BIOFILM ORGANIC ENRICHMENT IN VITRO

Aurelia Manuela Moldoveanu

FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
„OVIDIUS” UNIVERSITY, CONSTANTZA

Summary

Biofilms are usually found on solid substrates submerged in or exposed to
an aqueous solution, although they can form as floating mats on liquid surfaces and also on
the surface of leaves, particularly in high humidity climates. For this study the experimental
system used was represented by 100 ml containers filled with seawater, used as culture
media in static conditions. The sample surfaces were hydrophile microscope slides
sterilized in sulfochromic mixture. The main factor investigated was organic matter
influence on bacterial cell growth in low quantities between 3 - 9 mg/L. After harvesting all
the sample surfaces were immediately analyzed with optic microscopy. The biofilms
formation begins after a few hours after the surface immersion and the bacterial growth and
attachment is continuous in the interval 24 h - 48 h when the exponential growth phase was
established, after this interval at 72 hours bacterial density is lower usually. The bacterial
density obtained was of $10^3$ and $10^4$ cells/mm$^2$.

Key words: biofilm, surfaces, organic matter, nutrients, cell density

Introduction

The formation of a biofilm begins
with the attachment of free-floating
microorganisms to a surface. The biofilm is
held together and protected by a matrix of
secreted polymeric compounds. This matrix
protects the cells within it and facilitates
communication among them through
biochemical signals (Azua et al., 2003).
Some biofilms have been found to contain
water channels that help distribute
nutrients and signaling molecules (Allison
et al., 1987; Allison, 2003).

In this aquatic environment the
undesirable colonization and accumulation
of organic molecules, microorganisms,
plants and animals on natural and artificial
surfaces form biofouling (Carlson, 1983;
Callow, 1996; Bachmann et al., 2005). This
complex process occurs in three main
stages. The first stage there is a rapid
formation of a conditioning film by
accumulation of organic molecules
(Characklis 1990; Lazar, 2003).

Bacterial colonization on abiotic
materials such as suspended particles, metal
surfaces and concrete or biotic surfaces was
thought to be one of the microbial survival
strategies because it provides
microorganisms with important survival
strategies, including 1) increased access to nutrients,
2) protection against toxins and antibiotics,
3) maintenance of extracellular enzyme
activities 4) shelter from predation (Zamea,
1994; Dang and Lovell, 2000). For these
reasons, surfaces in contact with water are
rapidly colonized by bacteria.

Although the presence of a previous
stage is not a prerequisite for a subsequent
stage, the order reflects the length of time
taken for attachment and the abundance of
each component (Roszak and Well, 1987;
Carvalho et al., 2010, Satheesh and Wesley,
2010).

The fact that many bacteria multiply
and are otherwise physiologically active in
very dilute nutrient solutions is manifest
from the abundance of bacteria in fresh
and seawater. The organic content of seawater is generally less than 5 mg/l due to the oligotroph characteristics of this water, yet during the storage of seawater in the laboratory the bacterial population usually increases also is considered that the concentration of food does not influence the rate of growth of bacteria except when the concentration is very low of 0.01 to 0.1% of organic matter (Hoppe et al. 1988).

Some marine environments contain only dilute substances that can be used for metabolism and growth. In contrast, natural surfaces tend to collect and concentrate nutrients by charge-charge or hydrophobic interactions (Penfold and Norris 1912; Kinner, 1983; Patel, 2003).

The aim of this study was the determine influences of different types of organic matter in the first phases of bacterial colonization and biofilm formation in laboratory static conditions.

**Materials and methods**

In order to observe the role of organic substance on biofilms formation in static conditions some experiments were realized by using containers with fresh seawater as culture medium. The artificial surfaces (microscope slides) were previously degreased with 70% ethanol (Lazar et al. 2004) and sterilized by immersion in sulfochromic mixture (K₂Cr₂O₇/H₂SO₄) to avoid contamination with microorganisms and organic matter prior to the experiment (Mercier-Bonin, 2004).

