International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

Comparative Study of the Antibiofilm Effect of plant extracts and antibiotic on *Staphylococcus aureus ATCC 0352*

Dibyajit Lahiri
Department of Biotechnology
Assistant Professor
University of Engineering & Management
University Area, Plot III,New Town,Kolkata 700160

Moupriya Nag
Department of Biotechnology
Assistant Professor
University of Engineering & Management
University Area, Plot III,New Town,Kolkata 700160

Rina Rani Ray*
Department of Biotechnology
Associate Professor & Head
Maulana Abul Kalam Azad University of Technology
Haringhta, Nadia 741249, West Bengal

Abstract
The biofilm by sequestering the bacterial cells from stressful chemicals, provide extra protection to the bacteria.Thus biofilm associated infections are equipped with resistance to the host immune responses and antibiotic treatment.*Staphylococcus aureus,*a profuse producer of biofilm is a potent human pathogen and its biofilm mode of growth is tightly regulated by complex genetic factors.As day by day the antibiotics are being gradually ineffective against this bacteria, extensive researches are being made to find out some novel source of antibiofilm agent from nature. With this objective the ethanolic extracts of Neem (*Azardirachtica indica*)(A), Turmeric (*Curcumin longa*)(C), Tulsi (*Ocimum sanctum*)(O) , Mikania (*Mikania scadens*)(M) as alternative source of antibiofilm compound. Production of biofilm by *Staphylococcus aureus* ATCC 0352 was found to be enhanced in presence of glucose but was somehow inhibited in presence of NaCl. The MIC and MBC values were found to be highest in *Ocimum sanctum* and higher in sessile forms for all antibiofilm agent tested. The MBEC data implied that the planktonic cells were effectively killed after 24 hours of exposure to antibiotic Gentamycin and ethanolic extracts of plants, though the combined effect of three extracts namely M+A+C gave the best result. The scanning electron micrographs clearly indicated the destruction of biofilm on chitin flex adsorbed bacterial cells.The comparative study revealed that the plant extracts were more effective than gentamycin in inhibition of bacterial growth. The treated bacteria after cultivation in stress free growth media failed to show prominent revival even after 24 hrs, which confirmed the efficacy of the plant sources.

Keywords:Antibiotics, Biofilm, Plant extract,, *Staphylococcus aureus*

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

Introduction

Biofilm development is accomplished by the adherence of sessile communities of bacterial cells to the biotic or abiotic surface by a matrix of extracellular polymeric substances (1).The biofilms are found in adverse locations like water- air and water-solid interfaces (2,3) like abiotic surfaces,epithelia of multicellular organisms (Druschel.G.K.et al,2004).The reversible or irreversible adhesion of the biofilm to the surface is followed by the formation of microcolonies that plays a significant role in spreading pathogenicity.By adopting this sessile mode of life, biofilm-embedded microorganisms enjoy a number of advantages over their planktonic counterparts. The extracellular matrix is capable of sequestering theexogenous additives or toxinsand hereby provide the embedded cells resistance against environmental stress.The EPS chemically varies from the species to the organisms depending upon the quantity of polysaccharides,proteins and nucleic acid (2,3).

Staphylococcus aureus is one of the most common bacterial pathogens causing different human diseases . Its biofilm mode of growth is tightly regulated by complex genetic factors. In order to control the bacterial growth, removal of biofilm is warranted. Biofilm bound growth can be checked by the use of antimicrobial compounds including antibiotics.

The researches proved that antibiotic treatment can only eliminate planktonic forms of bacteria but the sessile forms develops resistance towards antibiotics and keep on propagating inside the biofilm (4). Biofilms promote bacterial persistence by resisting host immune responses and antibiotic treatment (5). Since the development of Extra Polysaccharide Matrix prevents the diffusion of antibiotic compounds into the biofilm, biofilm is considered as a target for pharmacological development. Recent studies have shown that natural agents having plant secondary metabolites can disrupt biofilm. Hence, researchers are focusing on herbal treatments to fight against biofilms. Moreover, plant extracts are considered to be the safest as they are naturally derived and do not show any significant adverse effects to the host tissues surrounding the biofilm.

