THE INFLUENCE OF *MYTILUS* EXTRACT ON BIOFILM CELLS ATTACHMENT

Aurelia Manuela Moldoveanu

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

Summary

Seawater has a low organic matter concentration in suspension. Marine bacteria attachment on surfaces is influenced by the presence of organic substances at the liquid – solid interface level that determines chemoattraction and chemotaxy towards the surfaces where bacterial cells form biofilms. This study was achieved in order to observe the influence of crude extract of *Mytilus* with natural organic substances on bacterial cells attachment and growth on artificial surfaces. The crude extract was made from viscera and muscles of *Mytilus galloprovincialis* in distillate water and was use in low quantities. Experiments were realized in static condition in 100 ml containers filled with sea water as culture medium. The variety of low quantities of organic extract determined a bacterial growth of $10^3$ cells/mm$^2$ and attachment on the sample surfaces had higher values especially at 7 mg/l and 9 mg/l additions. The main bacterial cells attachment was accomplish by bacilli type bacteria compare to the cocci, spirillum and filamentous forms with a lower density values and the total cell growth under the influence of the natural extract was between $42.6 \cdot 10^3$ cells/mm$^2$ and $78.8 \cdot 10^3$ cells/mm$^2$.

Key words: biofilm, static methods, *Mytilus*, extract concentration

Introduction

In aquatic environment the undesirable colonization and accumulation of organic molecules, microorganisms, plants and animals on natural and artificial surfaces form biofouling (Callow, 1996). This complex process occurs in three main stages. The first stage is a rapid formation of a conditioning film by accumulation of organic molecules.

Then during the second stage, microbial biofilms develop on the conditioning film. The final stage of biofouling involves settlement and growth of macrofouling organisms (Characklis 1990; Lazar, 2003).

Many marine environments contain only dilute substances that can be used for metabolism and growth (Raja et al., 2009 Khare and Kumar 2010). In contrast, natural surfaces tend to collect and concentrate nutrients by charge-charge or hydrophobic interactions (Penfold and Norris 1912; Kinner, 1983; Patel, 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 advantages, including 1) increased access to nutrients, 2) protection against toxins and antibiotics, 3) maintenance of extracellular enzyme activities 4) shelter from predation (Zarnea, 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. As bacteria are primary colonizers, they can positively or negatively affect settlement of macrofouling organisms (Roszak and Well, 1987; Carvalho et al., 2010). Many studies have been carried out
to explore how microbial biofilms and specific strains of bacteria affect settlement and growth of invertebrate larvae (Heukelekian and Heller, 1940; Hagstrom et al., 1984) but there have been relatively few studies on marine algae and marine invertebrates influence on heterotroph bacteria.

The crude extract of marine organisms are an important source of organic matter for the bacterial metabolism in situ to there contribution to dissolved organic matter concentration in seawater (Watermann, 1999; Chambers et al. 2006).

The crude extracts are a surce of carbon present in any type of aquatic environments that naturally supply the water rapidly use by the heterotroph bacteria.  

In this study was performed in order to determine the role of crude organic matter extract on bacterial cells attachment and growth on artificial surfaces in static laboratory conditions.

**Material and methods**

In order to observe the role of organic substance on biofilms formation in static conditions were realized for the experiments 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).

*Mytilus galloprovincialis* is known as Mediterranean mussel (Celik et al. 2009; Gavrilovic et al. 2011). The crude mussel extract was made of *Mytilus galloprovincialis* from muscles and viscera that were cut into small pieces mixed with in distillate water and later homogenized with the aid of a Potter Elvehjem homogenizer device (Fig.1).

After preparation the extract was sterilize at 120°C for 20 minute in the autoclave to avoid sample contamination with organic matter and bacteria prior to the experiments. In order to obtain biofilms on the smooth surfaces of the microscope slides, fresh seawater from the Black Sea (Eforie Nord) was used as culture medium littoral, kept in as shortly as possible in sterile containers and enriched with 0.1% crude *Mytilus* extract in distillate water filtered with 0.22µm Millipore membrane filters (Fig.2).

