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## Ecology and biotechnological potential of bacterial community from three marine sponges of the coast of Rio de Janeiro, Brazil

FÁBIO V. DE ARAÚJO<sup>1</sup>, MARCELLE C.M. NETTO<sup>1</sup>, GUSTAVO P. AZEVEDO<sup>1</sup>, MARCELLY M.A. JAYME<sup>2</sup>, MONICA C. NUNES-CARVALHO<sup>3</sup>, MARIANA M. SILVA<sup>1</sup> and FLÁVIA L. DO CARMO<sup>4</sup>

<sup>1</sup>Departamento de Ciências, Faculdade de Formação de Professores, Universidade do Estado do Rio de Janeiro, Rua Dr. Francisco Portela, 1470, Patronato, 24435-005 São Gonçalo, RJ, Brazil

<sup>2</sup>Departamento de Microbiologia, Imunologia e Parasitologia, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rua São Francisco Xavier, 524, 3º andar, Maracanã, 20550-900 Rio de Janeiro, RJ, Brazil

<sup>3</sup>Departamento de Bioquímica, Instituto de Química, Universidade Federal do Rio de Janeiro, Cidade Universitária, Av. Athos da Silveira Ramos, 149, 21044-020 Rio de Janeiro, RJ, Brazil

<sup>4</sup>Departamento de Microbiologia Geral, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, Cidade Universitária, 21941-590 Rio de Janeiro, RJ, Brazil

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### ABSTRACT

Marine sponges have been a large reservoir of microbial diversity, with the presence of many species specific populations as well as producing biologically active compounds, which has attracted great biotechnological interest. In order to verify the influence of the environment in the composition of the bacterial community present in marine sponges and biotechnological potential of bacteria isolated from these organisms, three species of sponges and the waters surrounding them were collected in different beaches of Rio de Janeiro, Brazil. The profile of the bacterial community present in sponges and water was obtained by PCR-DGGE technique and the biotechnological potential of the strains isolated by producing amylase, cellulase, protease and biosurfactants. The results showed that despite the influence of the environment in the composition of the microbial community, studied marine sponges shown to have specific bacterial populations, with some, showing potential in the production of substances of biotechnological applications.

**Key words:** bacterial diversity, biosurfactants, enzymes, marine sponges, PCR-DGGE.

### INTRODUCTION

Microorganisms can be found in different habitats in marine environments, since inanimate surfaces through the formation of biofilms until living in symbiosis with macroorganisms such as, for instance, sponges (poriferous) (Egan et al. 2008). In this association, sponges provide shelter and

nutrients and gain the benefit of biologically active metabolites produced by symbiotic microorganisms. Some authors suggest the term “sponge-specific” to define the association between the sponge and its microbial community as it has been shown that this community in the sponge is different from that present in seawater (Schmitt et al. 2012).

These microbial community associated to marine sponges have drawn an immense attention as

Correspondence to: Marcellly Miranda Aybal Jayme  
E-mail: [marcelllyjayme@yahoo.com.br](mailto:marcelllyjayme@yahoo.com.br)

a source for new secondary metabolites with important biotechnological properties, because of their wider biochemical accessibility, stability and higher activity than terrestrial counterparts (Kiran et al. 2014, Skariyachan et al. 2014).

Among these properties we can highlight the production of enzymes that are used to catalyze reactions in various industries with low environmental impact and cost of production (Beloqui et al. 2008, Ortega-Morales et al. 2008, Esteves et al. 2013) and biosurfactants, surface active compounds having both hydrophilic and hydrophobic domain that allows them to exist preferentially at the interface between polar and non-polar media, thereby reducing surface and interface tension, widely used in industrial and environmental applications (Banat et al. 2010, Sachdev and Cameotra 2013).

To better understand the ecology of sponge-bacteria association and evaluate the biotechnological potential of the microorganisms associated, our study aimed to compare the differences in bacterial communities present in sponges and in the marine water surrounding them and the capacity of strains isolated from sponges to produce different enzymes and biosurfactant.

## MATERIALS AND METHODS

### SAMPLING AREA

Sponges were collected in Itaipu beach (S-22.97, W-43.05), Niterói – RJ, Vermelha beach (S-22.95, W-43.16), Rio de Janeiro – RJ and Tartaruga beach (S-22.75, W-41.90), Armação dos Búzios – RJ.