As these plant productsdo not contain any nutritional constituents and are effective in very small amount, usage of them as antibiofilm agent would add economy to the entire process. Moreover, their efficacy as potent antibiofilm agent, due to the presence of bioactive compoundsin them, could be successfully utilised for removal of biofilm.

In the present study, four plant parts were used, individually and in combination to remove the biofilm of *Staphylococcus aureus*ATCC 0352, and experiments were made to check if these antibiofilm plant products are bacteriostatic or bacteriocidal.

Materials and Methods

Microorganism

Staphylococcus aureus ATCC 0352, a Gram-positive biofilm forming bacterial strain was used in this study. The strain was streaked upon Luria Bertani Agar (LB Agar) plates and was incubated at 37^0C for 24 hours. One of the colonies obtained were cultivated in 250 ml Erlenmeyer flask containing 50 ml of LB broth at 37^0C for 24-72 hrs.

Estimation of Biofilm by Crystal Violet Assay.

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

The bacterium was grown in normal culture medium for 72 hours in test tubes. Each tube containing growth medium of different hour was diluted (1:100) with sterile saline. The flat bottom tissue culture plates (96 wells) were filled with 200µl of each type of diluted cultures individually. The culture plates were incubated at 37°C for 24 hours. After incubation, preceded by a gentle tapping of the plates,the wells were washed with 200 µl of phosphate buffer saline (pH 7.2) for four times to remove free-floating bacteria. Biofilms which remained adherent to the walls and the bottoms of the wells were fixed with 2% sodium acetate and stained with 0.1% crystal violet. Excess stain was washed with deionized water and the plates were dried properly. Optical densities (OD) of stained adherent biofilm were obtained with a UV visible spectrophotometer (Shimadzu, Japan) at wavelength of 540 nm. The OD values of non inoculated sterile medium were taken as (6)control .

Effect of sugar and salt on biofilm formation

The formation of Biofilm by the strain was analysed through microplate assay method using various concentration of sodium 5 to 7.0%,w/v),glucose (0.25 to 10.0%,w/v).To determine the synergistic effect of these individual components, they are mixed together in conditions like glucose to be applied double wt/vol than sodium chloride and in other case sodium chloride was applied double than that of glucose. The experimental set ups are to be kept at temperature of 35^0C for a period of 72 hours.(7)

Determination of and Minimum Bactericidal Concentration (MBC) of the planktonic cells of *Staphylococcus aureus.*

Four plant parts taken into consideration namely from rhizome of *Curcuma longa*,leaves of *Mikania scadens*,*Azardirachtica umsanctum*were collected. Their respective ethanolic extracts were made by pulverizing the rhizome of turmeric and dried leaves of rest three plants followed by mixing 4gms of each of them with 10 ml of 100% alcohol for 24 hours followed by centrifugation at 10,000 rpm for 10 min.

The minimum inhibitory concentration of the plant extracts and gentamycin (antibiotic) were determined both for the planktonic and sessile group of organismsby disc diffusion method **(**8). The broth dilution by 2,3,5-triphenyl tetrazolium salt (TTC) method was used for the determination of Minimum Bactericiadal Concentration (10)

Inhibition of Biofilm Formation (Minimum Biofilm Eradication Concentration-MBEC Assay)

The antibiofilm effect of the plant sources and Gentamycin on *S.aureus* ATCC 0352 was done by microdilution method of *Tang.et.al* 2011(The concentration of the plant extracts and antibiotic were taken with respect to MBC. taking phosphate buffer saline (pH 7.2)as and the medium was alone taken as positive,non-treated and blank controls.