The natural sea water was organic matter influence on the marine bacteria attach to the artificial surfaces, seawater was enriched with low concentrations of 0.1% organic matter of five different types: amino-acid mixture, yeast extract Difco, bactotryptone Difco, glucose Merck and starch Merck. The organic solutions were prepared by adding 100 mg of organic matter in sterile containers 100 ml distillate water and after mixture low quantities of 3 mg/L, 5 mg/L, 7 mg/L and 9 mg/L were added to the containers with fresh seawater (fig.1). All the substances were filtered before use with membrane filters 0.22 μm Millipore.

The slides were stain without fixation immediately after harvesting with one drop of 0.1 % Methylene Blue by capillarity staining between slide and cover slips.

In the experiments the Henrici Slide Technique was used as culture method for obtaining bacterial biofilm on the smooth surface of glass by submersion of the surfaces in seawater as show by www. Biofilms ONLINE.com 2008, and in order to avoid deposing debris and extra bacterial cell attach the surfaces were placed on the diagonal in the containers as use previously by Moldoveanu (2011).

In order to obtain data about the first phases in biofilms formation under nutrient influence as a limiting factor, these were investigated for two hours to 24 hours at a two hours interval and with the from 24 hours to 72 hours for the of the slides harvesting at four hours intervals.

The slides were analyzed under bright field light at the Hund Wetzlar Microscope with 100× objective and 10× ocular (Hulea, 1969, Yuehuei, 1997). The number of bacteria was determined by means of the 10m×10mm micro-ocular grid (macroscopically), investigating 10 microscopic fields per harvested slide and with three repetitions for interval time (Fry, 1990).
Results and Discussions

The biofilms formed on the collected surfaces from the containers with littoral seawater were analyzed by optic microscopy and emphasized the existence of successive phases in biofilms formation. The marine bacteria have various types in the first phases and tend to form microcolonies and secrete the exopolysacharide matrix.

In the control probe the biofilms are formed in the natural seawater source and the bacteria attach in the normal conditions without any organic addition that can determine modification in the growth and cell density on the artificial surface of the glass slides.

The bacterial cell density values indicate that biofilms had an important cell growth and attachment for the different types of organic matter used as enrichment source. For the bacterial cell growth under the influences of organic matter (three types of protein and two types of carbohydrates sources) the main interval of growth of two, 24, 48 and 72 hours were chosen compare with the control probe (fig. 2).

In the control probe for the amino acids mixture after two hours the bacterial cell density was $0.98 \times 10^3$ cells/mm$^2$ after 24 hours this values is double with a value of $3.27 \times 10^3$ cells/mm$^2$ at 48 hours the density reaches the value of $5.25 \times 10^3$ cells/mm$^2$ and after 72 hours at the end of the interval the density value was lower of $4.91 \times 10^3$ cells/mm$^2$ (fig. 2).

The addition of various concentration of amino acids mixture determined a bacterial cell growth was between 1.12 and 1.24 $\times 10^3$ cells/mm$^2$ for the first two hours, of $4.28 - 6.91 \times 10^3$ cells/mm$^2$ at 24 hours, between $5.25 - 10.12 \times 10^3$ cells/mm$^2$ after 48 hours and a bacterial cell final decreases at 72 hours between 5.41 and 8.72 $\times 10^3$ cells/mm$^2$.

The second substance added was the tryptone and in the control probe after two hours the bacterial cell density was $0.56 \times 10^3$ cells/mm$^2$ after 24 hours this values is double with a value of $3.24 \times 10^3$ cells/mm$^2$ at 48 hours the density reaches the value of $5.11 \times 10^4$ cells/mm$^2$ and after 72 hours at the end of the interval the density value was lower of $4.23 \times 10^3$ cells/mm$^2$ (fig. 3).

