INTERNATIONAL JOURNAL OF CONSERVATION SCIENCE

ISSN: 2067-533X Volume 10, Issue 3, July-September: 485-492 www.ijcs.uaic.ro

BIOFILM STRATEGY FOR THE STREPTOSPORANGIUM SP. SURVIVAL IN THERMO-INDUCTION FROM 50 TO 75°C

Nik Him NIK RAIKHAN*

Faculty of Chemical Engineering, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor, Malaysia

Abstract

Malaysian locally isolated Streptosporangium roseum FETL-88 (Sr-FETL88) survived thermo-induction from 50°C to 75°C and produced very stable biofilm formation at 75°C. Therefore, we are projecting our novel finding of the thermal-barrier-biofilm that help promoted for the cell survival. SEM has shown that Sr-FETL88 was flagellated; having biofilm formation other than producing positive black colonies on Congo Red Medium (CRM). We studied the thickness of the biofilm with temperature increment by using SEM. The thickest of biofilm formation of 2.04±0.03mm was recorded with temperature of 75°C. Biofilm components tested to reveal the highest content of EPS (55%, 5.0 X 10^{10}cell/mL), lipid (25%, 17.8% of g/L dry weight) and various proteins (20%, 35.0g/mL). We have confirmed the findings by using ATR-FTIR spectra result. CLSM analysis of 24 hour biofilms of Sr-FETL88 grown at 75°C has been done with single section (z = 1μm) through a micro-colony growth of rod-shaped cells with the EPS stained with LPA lectin in green fluorescence. It is confirmed that the biofilm has been used by our Sr-FETL88 as a thermal-barrier to survive 75°C, a rare growth temperature for a Malaysian locally isolated species.

Keywords*: Biofilm; Streptosporangium roseum; Thermo-barrier; Protein thermostability*

Introduction

Sessile growth performed by microbial population has been developing many understandings on the characteristic and special formation of the growth which is better known as bacterial biofilm. The major feature observed on the biofilm formation is; it starts from free-floating cells growing into 'slime' due to cell-cell attachment supported by the surroundings such as the cell itself, some matrix, polymers, bioreactor wall even living and non-living thing. Biofilm arrangement is well promoted by lots of biochemical processes and its regulation. According to Garretta *et al.* [1]; "Although descriptions of biofilms have varied over the years, the fundamental characteristics are frequently maintained". The attachment or also called 'sticky effect' of one cell to the respective matrix, polymer or surface has been termed as 'adhesion' by Garretta *et al.* [1], and attachment of one cell to another cell was named 'cohesion'. The importance of this biofilm has been described in many journals and was commonly described as the cause of most infections especially to little kind of upper respiratory tract infections [2]. In this paper, we are projecting the biofilm as a way for Streptosporangium roseum FETL-88 (Sr-FETL88) cells get to survive thermo-induction from 50°C to 75°C and grew abundantly; which is very important to be reported scientifically. The ability to produce

* Corresponding author: raikhan7952@salam.uitm.edu.my

biofilm in broth medium has been visualized during our preliminary study on effect of temperature on the Sr-FETL88 cell size.

Materials and methods

Reagent, chemicals and pH

All material and chemicals were collected from various sources and quality as listed. Bisphenol A (BPA, R&M Chemicals, 98%), bacteriological peptone (ultrapure, protein = N x 6.38 ≥76.5%), ammonium phosphate monobasic (79546, ≥98%, Sigma-Aldrich), glycerol (Merck), Pseudomonas agar (Microbiology), nutrient agar (Merck), nutrient broth (Merck), potassium dihydrogen phosphate (KH_2PO_4, V90004, Vetec™ reagent grade, 99%), sodium hydroxide (NaOH, Fluka), yeast extract (Merck Milipore, granulated, for microbiology), 2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonic acid (Sigma-Aldrich), chitosan (low molecular weight, Sigma-Aldrich 448869), sodium chloride (NaCl, Sigma), sodium alginate (Sigma-Aldrich, W201502), calcium chloride (granular, ≤7.0mm, ≥93.0%, Sigma-Aldrich), glucose (Sigma-Aldrich, CAS Number: 50-99-7), peptone solution (Sigma-Aldrich, 42587), glacial acetic acid (Sigma-Aldrich, CH_3CO_2H),

Microorganism and growth medium

Streptosporangium roseum FETL-88 (*Sr*-FETL88) has been isolated from a hot spring in Malaysia with isolation temperature at 50°C. Growth medium contained 1.25% (w/v) glucose, 1.25% (w/v) yeast extract, 0.80% (w/v) NaCl [3] at pH = 6.0, shaking rates 200rpm and temperature varies from 50, 55 to 75°C.