After enumeration of the treated strain by haemocytometer ($1X10^6$ CFU/ml) it was added in the wells (96-well plate) leaving thecontrol well withun-inoculated media only and was incubated at 37^0C for 72 hours .The properly diluted extracts of plants and antibiotic was added in the wells and was incubated for 37^0C for 24 hours. The inhibition of viable Biofilm forming cellswere quantified using 3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay at a wavelength of 570

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

nm in ELISA plate reader (2018 GEN-NET).The number of viable bacterial cells were measured depending upon the ability of the living cells to convert yellow tetrazolium salt to purple formazon product. The percentage inhibition was calculated using the equation [1-($A_{570}$of the test /A_{570} of non-treated control)x100](10)

Determination of Viability count of the Biofilm forming *Staphylococcus aureus* cells

The biofilm by *Staphylococcus aureus*was developed on chitin flakes adopting the modified method ofAnderl*et.al* 2009. The bacterial cells grown in Luria Bertani Agar (LBA) slants at 37^0C for 18 hours,were sub culturedin Luria Bertani Broth (LB) medium enriched with 2%(w/v) Sodium chloride and 4% (w/v) Glucose to obtain a concentration of $1X10^6$ CFU/ml corresponding to a growth O.D of 0.05 at 600 nm. At this point of growth it was again inoculated into same mediumcontaining chitin flakes at 37^0C for a period of 72 hours. After thorough washing with 0.1% normal saline to remove the non-adherent cells, the number of viable cells in biofilm was determined by drop plating after suspending the biofilm cells in 0.9 mL of saline.(11)

Determination of biofilm structures

The biofilm structures were confirmed upon the chitin flakes using scanning electron microscopy following standard protocols(12)

Statistical analysis

The results obtained were expressed as mean ±SE. The experiments were performed in duplicate and two tailed unpaired t-test was used for statistical analysis.

Results and Discussion

Staphylococcus aureus was found to be a good producer of biofilm and after 72 hours of cultivation it produced profuse amount of biofilm (Fig 1). Since environmental factors, like glucose, salinity have been reported to affect biofilm formation, to evaluate the effects of NaCl on the biofilm formation, the strain was cultivated for 72 hrs in presence of these additives. Although the maximal amount of biofilm was found to be produced in presence of 1.6 to 4.8% NaCl in *S. Aureus* (13), 4% - 6% in *S. aureus* ATCC13565 (13)in the present strain NaCl did not show any enhancing effect. These results indicated that the salt present in processed food might not increase the biofilm formations of *S. aureus* ATCC 0352. Lee *et al*, 2014). On the other hand , glucose was found to be a facilitator of biofilm production in present strain and there was a trend in increase of biofilm formation with the increase in sugar concentration, which was similar to that found in *Escherichia coli 185p* (14). It was found that glucose was found to be more effective in inducingbiofilm formation than NaCl, and a combination of 2% NaCl +4% Glucose resulted in the highest biofilm production (Table 1). This effect was possibly due to the activation of some specific genes regulating transcription.

Table 1: Effect of Sugar & Salt concentration on the formation of Biofilm

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

	2% Glucose	4% Glucose	8% Glucose	10% Glucose	2% NaCl	4% NaCl	8% NaCl	10 % NaCl
OD	0.736±0.207	0.762±0.0.13	0.708±0.008	0.812±0.013	0.542±0.024	0.466±0.011	0.222±0.023	0.116±0.0207
	2% NaCl +4% Glucose	4% NaCl +8% Glucose	8% NaCl +16% Glucose	10% NaCl +20% Glucose	4% NaCl +2% Glucose	8% NaCl +4% Glucose	16% NaCl +8% Glucose	20% NaCl +10% Glucose
OD	0.944±0.018	0.822±0.008	0.794±0.008	0.714±0.011	0.626±0.015	0.608±0.029	0.584±0.011	0.404±0.015

Since the MIC has been used as a gold standard for determination of antimicrobial sensitivities for animal and human pathogenic bacteria (4)susceptibility of the working strain to antibacterial agents were tested, which showed that MIC value was found to be highest in *Ocimum sanctum*(Table 2), possibly due to the presence ofsome active compounds with antimicrobial activities like Saponins, glycosides and flavonoids (15). The MIC was higher in sessile forms for all antibiofilm agent tested as biofilms confer tolerance to antimicrobial agents. The highest MBC was found incase of *Ocimum sanctum*. (Table 2). Since the values of MBC were found to be more than the four times of respective MIC values, it could be concluded that all these antibiofilm compounds were bacteriostatic not bacteriocidal .