The addition of various concentration of amino acids mixture determined a bacterial cell growth was between 1.12 and 1.24 $\times 10^3$ cells/mm$^2$ for the first two hours, of $4.28 - 6.91 \times 10^3$ cells/mm$^2$ at 24 hours, between $5.25 - 10.12 \times 10^3$ cells/mm$^2$ after 48 hours and a bacterial cell final decreases at 72 hours between 5.41 and 8.72 $\times 10^3$ cells/mm$^2$.
A third substance used to observe bacterial cell growth was the yeast extract and in the control probe after two hours from imersion the bacterial cell density was $0.63 \cdot 10^3$ cells/mm$^2$ after 24 hours this values is double with a value of $3.19 \cdot 10^3$ cells/mm$^2$ at 48 hours the density reaches the value of $5.22 \cdot 10^3$ cells/mm$^2$ and after 72 hours at the end of the interval the density value was lower of $4.33 \cdot 10^3$ cells/mm$^2$ (fig. 4).

The protein substances have major influence on the bacterial cell growth and attachment during the first phase of the biofilm formation.

In order to observe the influences of carbohydrates on the bacterial cells at first glucose was used and in the control probe after two hours from imersion the bacterial cell density was $0.87$ and $0.92 \cdot 10^3$ cells/mm$^2$ for the first two hours, of $3.92 - 5.23 \cdot 10^3$ cells/mm$^2$ at 24 hours, between $5.12 - 7.32 \cdot 10^3$ cells/mm$^2$ after 48 hours and bacterial cell final decreases at 72 hours between $5.55$ and $7.5 \cdot 10^3$ cells/mm$^2$.

The second carbohydrate added was the starch and in the control probe after two hours after imersion the bacterial cell density was $0.73 \cdot 10^3$ cells/mm$^2$ after 24 hours this values is $3.22 \cdot 10^3$ cells/mm$^2$ at 48 hours the density reaches the value of $5.52 \cdot 10^3$ cells/mm$^2$ and after 72 hours at the end of the interval the density value was lower of $4.21 \cdot 10^3$ cells/mm$^2$ (fig. 6).
The addition of various concentration of starch determined a bacterial cell growth was between 0.88 and 1.52 \( \cdot 10^3 \) cells/mm\(^2\) for the first two hours, of 3.92 - 5.65 \( \cdot 10^3 \) cells/mm\(^2\) at 24 hours, between 5.12 -7.02 \( \cdot 10^3 \) cells/mm\(^2\) after 48 hours and a bacterial cell final decreases at 72 hours between 4.89 and 7.61 \( \cdot 10^3 \) cells/mm\(^2\).

In late phases the biofilm cells have a thick bacterial wall due to a high metabolism activity and attachment. The bacterial cells attach on the artificial surfaces absorbed a large quantity of stain as well. This is observed especially in the case of the amino acids high concentration of 9 mg/L (fig.7).

**Conclusions**

The use of different concentrations of organic matter from determined large differences between \( 10^3 \) – \( 10^4 \) cells/mm\(^2\) in the bacterial density.

Marine heterotrophic bacteria prefer the substances with a low molecular weight like amino acids and glucose due to their rapid availability for bacterial cell metabolism.

The substances with a high molecular weight are use more slowly and by a low number of bacteria due to their low availability and the necessity to use enzyme in their degradation.

The use of high quantities of organic matter has determined a high bacterial cell growth and attachment due to substances rapid availability for the marine bacteria even in laboratory static conditions.

**References**


Carvalho C. C. R. and Fernandes P., Production of Metabolites as Bacterial Responses to the Marine Environment, Mar. Drugs, 8, 705-727, 2010.

Characklis W.G., McFeters G.A., Marshall K.C., Physiological ecology in biofilm...


Zarnea Gh. General Microbiology, Microbial ecology, 5, Romanian Academy publish. house, Bucharest, 1994.