The additions of crude extract were of 3 mg, 5 mg, 7 mg and 9 mg per liter of fresh seawater in order to obtain a multiplication under normal concentrations of the organic matter similar to those in situ conditions.

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 depositing 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 2 hours to 24 hours at a 2 hours interval and with the from 24 hours to 72 hours for the of the slides harvesting at 4 hours intervals.

The slides were analyzed under bright field light at the Hund Wetzlar Microscope with 100X objective and 10X ocular (Hulea, 1969, Yuehuei, 1997). The number of bacteria was determined by means of the 10mm X 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 bright field analysis of the biofilms formed on the collected surfaces from the containers with littoral seawater emphasized the existence of successive phases for the formation of biofilms, which display an important increase of bacterial density after a period of only three days and the biofilm formed visible without fixation (Fig.3).

The marine bacteria have various types in the first phases and tend to form microcolonies and secrete the exopolysacharide matrix.

The main cell attach on the artificial surfaces were de bacilli, which form the main part on the biofilm on the artificial surfaces, but also a large number of spirillum and cocci bacteria can be fount in the biofilms layers.

In the control probe after only two hours after imersion the bacterial cell density was $0.85 \cdot 10^3$ cells/mm$^2$ after 6 hours this values is double with a value of $1.91 \cdot 10^3$ cells/mm$^2$ at 14 hours the density reaches the value of $2.84 \cdot 10^3$ cells/mm$^2$ a first pick is reaches at 24 hours when the density is $3.86 \cdot 10^3$ cells/mm$^2$ at 48 hours the pick is $5.28 \cdot 10^3$ cells/mm$^2$ the highest value in the control probe and after 72 hours at the end
of the interval the density value was lower of 3.98 \times 10^3 \text{cells/mm}^2 (Fig.4).

The values obtain shown that the density values in normal condition determined an optimal cell growth and attachment of the marine bacteria and the equation of growth was high of 0.32 and also the linearity coefficient had a medium value of 0.74, this values show a medium cell growth linear tendency.

In a first probe with organic addition a low quantity of crude organic extract was added to the seawater to observe the influence of low addition of organic mater on marine bacteria compare to the standard control probe without organic mater additions.

![Fig. 5 The 3 mg/l addition](image)

The addition of 3 mg/l of crude extract determined after two hours of immersion a bacterial cell density of 1.15 \times 10^3 \text{cells/mm}^2 and after 6 hours this values is double with a value of 2.25 \times 10^3 \text{cells/mm}^2, at 14 hours the density reaches the value of 3.24 \times 10^3 \text{cells/mm}^2 and a first pick is reaches at 24 hours when the density is 4.94 \times 10^3 \text{cells/mm}^2, also at 48 hours a second pick is observed with a density value of 6.42 \times 10^3 \text{cells/mm}^2 this id the highest value in the first probe and after 72 hours at the end of the interval the density values were lower of 5.94 \times 10^3 \text{cells/mm}^2 (Fig.5).

The values determined that additions of low quantities determined an optimal cell growth and attachment and the equation of growth was high of 0.49 and also the linearity coefficient had a higher value of 0.88, due to a large linearity of the bacterial density values.

A second addition with 2 mg/l higher than the first one was made for the bacterial investigation at a medium quantity addition.

In the probe with a 5 mg/l addition of extract after two hours after immersion the bacterial cell density was 1.52 \times 10^3 \text{cells/mm}^2 after 6 hours this values is double with a value of 2.28 \times 10^3 \text{cells/mm}^2 at 14 hours the density reaches the value of 3.98 \times 10^3 \text{cells/mm}^2 a first pick is reaches at 24 hours when the density is 5.57 \times 10^3 \text{cells/mm}^2 at 48 hours the pick is 7.21 \times 10^3 \text{cells/mm}^2 the highest value in the control probe and after 72 hours at the end of the interval the density values are lower of 5.94 \times 10^3 \text{cells/mm}^2 (Fig.6).