Table 2:MIC and MBC value of planktonic and sessile group of cells.

Antibiofilm Agent	Planktonic Cells		Sessile Cells	
	MIC (µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
Mikania scadens	0.25	9	0.50	132
Azardirachtica indica	0.16	9	0.52	128
Curcuma longa	0.20	4	0.70	128
Ocimum sanctum	0.06	4	0.80	120
Gentamycin	0.20	4	0.56	70

The minimum biofilm eliminating concentration (MBEC) assay also revealed the viable count of the cells when observed at a wavelength of 590 nm by challenging with triple combination of plant extract (A+M+C; 0.165±0.009) was found to have highest reduction rate of 73.5% whereas biofilm treated with antibiotic Gentamycin showed greater number of viable cells(0.354±0.02) and showed reduction of only 43.1%. It was seen that the planktonic cells were effectively killed after 24 hours of

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

exposure to antibiotic Gentamycin and ethanolic extracts of plants.This implied that the antibiotic failed to act upon the cells after the formation of biofilm as it formed an impermeable EPS that could prevent the penetration of the drug.(16,17).Although natural sources individually proved to be potent anti biofilm agent, a combination of three plant parts could successfully eliminate the biofilm (Table 3) .

Table:3 MBEC assay showing Percentage Reduction of the Biofilm forming cells when treated with various antibiofilm agents

OD at A_{590}	**Control**	**A**	**M**	**O**	**C**	**M+A+C**	**G**	**A+M**	**A+C**	**M+C**
	0.622± 0.194	0.254+ 0.01	0.227± 0.02	0.269± 0.006	0.269± 0.026	0.165± 0.009	0.354± 0.02	0.312± 0.029	0.215± 0.011	0.225± 0.01
Percentage reduction		59.2	63.65	56.8	56.8	73.5	43.1	49.9	64.6	59.1

M: *Mikania scadens,***A:** *Azardirachtica indica,* **O**:*Ocimum sanctum,* **C**:*Curcuma longa,* **G***:* Gentamycin

A gradual trend of cell number reduction was noted (Table 4) after the treatment with antibiofilm agent was found in the cells of *Staphylococcus aureus* (Table 4). The reduction was maximum in the cells exposed to the challenge of a combination of three antibiofilm plant extracts whereas cell reduction was not so prominent in gentamycin treated cells. The comparative data indicate the failure of antibiotic to act as effective antibiofilm compound

Table 4:Viability Count of Sessile Group of *S.aureus ATCC 0352* in the presence of the Challenge of Plant extract and Gentamycin(antibiotic)

Hrs	**No. of Ce**									
	M	**A**	**O**	**C**	**G**	**M+A+C**	**M+C**	**A+C**	**M+A**	**C**
0	7.31±0.01	7.32±0.01	7.31±0.011	7.3±0.005	7.29±0.011	7.32±0.012	7.34±0.011	7.33±0.011	7.33±0.001	7
2	7.31±0.005	7.29±0.015	7.28±0.005	7.27±0.011	7.28±0.011	7.28±0.010	7.33±0.005	7.31±0.011	7.33±0.011	7
4	7.28±0.005	7.28±0.015	7.27±0.005	7.26±0.020	7.27±0.005	7.27±0.011	7.34±0.005	7.31±0.005	7.32±0.005	
6	7.26±0.012	7.28±0.10	7.27±0.012	7.26±0.020	7.28±0.005	7.22±0.012	7.32±0.005	7.3±0.005	7.32±0.005	7
24	7.25±0.011	7.27±0.012	7.26±0.005	7.25±0.032	7.27±0.011	7.2±0.005	7.31±0.011	7.29±0.011	7.31±0.005	7

Table 5:Revival of the Organism after allowing the organism to grow without the challenge of antibiofilm agent

Hrs	**No. of Cells (CFU)**									
	M	**A**	**O**	**C**	**G**	**A+M**	**A+C**	**M+C**	**M+A+C**	**CONTROL**
0	7.3±0.001	7.31±0.005	7.33±0.011	7.3±0.001	7.31±0.001	7.35±0.012	7.36±0.005	7.36±0.005	7.31±0.001	7.34±0.003