### COLLECT

*Aplysina fulva* and *Amphimedon viridis* in Tartaruga beach and *Hymeniacidon heliophila* in Itaipu, Tartaruga and Vermelha beaches were collected through freediving, using a spatula to remove them from the substrate. After collected, sponges were packed in sterile bags with local sea water

volume and preserved on ice until processing in the laboratory. Water from the collection sites were also collected with sterile plastic bags and stored under refrigeration too.

### SAMPLE PROCESSING

In laboratory, sponges were washed with sterile distilled water to remove sediment particles, some small invertebrates associated or other impurities present in their bodies. Aseptically, with the aid of tweezers and a scalpel, portions of 0.5g of each sponges were removed.

To one of this portions 4.5 mL of sterile saline (0.85% NaCl) was added to obtain 1:10 dilution. This final solution was macerated in reinforced glass tube. The final homogenate was poured into a sterile beaker and aliquots with decimal dilutions (1:10, 1:100 and 1:1000) were inoculated by spreading plate method in marine agar and incubated at 25°C for 1 week (Apha 2000).

After incubation period, all colonies that presented differences in its color, edge, surface, elevation, shape, brightness and size were selected and re-isolated on Marine Agar to obtain pure culture. These strains were subjected to enzymatic and biosurfactant production tests.

### MOLECULAR ANALYSIS

Another three 0.5g sponges portions were subjected to DNA extraction using the FastDNA SPIN Kit for Soil from BIO101 (California, USA), but instead of soil was added the 0.5g of sponge. After extraction 10x TE was added to give a concentration of 1x TE, and the material was stored at -20°C. The quality of extracted DNA was evaluated in 0.8% agarose gel, applying 10µl of the sample with 5µl of dye for electrophoresis of DNA. Gels were subjected to electrical current 80V in 1x TBE buffer for 2 hours and stained with ethidium bromide, visualized by UV transillumination and photographed with a digital camera.

The DNA amplification was made by PCR with primers for the gene rDNA16S bacteria. The selected primers were U968f-GC1 ("clip" + 5' AAC GCG AAG AAC CTT AC 3') and L1401r (5' GCG GTA TGT CAG CAA CC 3') (Nubel et al. 1997).

The 50µL reactions were performed by mixing the enzyme Taq polymerase buffer 1x, 2.5µM MgCl<sub>2</sub> (Invitrogen), 200µmol of each dNTP, 20µmol of each primer (Promicro), 1% formamide, 5 mg BSA (Sigma), 2.5U Taq polymerase (Invitrogen) and sterile Milli-Q water. In each reaction were applied 1µL DNA.

The PCR program started with the cycle of DNA strands denaturation for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and extension at 72°C for 10 min.

All PCR products were subjected to electrophoresis on 1.2% agarose gel, applying 10µL of the sample with 5µL of dye for DNA electrophoresis. Gels were then subjected to electrical current 80V in 1x TBE buffer for 2 hours and stained with ethidium bromide, visualized by UV transillumination and photographed with a digital camera.

DGGE were performed with equipment "Dcode TM Universal Mutation Detection System" (Bio-Rad Richmond, USA). PCR products were obtained using the 16S rDNA primers, where a GC clamp described by Muyzer et al. (1993) (5'CGCCCGCCGCGCGGCGGGCGGGGCGGGGCACGGGGGG3') was added to U968 primers.

20µL of the products plus 20µL of the dye for DNA were then applied directly on a polyacrylamide gel in 0.5x TAE buffer containing a linear gradient of denaturant from 45 to 70%. Electrophoresis was performed with 75V / 60°C / 16h. After the run the gel was removed from the plate and stained for 40 minutes with Syber Green I (Molecular Probes)

and observed in UV, using a STORM system (Amersham) image capture.

Images of gels were used for the construction of a simple binary array where more defined bands were selected and analyzed for their presence or absence in each sample. The matrices constructed from the 16S gels were analyzed with Statistica 5.1 software (Starsoft Inc., USA), which was used for construction of dendrograms UPGA by using the coefficient 1-r Person.

#### ENZYMATIC TESTS

To detect amylolytic and proteolytic activity of casein were used the methods described by Williams et al. (1983) and for the detection of celullolytic activity was used the method described by Bairagi et al. (2002).