Liquid-interface of nylon scouring pad assay

Modification of method by Taj *et al.* [4] has been applied to the use of 2mm thick of nylon scouring pad; 1.0X1.0cm^2. The concept of this assay is to measure the thickness of the biofilm adhered to nylon scouring pad after 24 hours of experimental procedure (pH = 6.8, temperature of 50, 55 to 75°C). The medium used was the growth medium with 6.0% (v/v) inoculum (5.0x10^8cells/mL). Nylon cuts were sterilized and added into the fermentation medium in ratio of 3:50 mL growth medium. The *Sr*-FETL88 biofilm formation was visualized under SEM and recorded in millimeter (mm) thickness and surface covered area over 1.0X1.0cm^2 using method by Nik Raikhan and Khairul Izwan [5].

Congo red assay method

The main ingredient of the Congo red medium was 37.0g/L of brain heart infusion broth (BHI, Oxoid Ltd, Hampshire, England), 50.0g/L sucrose, 10.0g/L Agar No 1 (Oxoid Ltd, Hampshire, England) and 0.80g/L of Congo red (BDH Chemical Ltd, Poole, England). The method by Yasmeen *et al.* [4] has been modified to adapt to the need of biofilm assay with *Sr*-FETL88. The Congo red stain was sterilized (121°C for 15 minutes) separately from the medium components and supplemented to the agar at 55°C. Thickness of the agar was 5mm prepared in glass plates to avoid expression of unwanted gasses at 75°C. The production of black colonies with a dry crystalline consistency by the organisms was taken to indicate biofilm production as non-biofilm-producing strains develop red colonies [6].

Scanning Electron Microscope (SEM).

24 hour culture of *Sr*-FETL88 biofilm was fixed on McDowell-Trumph Fixative prepared in 0.1M (pH = 7.2) phosphate buffer (24 hours, 4°C) [3]. The fixation was washed 3 times with phosphate buffer (10 min interval). The culture was fixed using 1% osmium(VIII) oxide (OsO_4) for 2 hours followed by double washing with dH_2O (10min interval). Dehydration process was executed using 50% ethanol (15min), 75% ethanol (15min), 95% ethanol (2 times, 15 min) and 100% ethanol (3 times, 30 min) [3]. The tissue was submerged in ethanol during movement to Critical Point Drying and was kept in for 24 hours. Dried specimen was attached to the specimen stub using double sided tape and was covered with gold and ready to be viewed under SEM (Leica, Cambridge).

Transmission Electron Microscope (TEM)

TEM was done to detect the appearance of flagella structure on the *Sr*-FETL88. The 24 hour culture of *Sr*-FETL88 biofilm was fixed on McDowell-Trumph Fixative prepared in 0.1M (pH = 7.2) phosphate buffer (24 hours, 4°C) [3]. The fixation was washed 3 times with phosphate buffer (10min interval). The culture was fixed using 1% osmium(VIII) oxide (OsO4) for 2 hours followed by double washing with dH_2O (10min interval). Dehydration process was executed using 50% ethanol (15min), 75% ethanol (15min), 95% ethanol (2 times, 15 min) and 100% ethanol (2 times, 30min) and 100% acetone (2 times, 10 min). The culture was kept in the rotating machine for 30min [3]. The second rotation was done with mixture of Spurr resin; overnight and the last one with another mixture of Spurr resin for 5 hours on the rotating machine.

FTIR-ATR Spectroscopy and Confocal Laser Scanning Microscopy

Attenuated Total Reflectance - Fourier Transform InfraRed (ATR-FTIR) spectroscopy was used to confirm EPS, proteins and lipid contents in *Sr*-FETL88 biofilm as well as to study the in situ and in real time and biochemical properties of biofilms. Method was from Andersson *et al.* [7]. Confocal Laser Scanning Microscopy (CLSM) to determine the biofilms communities adhered to a surface was done with modification of method by Fernanda *et al.* [8]

Biofilm Content Test

Content of the biofilm was tested using Bradford protein assay for protein [9], crystal violet (CV) assay for exopolysaccharides [10], lipid assay for lipid content [12] and glycogen test for glycogen content [12].