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

2	7.3±0.011	7.3±0.013	7.32±0.001	7.3±0.015	7.31±0.001	7.36±0.013	7.35±0.001	7.35±0.005	7.3±0.001	7.36±0.032
4	7.29±0.001	7.3±0.005	7.32±0.001	7.31±0.005	7.32±0.18	7.34±0.011	7.32±0.012	7.34±0.018	7.29±0.005	7.4±0.005
6	7.29±0.001	7.28±0.018	7.31±0.005	7.3±0.001	7.32±0.001	7.33±0.010	7.32±0.013	7.34±0.001	7.26±0.001	7.42±0.001
24	7.28±0.005	7.28±0.011	7.29±0.013	7.27±0.003	7.33±0.011	7.33±0.005	7.31±0.005	7.33±0.002	7.25±0.012	7.45±0.012

On the other hand ,the growth kinetics of *S aureus* indicated that the antibiofilm agent treated cells did not show prominent revival after 24 hours of growth , even when these challenges were withdrawn, which suggested that these plant extracts could be used for
Scanning Electron micrographs of the biofilm produced by the working strain on chitin flakes (adherent) (Fig 1) and the destruction of biofilm (Fig 2) after treatment with plant extract (M+A+C) confirmed the antibiofilm nature of the plant products.

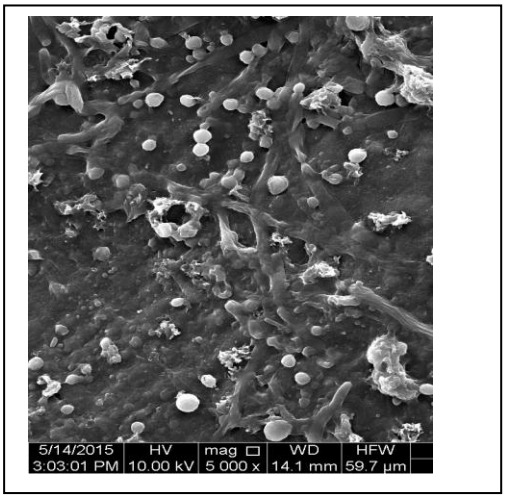

Fig 4:SEM confirming the formation of Biofilm on chitin flakes at 500 X

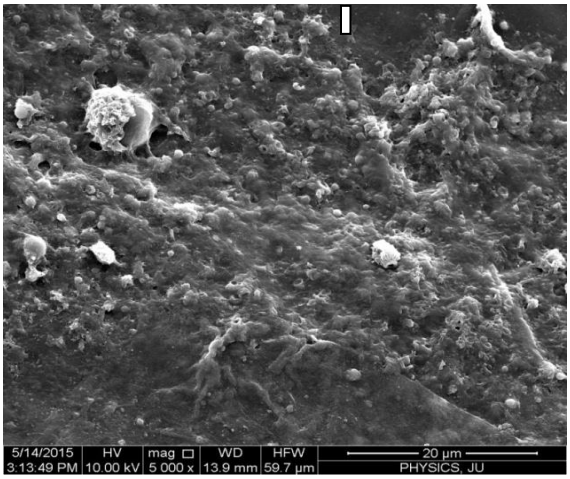

Fig 5:SEM confirming the eradication of Biofilm on chitin flakes on applying triple combination of plant extract at 500 X

Conclusion

The result showed that the plant extracts proved to be most effective source to check the biofilm producing activities of *Staphylococcus aureus* ATCC 0352 and was proved to be more promising than that of common antibiotic gentamycin. Considerable reduction in the sessile group after the treatment with a combination of different plant products made it more effective for using for

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

destruction of the potent human pathogen. Moreover the very slow and inconspicuous rate of bacterial revival after withdrawal of antimicrobial stress of plant extracts confirmed its efficacy in controlling bacterial propagation successfully.