The enzymatic activity was determined by the ratio between the diameter of substrate degradation halo (H) and the diameter of the colony tested (C). This relationship is called enzymatic Index (I)<sup>2</sup>.  $(I)^2 = H/C$ .

The strains with enzymatic index over twenty milimeters (2cm) were considered strains with a high potential to degrade the substrate offered for testing (Lealem and Gashe 1994).

#### BIOSURFACTANT TEST

Strains were inoculated into 50 mL flasks containing 20 mL of mineral medium supplemented with 2% sucrose. The flasks were incubated under shaking (150 rpm) at 30°C for 24h and 48h. To evaluate the emulsification index, 5 mL of supernatant and 10% of diesel oil were placed into sterile tubes, and stirred in vortex for 20s and after 20s manually (Carmo et al. 2014). After 24h and 48h of this process, to enable the elimination of unstable emulsions, the reading of the emulsion index (EI) were carried out. EI was determined by the ratio between the height of the emulsified layer and the height of the total solution (Ramos et al. 2010).

## MOLECULAR IDENTIFICATION (SEQUENCING)

The strains that present the best results in the tests realized were identified by sequencing and had their genomic DNA extracted by thermal shock and purified with the Qiaquick PCR Purification Kit (Qiagen®). After purification, the DNA underwent polymerase chain reaction for amplification of bacterial 16S rRNA using primers 27FB and 1492RAB. The polymerase chain reaction was performed containing Taq polymerase 5U, dNTP (25mM), 10X buffer, MgCl<sub>2</sub> (50 mM), 1µL of each primer to 100pmol, 1µL of DNA and sterile MilliQ water to complete 50µL. The mix was then forwarded to the thermal cycler programmed with 5' at 94°C, followed by 35 cycles of denaturing temperature at 94°C for 1'30'', annealing temperature at 52°C for 1'30'', 72°C extension temperature for 2' and a final soak at 72°C for 15'. The samples were cooled to 4°C at the end. The amplicons were sent to the Genome Laboratory of the Department of Biochemistry at UERJ for performing the sequencing.

The sequencing was performed containing Big Dye Terminator v 3.1 sequencing buffer, 5x sequencing buffer, Primers 27 and 532FB and 907 and 1492RAB (3.2 pmoles/µl), 150ng of DNA, sterile MilliQ water to complete 10µL. The mix was then forwarded to the sequencer 3500 Genetic Analyser (Applied Biosystems™, ThermoScientific) programmed with 28 cycles of denaturing temperature at 95°C for 1', annealing temperature at 95°C for 15'', 50°C extension temperature for 15'' and a final soak at 60°C for 2'.

## RESULTS AND DISCUSSION

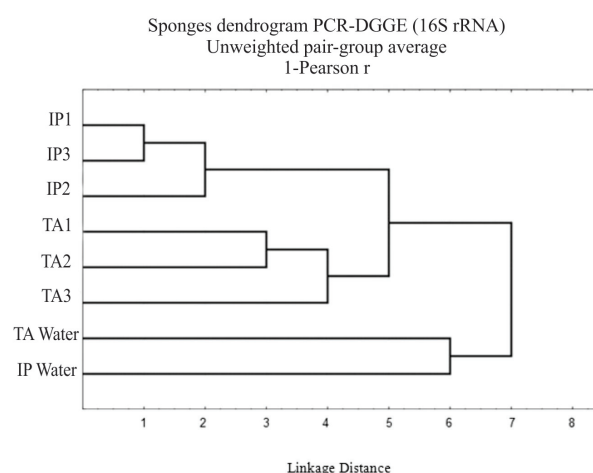
### MOLECULAR AND ECOLOGY ANALYSIS

To verify the differences in bacterial communities present in the same sponges species from different regions and in the marine water surrounding them

we compared by DGGE the microbial communities of *H. heliophila* collected in Itaipu, Niterói, RJ and Tartaruga, Búzios, RJ.

Despite the dissimilarities in relation to water, the formation of different groups between sponges of the same species sampled from different locations, suggests an environmental influence on the composition of the bacterial communities found in these organisms (Figure 1).