Relation between biofilm thicknesses with temperature increment on fixed cell concentration

Study on the biofilm thickness was performed with liquid-interface of nylon scouring pad assays at 50-75°C. The nylon was cut into 2.0mm thickness and a size of $1.0X1.0cm^2$. Correlation between biofilm thickness, temperature and specific growth rate was studied.

Results and discussion

Confirmation of biofilm producer

Biofilms which commonly look like slimy layer of growth are a group of microbial mono-species or multi-species representing the most successful colonization among microorganisms. They are very ubiquitous in most selective nature and have been responsible for quite many contamination, colonization and integration. *Sr*-FETL88 has been confirmed as mono-species biofilm producer and didn't rely on other species to start an adhesion from planktonic cells. *Sr*-FETL88 has produced black colonies with dry crystalline on Congo red medium (Fig. 1).

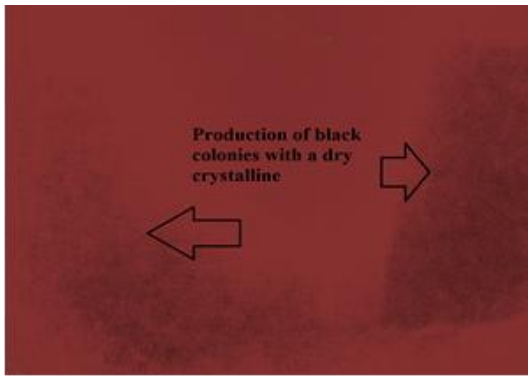


Fig. 1. Congo red medium test to support biofilm production

This characteristic has confirmed the cell ability to perform biofilm integration. The production of black colonies with a dry crystalline is consistence to indicate biofilm production as non-biofilm-producing strains will develop red colonies instead of black colonies [6, 14].

Confirmation of the EPS, proteins and lipid contents in *Sr*-FETL88 biofilm has to be done to characterize the contents and we used ATR-FTIR for the purpose. The confirm content of *Sr*-FETL88 biofilm 53% EPS, 32% various proteins and 15% lipid. The spectra result is depicted in Figure 2. EPS has been circled in green.

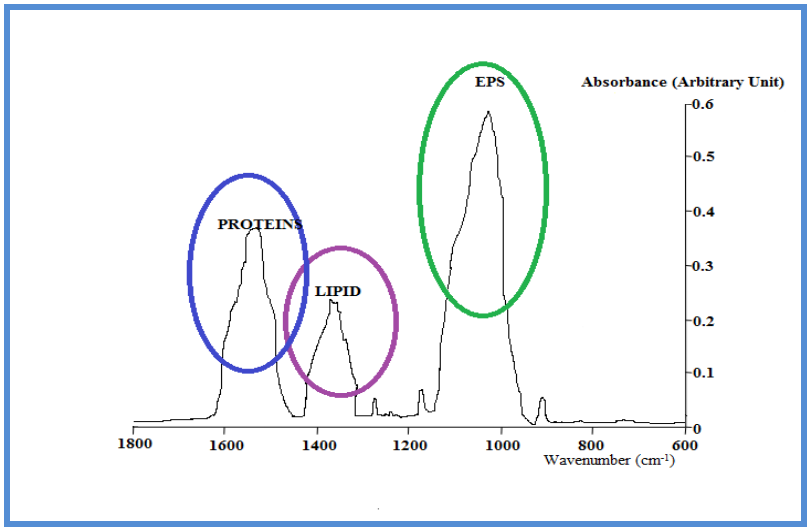


Fig. 2. Confirmation of EPS, proteins and lipid contents in *Sr*-FETL88 biofilm from ATR-FTIR spectra result.