References

1. Baker B.J., Banfield J.F(**2003**), *FEMS Microbiol Ecol,* Microbial communities in acid mine drainage. 44: 139.
2. Flemming, H.-C., Wingender, J., Griegbe, T., & Mayer, C. (2000). *Harwood Academic Publishers*,Physico-chemical properties of biofilms. *Biofilms: recent advances in their study and control.Amsterdam:*, 19-34.
3. O'toole, G. A. (2003). *Journal of bacteriology*,To build a biofilm.*, 185*(9), 2687-2689.
4. Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Science, Bacterial biofilms: a common cause of persistent infections. 284(5418), 1318-1322.
5. Raad R.O., Heard S.O., Thornby J.I., Wenker O.C., Gabrielli A. (**1999**), *N Engl J Med*. A comparison of two antimicrobial-impregnated central venous catheters. 340:1.
6. Ray R.R. (2015),Trends in Life Science,Biofilm Mediated Sequestering Of Metal Ions By *Klebsiella variicola* PRBC 14.Volume 4 (p)2319–5037
7. Dos Santos Goncalves M, Delattre C, Balestrino D, Charbonnel N, Elboutachfaiti R, et al. (2014), PLoS ONE, Anti-Biofilm Activity: A Function of Klebsiella pneumonia Capsular Polysaccharide. 9(6): e99995. doi:10.1371/journal.pone.0099995)
8. AL-Saadi`, Zainab Nashaat (2016**),**American Journal of Biomedical and Life Sciences,Estimation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Cell-Free Extracts of *Bifidobacterium* Species Against Methicillin-Resistant *Staphylococcus aureus in vitro.*, Volume 4, Issue 5, October 2016, Pages: 75-80
9. Mohammadzadeh A, Farnia P, Ghazvini K, Behdani M, Rashed T, Ghanaat J.(2006)J Med Microbiol, Rapid and low-cost colorimetric method using 2,3,5-triphenyltetrazolium chloride for detection of multidrug-resistant Mycobacterium tuberculosis. . 2006 Dec;55(Pt 12):1657-9.
10. Tang HJ, Chen CC, Ko WC, Yu WL, Chiang SR, Chuang YC(2011).Int J Antimicrob Agents, In vitro efficacy of antimicrobial agents against high-inoculum or biofilm embedded methicillinresistant Staphylococcus aureus with vancomycin minimal inhibitory concentrations equal to 2 μg/ml (VA2-MRSA).. 2011; 38(1):46–51).
11. Gomes F., Teixeira P, Ceri H. & Oliveira R. (2012). Indian J.Med. Res. 135542-547).
12. Baishya Rimashree, Bhattacharya Anusuya, Mukherjee Madhurima, Lahiri Dibyajit, Banerjee Soma, *Materials Today: Proceedings* ,Establishment of a simple reproducible model for antibiotic sensitivity pattern study of biofilm forming *Staphylococcus aureus in 3 (2016) 3461–3466*
13. Yong Lim, Malabendu Jana, Thanh T. Luong, and Chia Y. Lee(2004)JB Control of Glucose- and NaCl-Induced Biofilm Formatioby *rbf* in *Staphylococcus aureus*J Bacteriol. 2004 Feb; 186(3): 722–729.doi: 10.1128/.186.3.722-729.2004
14. Khangholi Mahdi andJamalli Ailar **(2016)** Jundishapur J Microbiol. 2016 Sep;. The Effects of Sugars on the Biofilm Formation of *Escherichia coli 185p* on Stainless Steel and Polyethylene Terephthalate Surfaces in a Laboratory Model. 9(9): e40137

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal

15. Kubmarawa, D, Ajoku GA, Enwerem NM, Okorie DA. Afr. J. Biotechnol. 2007, 6(14), 1690-1696
16. Anderl J. N., Franklin M. J & Stewart P. S (2000). Antimicrob. Agents Chemother. 441818–1824.
17. Ceri H., Olson M. E., Stremick C., Read R. R., Morck D. & Buret A(1999).. J. Clin. Microbiol. 371771–1776.

Received: 8 September Revised: 15 September Accepted: 20 September
Index in Cosmos
October 2018 Volume 8 Number 10
UGC Approved Journal