It could be observed that the bacteria present in the Itaipu sponges were grouped more strongly than the group formed by the bacterial communities found in *H. heliophila* of Búzios. Despite this small difference between these groups; these are even more similar to each other than in relation to the bacterial communities present in waters where they occur; showing that these organisms have specific populations, which reinforces the idea that the sponges create favorable habitats for the establishment and evolution of bacterial populations, thus contributing to increasing the diversity of microorganisms, going according to several studies showing groups of bacteria specific in porifera (Schmitt et al. 2012, Kamke et al. 2010, Webster and Taylor 2012). In Schmitt et al.



**Figure 1** - Dendrogram generated using Statistica software (using Pearson and UPGA similarity coefficient) to analyze the DGGE bands profiles of samples of water and sponge *H. heliophila* of Itaipu (IP) and Tartaruga (TA) beaches.

(2012), 32 species of sponges in several oceans and seas (Indian and Pacific oceans, Red Sea, Mediterranean, Caribbean, New Zealand and the Great Barrier Reef), were analyzed for their 16S rRNA and performed the comparison of bacterial communities in broader taxonomic levels (phyla and order) revealing a large overall similarity, but no correlation with the phylogeny of the sponge.

However, the bacterial species in different sponges are more closely related among themselves than bacteria from sea water where they are. The geographic variation observed between the microbial communities of the sponges of the same species is due probably to the fact that sponges are filterers. Thus, the microbiota present in the local water will have a contribution in the microbial community present in the sponges.

#### ENZYMATIC TESTS

A total of 38 strains were isolated from sponges; 14 from *A. fulva*, 8 from *A. viridis* and 16 from *H. heliophila*. From these total, 19 showed enzymatic activity for at least one of the three tested substrates: starch, cellulose and casein. Lealem and Gashe (1994) suggest a value of  $EI \geq 2$  as high potential degradation. Thus, among the 38 strains isolated, only 11 (28.9%) show this potential; 1 (2.7%) for starch, 1 (2.7%) for cellulose and 9 (23.5%) for casein. Only one strain isolated from *H. heliophila* (Hh9) was able to degrade the three substrates (Table I).

Baker (1998) says that bacteria is not only important in the nutritional process of sponges, but also in the degradation and transformation of marine plant biomass. Part of these bacterial isolates appear to take part in such ecological functions.

Nunes et al. (2011) demonstrated high potential degradation of macrophytes by bacterial cellulases, where cellulolytic activities presented the highest degradation halos as compared to the other substrates tested, indicating that the need to

**TABLE I**  
**Values of Enzymatic Index (EI) for strains isolated from sponges for the different substrates tested.**

Strains	Amylase	Cellulase	Caseinase
Af. 2	-	-	4.0*
Af. 3	-	-	1.4
Af. 4	1.4	-	-
Af. 5	-	-	3.3*
Af. 6	-	-	2.4*
Av. 2	-	-	1.3
Av. 3	-	3.0*	1.7
Av. 4	-	-	1.8
Av. 5	-	-	2.0*
Av. 6	-	-	2.4*
Av. 7	-	-	2.9*
Hh. 1	1.1	-	1.4
Hh. 3	1.2	-	-
Hh. 6	1.3	-	2.7*
Hh. 7	-	1.4	1.4
Hh. 9	2.3*	1.4	1.5
Hh. 10	-	-	4.6*
Hh. 12	-	1.2	-
Hh. 13	-	-	2.1*

Af. = *Aplysina fulva*, Av. = *Amphimedon viridis*, Hh. = *Hymeniacidon heliophila*. \* = Values of  $EI \geq 2.0$ ; Values of  $EI < 2$ ; (-) negative result, absence of halo.

decompose plants and seaweeds as a carbon source for metabolism influenced the high productivity of cellulase. In our work cellulolytic activity was found only in 4 (21%) strains.

Mohapatra et al. (2003) and Feby and Nair (2010) found amylase as one of the most predominant enzyme produced by bacteria associated to sponges. Our results do not corroborate these data. Caseinase, a protease, was the most predominant enzyme detected in our study, present in 84.2% (16 strain) of the 19 that showed some enzymatic activity. The higher values of EI was found to caseinase too. Proteases occupy a central position with respect to their commercial applications. They are the most important hydrolytic enzymes and have been extensively studied since the advent of enzymology.



Between the three sponges species presently studied, differences were observed in the number of isolates and their enzyme activity. This could be due to the differences in their physiological requirement and the ambient variation in the quality and quantity of the organic matter.