Planktonic Cells

After detail investigation on planktonic or free cell of *Sr*-FETL88, we have successfully engaged its flagella on the 18 hours cells. Fig. 3 depicted 2 flagella spotted at the cell outer layer of the *Sr*-FETL88. This finding confirmed the ability to float and attached on surfaces and the scouring pad we used as a base to study the mechanism. 'Any natural or synthetic surface is covered by the constituents of the local environment, electrolytes, water and organic materials form a film before the arrival of the organisms which neutralize the charge over the surface (conditioning) that prevents the approximation between bacterial cells fungi and so begins adherence, these organic compounds can serve as nutrients for these microorganisms' [3]. Microbes with flagella have been related to the ability of producing biofilms. The cultural morphology of biofilm-forming bacteria is commonly distinct and doesn't appear the same as those strains which do not form biofilms. In this study, we have been observing cells of Sr-FETL88 attaching themselves to the scouring pad and the shake flasks solid surfaces. We believed that the cells have been forming a micro-aggregation to initiate the process. TEM has proved the *Sr*-FETL88 is flagellated (Fig. 3). Flagella-driven motility is shown as the 'equipment' for lots of biofilm producers to initiate a contact within the systems and towards the solid surfaces. Chen *et al.* [13] has reported *E.coli* which is lacking of flagella is not with ability to produce biofilm.

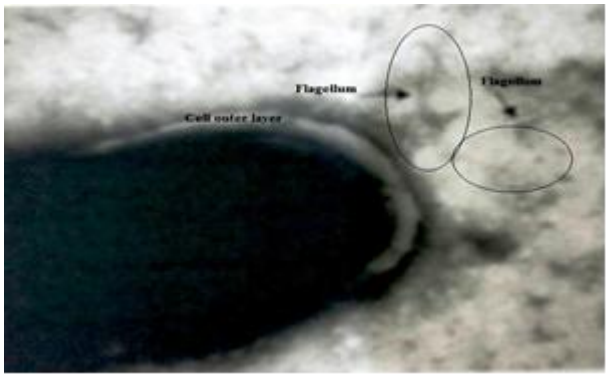
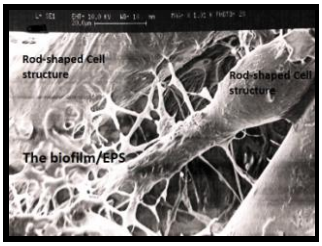
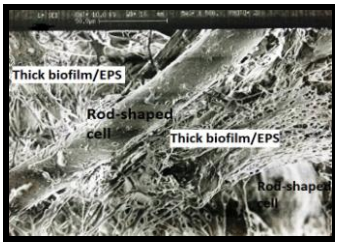
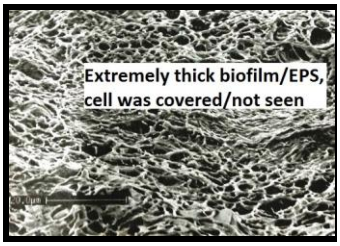


Fig. 3. Flagella were detected on *Sr*-FETL88 confirming ability to float and attach to surfaces.

Bacterial Adhesion

The bacterial adhesion and physical characteristic of *Sr*-FETL88 biofilm production during the thermo-induction has been investigated using the Scanning Electron Microscope (SEM). The planktonic cells will first develop a thin layer that is adsorbed to the surface using some electrostatic forces to attract more cells towards the layer. This form is layer is commonly reversible therefore the absorption may lose the static and letting go the forces making it planktonic cells again. *Sr*-FETL88 has used the repulsive force on the flagella together with the lipopolysaccharide in outer layer of cells. The EPS reported earlier in Fig. 2 has been confirmed to be enhancing the process towards irreversible adhesion until cells received enough interaction using the chemical signals through the flagella. Once the minimum number is achieved, the cells divided and colonized the surface of the scouring pad suggesting that the microbial population density has formed minimum critical biofilm layers successfully. Table 1 shows the details of the formation of biofilm at 55°C, 65°C and 75°C. The thickness of the layers varies with the temperature used.