![Fig. 6 The 5 mg/l addition](image)
In the probe with a 7 mg/l addition of extract after two hours after immersion the bacterial cell density was \(2.02 \cdot 10^3\) cells/mm\(^2\) after 6 hours this value is double with a value of \(4.19 \cdot 10^3\) cells/mm\(^2\) at 14 hours the density reaches the value of \(5.21 \cdot 10^3\) cells/mm\(^2\) a first pick is reaches at 24 hours when the density is \(7.26 \cdot 10^3\) cells/mm\(^2\) at 48 hours the pick is \(8.38 \cdot 10^3\) cells/mm\(^2\) the highest value in the control probe and after 72 hours at the end of the interval the density values are lower of \(7.89 \cdot 10^3\) cells/mm\(^2\) (Fig.7).

The values obtained shown that the medium salinity values determined an optimal cell growth and attachment and the equation of growth was high of 0.40 and also the linearity coefficient had a higher value of 0.76, this values show a medium cell growth linear tendency compare to lower additions.

The last concentration of organic extract use was lower that 10 mg/l due to bacterial low reaction to organic matter after this concentration.

In the probe with a 9 mg/l addition of extract after two hours after immersion the bacterial cell density was \(2.51 \cdot 10^3\) cells/mm\(^2\) after 6 hours this values is double with a value of \(4.71 \cdot 10^3\) cells/mm\(^2\) at 14 hours the density reaches the value of \(5.21 \cdot 10^3\) cells/mm\(^2\) a first pick is reaches at 24 hours when the density is \(7.26 \cdot 10^3\) cells/mm\(^2\) at 48 hours the pick is \(8.38 \cdot 10^3\) cells/mm\(^2\) the highest value in the control probe and after 72 hours at the end of the interval the density values are lower of \(7.89 \cdot 10^3\) cells/mm\(^2\) (Fig.8).

The values obtain shown that the medium salinity values determined an optimal cell growth and attachment and the equation of growth was high of 0.40 and also the linearity coefficient had a higher value of 0.76, this values show a medium cell growth linear tendency compare to lower additions.

In figure 9 the control probes with out organic matter supply determined a bacterial cell density of \(42.6 \cdot 10^3\) cells/mm\(^2\) on the artificial surfaces. After the supply of sea water with 3 mg/l of amino acid mixture the bacterial cell attachment reached the value of \(56 \cdot 10^3\) cells/mm\(^2\). A high concentration of 5 mg/l determined a bacterial cell density of \(61 \cdot 10^3\) cells/mm\(^2\).
The use of even higher concentrations of 7 mg/l of tryptone reached the value of 71.7 \( \cdot 10^4 \) cells/mm\(^2\) and at 9 mg/l was the bacterial cell density was 78.8 \( \cdot 10^3 \) cells/mm\(^2\).

The total bacterial growth shows large differences between the control probe and the probe of organic addition with the highest density in the case of the 9mg/l addition (Fig.10).

![Fig. 10 Bacterial cells](image)

Conclusions

The use of different concentrations of organic matter from a natural crude extract has determined large differences between \(10^3 - 10^4\) cells/mm\(^2\).

The use of natural organic extract can represent a more appropriate organic source for bacterial growth even in laboratory conditions compare to additions of artificial substances.

The use of high quantities of organic extract 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.


Lazar V., Herlea V., Cernat R., Balotescu M., Bulai D., Moraru A., General Microbiology, Laboratory Protocols, Bucharest University, 2004
Penfold W. J and Norris D., The elation of concentration of food supply to the generation of bacteria, Biology of Bacteria, J. of Hygiene, 527-531, 1912

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