#### BIOSURFACTANT PRODUCTION

According Dhasayan et al. (2015), the sponge-associated bacteria for the biosurfactant production are scarcely reported; thus, the present study is contributing to increase this knowledge.

Of the 38 isolates obtained, 17 showed some emulsifying activity, a peculiar feature of the biosurfactants in general (Table II).

The presence of bacteria producing enzymes in sponges was reported early by some authors (Mohapatra et al. 2003, Feby and Nair 2010). These enzymes have an important role in helping sponges nutrition hydrolyzing complex molecules present in marine environment that sponges cannot directly absorb (Marx et al. 2007).

Six strains (35.29%) were able to emulsify 80 to 100% of the substrate used for testing (diesel oil), in 24 hours of growth. Some colonies maintained their emulsion index or increased up to 100% in 48h of growth.

Our results are quite satisfactory once Dhasayan et al. (2015) obtained of a total of 101 sponge-associated bacteria isolated, only 29 with positive result for biosurfactant production; and among these 29 positive isolates, just four presented high emulsification activity. Moreover, corroborate Jennings and Tanner (2000) results that compared the amount of microorganisms producers of biosurfactants isolated from environments contaminated and not contaminated with hydrocarbons and observed a higher percentage of biosurfactants producers in uncontaminated environments. The authors concluded that environments never exposed to contamination also

**TABLE II**  
**Values of Emulsification index for the bacterial isolates from the sponges studied.**

Strain	24 h	48 h	Strain	24h	48h
Af. 1	0%	0%	Av. 6	60%	100%
Af. 2	0%	0%	Av. 7	60%	60%
Af. 3	20%	20%	Av. 8	0%	0%
Af. 4	0%	0%	Hh. 1	80%	80%
Af. 5	0%	0%	Hh. 2	40%	40%
Af. 6	0%	0%	Hh. 3	0%	0%
Af. 7	20%	60%	Hh. 4	80%	80%
Af. 8	80%	80%	Hh. 5	0%	0%
Af. 9	40%	40%	Hh. 6	0%	20%
Af. 10	0%	0%	Hh. 7	100%	100%
Af. 11	0%	20%	Hh. 8	80%	80%
Af. 12	0%	40%	Hh. 9	0%	0%
Af. 13	0%	0%	Hh. 10	0%	0%
Af. 14	80%	100%	Hh. 11	0%	0%
Av. 1	40%	80%	Hh. 12	0%	0%
Av. 2	0%	0%	Hh. 13	0%	0%
Av. 3	20%	20%	Hh. 14	0%	0%
Av. 4	0%	0%	Hh. 15	0%	0%
Av. 5	60%	100%	Hh. 16	0%	0%

Af. = *Aplysina fulva*, Av. = *Amphimedon viridis* and Hh. = *Hymeniacidon heliophila*.

have microorganisms producing biosurfactants as the case of the environments studied in the present work.

An emphasis should be given to the study of these molecules, as they become increasingly promising as their use to replace highly aggressive chemicals to the environment, both in industry and in wastewater treatment, or remediation of contaminated sites (Carmo et al. 2014).

Four strains (Av.3, Av.5, Av.6 and Av.7) stood out among all isolates, because presented  $EI \geq 2$  for some of the substrates tested and also for having emulsification capacity between 20-100%. Despite the production of enzymes has been reported for microorganisms isolated from the three sponges specie studied, our best results were found in bacteria isolated from *A. viridis*. Bonugli-Santos

et al. (2010) describes marine fungi isolated from this species as producing laccase but we could not find in the literature reports of bacteria producing enzymes associated with this specie of sponge.

Strain Av.3 was identified by sequencing as *Bacillus pumilus*. According Jong et al. (2015), *B. pumilus* is a Gram-positive rod-shaped spore-forming soil bacterium but already isolated from marine environments. It is also used beneficially in the production of industrially relevant compounds, such as xylanases, lipases, and proteases.

Despite the geographic variation observed in their microbial community composition, *H. heliophila* occurring in two sites of the coast of Rio de Janeiro, Brazil, showed specific bacterial populations with some of them presenting potential for prospecting substances of biotechnological applications. The presence of bacteria with potential biotechnological application in the other species of sponges studied suggests that these organisms are a great source for the research of bioactive compounds.

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