak plate method were performed. Colony characteristics of potential strains of actinomycetes were observed (Table 1).

The active isolates were selected and then followed by secondary screening. All the experiments were done in triplicates. Out of 30 isolates, five isolates ($A_1$, $A_6$, $A_8$, $A_{26 \text{ and } A22}$) showed maximum activity against the test organisms. 23mm thee maximum zone of inhibition shown against *Pseudomonas* sp (Table 2).

Here, microorganisms from biofilm were transferred aseptically by rolling a segment of urinary catheter to nutrient agar medium (Fig 2). The colonies formed after incubation was reinoculated to fresh media and stored at 4ºC. Microorganisms obtained from biofilm were streaked on to Congo red agar plates (CRA) in which black colonies were obtained (Fig 3). This indicates the microorganisms are capable of developing biofilm.

As the Multidrug resistance persists, it results in the recurrent health care associated infections and there by death. Conventional antibiotics fail to inhibit these drug resistant pathogens as they are adapting. Here comes the importance of the bioactive compounds from actinomycetes. It is stated that the bioactive compounds from actinomycetes has high potential to be used in treating biofilm infections(15). Research reveals that the production of bioactive compounds by actinomycetes has many properties of which, their antioxidant properties is an important feature (16). In this study, crude extracts of bioactive compounds showed antibacterial properties, in which it's other activities to be studied in detail. It is reported that the test isolates from biofilm on implanted devices can be detected by using rolling plate method, Congo red agar method, tube test [6].

Microtitre plate method (MtP) performed so that the formation of biofilm was confirmed. It is a quantitative test in which 96 well flat bottom microtitre plates were used. As the organisms after incubation attached on to the well surfaces and the readings are made (6) (17). Tube assay or tube method is a qualitative test, again which confirms the growth of biofilm forming organisms after staining the tubes. In this study, by tube assay method biofilm matrix at the bottom of the test tubes obtained . Different tubes were inoculated with the microorganisms isolated from the biofilm confirmed the same. Black colour colonies obtained after performing CRA method indcates the organisms forms crystalline slime which ehhances the biofilm formation(17). As all the studies reveals that the bioactive compounds from actinomycetes possess uniqueness in its structure and function, it is possible to overcome the multidrug resistance. Biofilm, especially in association with medical implanted devices can be inhibited with the use of potential drugs of actinomycetes origin(15).

It has been previously reported that actinomycetes isolated from soil can inhibit the biofilm formation [7]. In this present study, actinomycetes isolated from soil could produce secodary metabolites which possess biological activity. According to the previous research [6],[17] microtitre plate method(gold-standard method) detected 25 strong biofilm formers and 45 moderate biofilm formers including *Staphylococcus* sp, followed by *E.coli* and also *Pseudomonas* sp. Here, in this study microtitre plate method, from the readings biofilm forming organisms were confirmed. In Tube assay strong , weak and moderate biofilm producers were studied according to the number of tubes formed the biofilm at the bottom of the test tubes [17]. Tubes lined with biofilm were compared with the control tube and considered positive. In this study biofilm forming organisms forms matrices at the bottom of the test tubes. Congo red agar test also indicated the test organism produces black colonies as they produces crystalline slime which enhances the biofilm formation. According to the research [9, 14, 18] it is proved that actinomycetes has the potential to inhibit the biofilm forming organisms through antibacterial assays. Actinomycetes isolated from soil, of which *Streptomyces* are the important group which serves as the huge source of antimicrobial drugs [19]. Here in this present communication, actinomycetes showed antimicrobial activity against biofilm forming bacteria which in future can be used to treat implant associated infections caused by biofilm forming pathogens [20].

| S.NO | Actinomycetes isolates | Colony morphology | Pigment |
|---|---|---|---|
| 1 | $A_1$ | Flat powdery, irregular | Red |
| 2 | $A_6$ | Medium , dotted and wavy, powdery | Medium yellow |
| 3 | $A_8$ | Puffy powdery, medium | Grey |
| 4 | $A_{22}$ | Leathery powdery, medium, dotted. | Light sandal |
| 5 | $A_{29}$ | Leathery powdered, Medium. | Light green |

**Table 1:**Colony characteristics of potential strains of actinomycetes

| S. no | Organisms | Actinomycetes isolates | Zone of inhibition |
|---|---|---|---|
| 1. | *E. coli* sp. | $A_{8,}A_{,26}$ | 13mm, 11mm |
| 2. | *Pseudomonas* sp. | $A_1$, $A_6$ | 23mm, 20mm |
| 3. | *Staphylococcus* sp. | $A_{22}$, $A_{26}$ | 12mm, 11mm |