Table 1: Physical Characteristic of *Sr*-FETL88 Biofilm under SEM Using Thermo-Induction Process

55°C	65°C	75°C
SEM proved the growth with increment of 5°C above the isolation temperature has been supported with mild biofilm formation.	The thickness of biofilm has increased significantly at 65°C	Biofilm has been spreading all over the surfaces of scouring pad and cells supporting the growth at 75°C
		

Synthesis of extracellular matrix

Table 2 shown CLSM analysis of 24 hour biofilms of Sr-FETL88 grown at 75°C. This result is significant with a result recorded by Fernanda *et al.* [8]. Quantitative values for different types of extracellular polymeric substances from *Sr*-FETL88 biofilm grown at 75°C has been checked and confirmed using crystal violet (CV) assay, Bradford assay, lipid test and glycogen test using various methods from different scientists. According to Pantanella *et*

al.[16], 'CV staining is one of the first methods adopted for biofilm biomass quantification". Table 3 shows quantitative values for different types of extracellular polymeric substances from Sr-FETL88 biofilm grown at 75°C. Percentage of protein content of the EPS was valued at 20% using Bradford protein assay [9]. Other polymeric substances were recorded as 0.5% for glycogen, 24.5% for lipid and 55% for exopolysaccharides with quantitative result were valued as very low, 17.8% of g/L dry weight and 5.0X1010cell/mL; respectively.

Table 2. CLSM analysis of 24 hour biofilms of *Sr*-FETL88 grown at 75°C

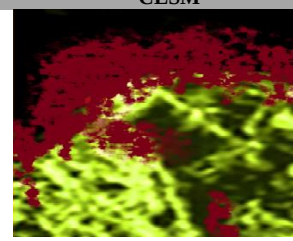
Details	CLSM
CLSM analysis of 24 hour biofilms of *Sr*-FETL88 grown at 75°C on scouring pad stained with fluorescently labeled lectin. Shown was the single section (z = 1 μm) through a microcolony growth of rod-shaped cells with the EPS stained with LPA lectin in green fluorescence followed by nucleic acid counterstained with EtBr (red). The scale box was 5 μm.	

Table 3. Quantitative Values for Different Types of Extracellular Polymeric Substances from *Sr*-FETL88 Biofilm Grown at 75°C

Types of Extracellular Polymeric Substances	Method of Detection	Quantitative Results	Percentage of total content (%)
Proteins	Bradford protein assay	35.0 g/ml	20
Exopolysaccharides	Crystal violet (CV) assay	5.0 X 10^{10} cell/ml	55
Lipid	Lipid assay	17.8% of g/L dry weight	24.5
Glycogen	Glycogen test	Very low, almost not detected	0.5

Maturation and dispersion

Table 4 show the correlation between biofilm thicknesses with temperature increment when nylon cut was maintained at 2.0 mm thickness and a size of 1.0 X 1.0 cm2. The result has significantly showed that thicknesses of the *Sr*-FETL88 biofilms have gradually increased with the increment of temperatures from 50-75°C. The thickest biofilm formation 2.04±0.03 mm was recorded with temperature of 75°C. We have recorded a very significant increment of biofilm thickness together with the specific growth rate as the temperature was increased. This proved that biofilm was produced abundantly with more cell numbers at all increasing temperatures. *Sr*-FETL88 survived thermo induction and obviously depends on the formation of the biofilm to steadily grow out of its normal growth temperature range (55-75°C). According to Hall-Stoodley *et al.* [15], they believed biofilm organization significantly depicted the mode of cell growth; somehow giving a positive interaction to wider survival rates in most hostile environments, disperse to form new niches including giving them significant, advantages in protection against environmental fluctuations such as humidity, temperature, pH, the concentration of nutrients and waste removal.

Table 4: Correlation between biofilm thicknesses with temperature increment

Temperature (°C)	Biofilm Thickness (mm)	Specific Growth Rate, μ (h^{-1})
50	1.01± 0.01	0.39
55	1.09 ± 0.03	0.40
60	1.56 ± 0.01	0.41
65	1.67 ±0.05	0.42
70	1.89 ± 0.09	0.48
75	2.04 ± 0.03	0.45

Conclusion

Research on microbial biofilm is well emphasized but was not valued as a strategy for lots of microorganism's heat survival. It was not even becoming important to any scope of research until the scientist found out the importance of some biofilms for the purpose of membrane like function in certain waste water treatment. In this finding, we have confirmed that the biofilm has been used as a thermo-barrier to survive the increment of the temperature from 50 to 75°C, a rare growth temperature for a Malaysian locally isolated species. The *Streptosporangium roseum* FETL-88 (*Sr*-FETL88) has been ackowldeged as a potential species for a production of sterile film for a waste water treatment scope therefore the finding reported in this journal will be an advance process for the whole waster water treatment possibility at moderately high temperature.

Acknowledgment

The author gratefully acknowledges the USM for the research to take place, the Ministry of Higher Education and RMC of UiTM for funding. Humongous thanks go to the team who has been supporting the work and this research

References

[1] T.R. Garretta, M. Bhakoob, M. Zhanga, Bacterial adhesion and biofilms on surfaces, **Progress in Natural Science, 18**(9), 2008, pp. 1049-1056.

[2] B. Prakash, B.M. Veeregowda, G. Krishnappa, Biofilms: A survival strategy of bacteria, **Current Science, 85**(9), 2003, pp. 1299-1307.

[3] N.H. Nik Raikhan, *Production of Hyperthermotolerant Lipase from Actinomycete Streptosporangium roseum*, **M.Sc. Thesis**, Universiti Sains Malaysia, Penang, Malaysia, 2003.

[4] T. Yasmeen, E. Farhan, A. Faisal, A. K. Shahana, Study on biofilm-forming properties of clinical isolates of *Staphylococcus aureus*, **The Journal of Infection in Developing Countries, 5**(6), 2012, pp. 403-409.

[5] N.H. Nik Raikhan, A.R. Khairul Izwan, *Novel Treatment of Heavily Oiled Wastewater Using Pseudomonas aeruginosa NR.22 Producing Usable Free Fatty Acids (FFA)*, **International Journal of Conservation Science**, **8**(3), 2017, pp. 537-544.

[6] T. Mathur, S. Singhal, S. Khan, D. J. Upadhyay, T. Fatma, A. Rattan, A., *Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods*, **Indian Journal of Medicine and Microbiology**, **24**, 2006, pp. 25-29.

[7] S. Andersson, G. K. Rajaro, C. J. Land, D. Gunnel, *Biofilm formation and interactions of bacterial strains found in wastewater treatment systems*, **FEMS Microbiology Letters, 283**(1), 2008, pp. 83-90.

[8] G. Fernanda, P. Sofia, T. Pilar, O. Oliveira, *Confocal laser scanning microscopy analysis of S. epidermidis biofilms exposed to farnesol, vancomycin and rifampicin*, **BMC Research Notes, 5,** 2012, pp. 244.

[9] M.M. Bradford, *A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding*, **Analytical Biochemistry, 72**, 1976, pp. 248-254.

[10] K. Chiba, K. Kawakami, K. Tohyama, *Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells*, **Toxicology in Vitro, 12**(3), 1988, pp. 251-258.

[11] T. Matsushita, S.I. Inoue, R. Tanaka, *An Assay Method for Determining the Total Lipid Content of Fish Meat Using a 2-Thiobarbituric Acid Reaction*, **Journal of the American Oil Chemists' Society, 87**(9), 2010, pp. 963-972.
[12] M.C.Timothy, J. H. Exton, *A rapid method for the determination of glycogen content and radioactivity in small quantities of tissue or isolated hepatocytes*, **Analytical Biochemistry, 71**(1), 1976, pp. 96-105.
[13] C.Y. Chen, C.F. Hofmann, B.J. Cottrell, T.P. Jr. Strobaugh, G.C. Paoli, L.H. Nguyen, X.H. Yan, G.A. Uhlich, *Phenotypic and genotypic characterization of biofilm forming capabilities in non-O157 Shiga toxin-producing Escherichia coli strains*, **PLoS ONE, 8**(12), 2007, Article Number: e84863. doi: 10.1371/journal.pone.0084863.
[14] T.D. Kaiser, E.M. Pereira, S.K.R. Dos, E.L. Maciel, R.P. Schuenck, A.P. Nunes, *Modification of the Congo red agar method to detect biofilm production by Staphylococcus epidermidis*, **Diagnostic Microbiology and Infectious Disease**, **75**(3), 2013, pp. 235-239.
[15] L. Hall-Stoodley, W.J. Costerton, P. Stoodley, *Bacterial biofilms: from the natural environment to infectious diseases*, **Nature Reviews. Microbiology**, **2**(2), 2004, pp. 95-108.
[16] F. Pantanella, P. Valenti, T. Natalizi, D. Passeri, P. Berlutti, *Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use*, **Annali di igiene : medicina preventiva e di comunità**, **25**(1), 2013, pp. 31-42.

Received: March 18, 2018
Accepted: August 24, 2019