**Table 2**: Well diffusion method

| S. no | Test isolates | OD at570nm | Biofilm formation |
|---|---|---|---|
| 1. | *E.coli* species | 0.135 | M |
| 2. | *Staphylococcus* species | 0.401 | S |
| 3. | *Pseudomonas* species | 0.264 | M |

**Table 3**: Microtitre plate assay (*3(Control) = 0.086,*3(Control) =0.173, M= Moderate, S=Strong)

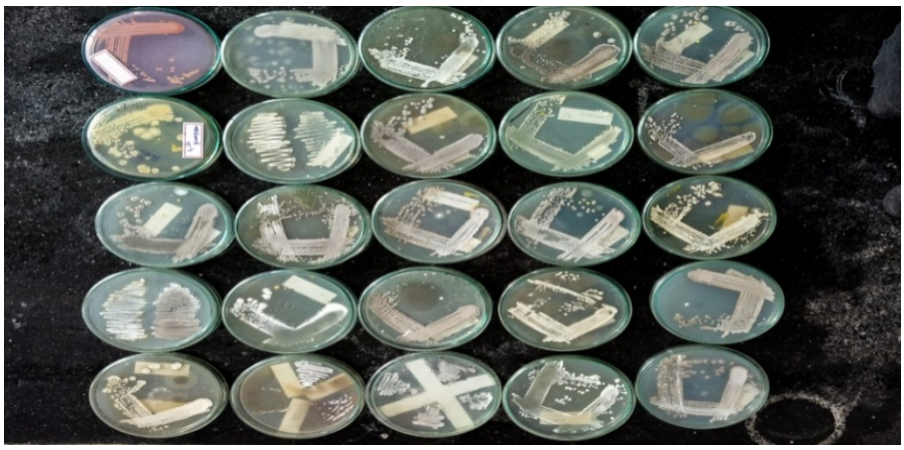


**Figure1**: Isolated Colonies of Actinomycetes

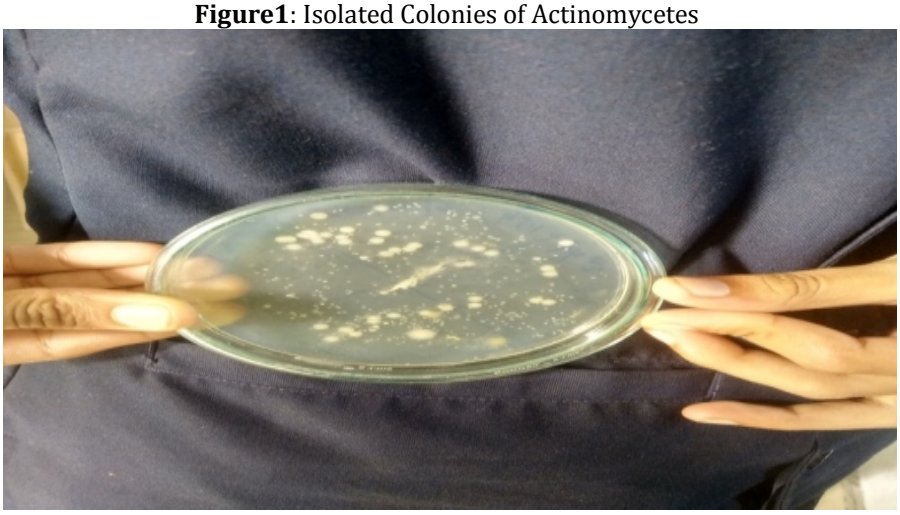


**Figure2**: Isolation of test organisms by rolling plate method

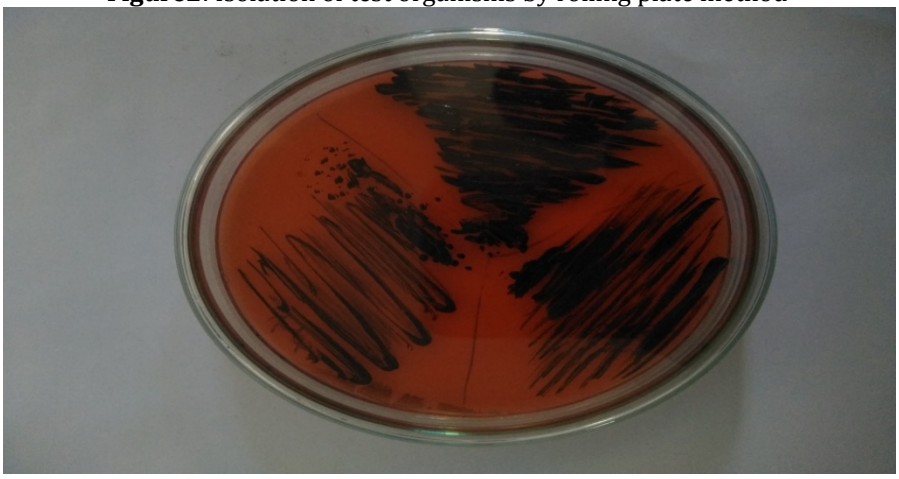


**Figure 3**: Congo red agar test


**Figure 4**: Tube assay (Biofilm detection)

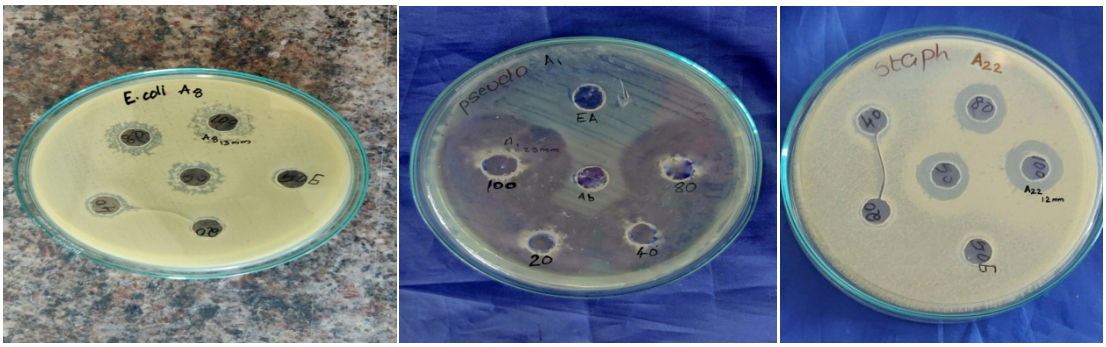
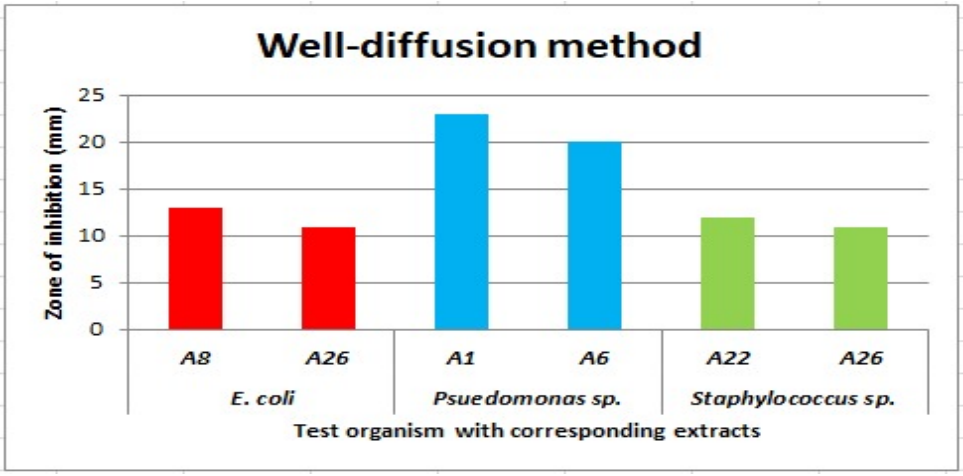
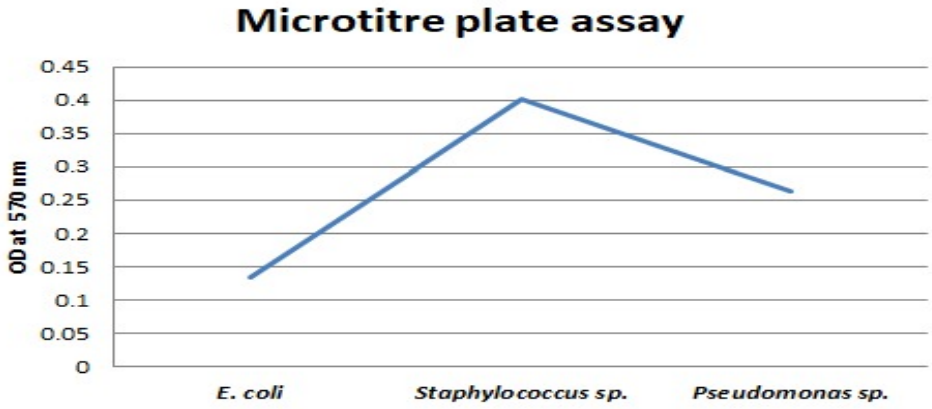



Plate A Plate B Plate C

**Figure 5:** Well diffusion method: Plate A:*E.coli,* Plate B: *Pseudomonas sp* Plate C: *Staphylococcus sp*




**Graph 1**: Antibacterial assay (highest zone of inhibition is shown by the *Pseudomonas* sp)




**Graph 2**: Microtitre plate assay

## CONCLUSION

This study explains that the test actinomycetes isolates posses the potential to inhibit the biofilm forming organisms which are multidrug resistant. Actinomycetes could be the promising source for the extremely active biopotential compounds which inhibit the biofilm especially which is formed on medical devices.

## REFERENCES


1. Flemming, H.C., Wingender, J.(2010). The Biofilm Matrix*: Nat Rev Microbiol* 8(9):623–633
2. Davies,D. (2003) Understanding biofilm resistance to antibacterial agents:*Nat Rev Drug Discov* 2(2): 114-122
3. Shriti, Singh., Santosh, Kumar. Singh., Indrajit, Chowdhury., Rajesh Singh (2017) Understanding the Mechanism of Bacterial Biofilms Resistance to Antimicrobial Agents.The Open Microbioogy Journal.11:53-62
4. Ian, L. Pepper., Terry, J. Gentry.(2015). Environmental Microbiology.(3)
5. Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F.(2007)
6. Sahra, Kirmusaoglu.(2019) The Methods for Detection of Biofilm and Screening Anti-biofilm Activity of Agents.
7. Hotam, S., Chowdhury.(2013) Antibacterial activity of *Actinomycetes* isolated from different soil samples of Sheopal. *J Adv Pharm Technol Res*. 4(2):118-23
8. Goodfellow M., Haynes J. A. (1984). Actinomycetes in marine sediments. In: Biological, Biochemical and Biomedical Aspects of Actinomycetes. Oritz-Oritz, L., Bojali, C. F. and Yakoleff, V. (eds.). Academic Press. New York, London. pp. 453-463
9. Saadoun,I., Hameed, K. M.,Moussauui, A.(1999) Characteriation and analysis of antibiotic activity of some aquatic actinomycetes. *Microbios*.99(394):173-9
10. Lam, K.,S. (2006) Discovery of novel metabolites from marine actinomycetes. *Curr. Opin. Microbiol:245-251*
11. Valli, S.,Suvathi, Sugasini., S, Aysha, O.S., Nirmala, P., Vinoth, Kumar P., Reena, A., (2012)Antimicrobial potential of Actinomycetes species isolated from marine environment. *Asian pac J Trop Biomed* 2(6): 469–473
12. Mojtaba, Mohseni., Hamed, Norouzi., Javad, Hamedi and Aboulghasem Roohi (2013) Screening of Antibacterial producing Actinomycetes from Sediments of the Caspian Sea. *Int J Mol Cell Med*. 2(2): 64–71.
13. Mounyr, Balouiri.(2016) Methods for *in vitro* evaluating antimicrobial activity: A review Vo 6( 2): 71-79
14. Sandeep, Rana., Menaka,Devi.Salam.,(2014) Antimicrobial potential of actinomycetes isolated from soil samples of Punjab, India. *J Microbiol Exp*1(2):63-68
15. Zohra, Khatoon., Christopher, D. McTiernan., Erik, J. Suuronen., Thien-Fah Mah b., Emilio, I., Alarcon A.(2018)Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. 4(12): 1067
16. Avilala, Janardhan., Arthala, PraveenKumar, Buddolla,Viswanath, D. V. R., Saigopal, and Golla Narasimha.(2014). Production of Bio active Compounds from Actinomycetes and Their Antioxidant Properties**.**
17. Afreenish, H., Javaid, U., Fatima, K., Maria, O., Ali K, Muhammad, I., (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. *Braz J Infect Dis* 15(4).
18. Sweetline, C., Usha, R., & Palaniswamy, M., (2012). Antibacterial Activity of Actinomycetes From Pichavaram Mangrove of Tamil Nadu. European Journal of Biological Sciences 2(4): 77-83
19. Angima Bichanga Kingsley., & Usha, R., (2018). Modification of Urinary catheters using antimicrobials from Streptomyces Sp ABK07 for urinary tract infection resistance . Asian J Pharm Clin Res 11 (7), 158-162.
20. Usha, R., & Angima Bichanga Kingsley., (2018) Streptomyces Sps-A promising source of antimicrobial agent. J Anal Pharm Res 7 (3), 323-328.



## CITATION OF THIS ARTICLE

Sreelakshmi K S and Usha R. Actinomycetes - A Promising Source of Anti Biofilm Agent. Bull. nv. Pharmacol. Life Sci., Vol 10[1] December 2020 : 162-167