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Evaluation of the biotechnological potential of bacterioplankton from Niterói coast, RJ

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ABSTRACT

Production of antibiotics and enzymes by microorganisms is well recognized for its economic benefits. Searching potentially exploitable microorganisms, strains of heterotrophic bacteria were isolated from marine and estuarine waters in Niterói-RJ, Brazil, and tested for the production of enzymes and antimicrobial substances. From the 76 isolated colonies, 09.21% showed antimicrobial potential, 63.16% showed enzymatic activity for at least one of the substrates tested and 91% showed multidrug resistance. Strains that presented the better results were identified by sequencing. The species identified are widely distributed in many different types of environments, having been isolated previously from marine environment. Our results suggest that marine and estuarine waters can be a source for bioprospecting bacteria with potential biotechnological uses.

1. Introduction

Bacterioplankton consist of bacteria that live suspended in the water column, with great role in the ecology of coastal ecosystems, participating in processes such as nutrient cycling, production and decomposition of organic matter and regulation of the main biogeochemical cycles, beyond the control of a substantial fraction of the overall carbon flux in a process that is known as "microbial loop" [1,2].

In coastal environments, multiple environmental factors can alter the composition of this community, including salinity, inorganic nutrient concentrations, turbidity and the concentration of organic labile compounds [1]. The anthropogenic effects, mainly through the discharge of sewage and chemicals such as pesticides, hormones and antibiotics widely used in human and veterinary clinics can interfere in these variables and introduce potential enteropathogens that can possess antimicrobials resistance genes. Considering that, in aquatic environments, horizontal transfer of genetic material is intense, the anthropogenic influence is a threat to public health, since opportunistic pathogens may acquire resistance to drugs through this mechanism [3].

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To be adapted and survive in the marine ecosystem, with all its variables, marine microorganisms accumulate bioactive secondary metabolites structurally unique, many not being found in terrestrial organisms. For this reason, researches of marine bacteria as a source of biotechnology are increasing, since these compounds have important applications with high economic value in the textile, pharmaceutical, and cosmetic industries, for example. In contrast to macroorganisms, microorganisms represent promising sources of natural products that have the advantage of viable and sustainable production of large amounts of secondary metabolites, at a reasonable cost by large-scale cultivation [4].

The coastline of Niterói, Rio de Janeiro, Brazil, has for years a large load pollution [5] contaminating its waters and thereby enabling the occurrence of species that produce bioactive compounds in their bacterioplankton community. Thus, this study aimed to isolate in this community, bacteria with potential application in biotechnology.

2. Methods

2.1. Collection

Over three years (September 2007 to September 2010), water samples were collected monthly in different beaches of the city of Niterói, RJ, some of them inside of Guanabara bay: Gragoatá (22°54′17″ S; 43°08′08″W), Boa Viagem (22°54′30″ S; 43°07′47″ W), Praia das Flechas (22°54′16.8″ S; 43°07′22″ W), Icaraí (22°54′37.4″ S; 43°06′37″ W), São Francisco (22°54′59.3″S; 43°05′42.2″W), Charitas (22°54′17″ S; 43°08′08″ W), Jurujuba (22°55′36″ S; 43°07′06.8″ W) and Adão e Eva (22°55′45.2″ S; 43°07′20.7″ W) and others as Prainha de Piratininga (22°57′11.5″ S; 43°05′52.1″ W), Praia de Piratininga (22°57′16.3″ S; 43°05′03.6″ W), Camboinhas (22°57′42″ S; 43°03′17.4″ W), Itaipu (22°58′13.3″ S; 43°02′43.8″W) and Itacoatiara (22°58′28″S; 43°01′55″W) located in oceanic region of Niterói.

2.2. Isolation of bacteria from bacterioplankton

Water samples obtained at each station were inoculated by spread plates technique [6] in Petri plates containing nutrient agar culture medium and incubated for one week at room temperature. Colonies with distinct morphological characteristics were isolated, purified and stored in tubes containing nutrient agar and covered with mineral oil.

2.3. Antimicrobial compounds production test

To test the production of antimicrobial compounds, an adaptation of the method described by Giambiagi-Marval et al. [7] was used.

The antimicrobial production potential of the strains tested was observed by their capacity to inhibit the growth of the bacteria type *Staphylococcus aureus* (ATCC29213) and *Escherichia coli* (ATCC25922) provided by the Molecular Microbial Ecology Laboratory of the Institute of Microbiology Professor Paulo de Góes (IMPPG) UFRJ.

The inhibition of growth was determined by the presence of a halo without bacteria type growing around the strain tested. The strains that showed this halo were considered positive and the inhibition was measured as weak (halo between 0–10 mm); medium (halo between 11–20 mm) and strong (halo greater than 20 mm).

2.4. Enzyme activity test

For the detection of amylolytic and proteolytic activity of casein, were used the methods described by Willians et al. [8] and for the detection of lytic activity, was used the method described by Bairagi et al. [9].

The enzymatic activity was determined by the ratio between the diameter of substrate degradation halo and the diameter of the colony tested. This relationship is called enzymatic index $(I)^2$.

The strains with enzymatic index over 20 mm were considered strains with a high potential to degrade the substrate offered for testing [10].

$$(I)^2 = \frac{H}{C} \geq 20 \text{ mm}$$

H = halo diameter; C = colony diameter

2.5. Antimicrobial susceptibility testing

The test for investigating antimicrobial susceptibility was performed according to standards established by the Clinical and Laboratory Standards Institute [11] using the diffusion method Kirby-Bauer disk. The colonies were tested for the following antimicrobial agents: amikacin 30 mg, ampicillin 10 mg, cefotaxime 30 mg, cefoxitin 30 mg, ciprofloxacin 5 mg, chloramphenicol 30 mg, tetracycline 30 mg, gentamicin 10 mg and cephalothin 30 mg. Multidrug resistance profile according Magiorakos et al. (2012) [12] is defined by the resistance to three or more classes of antimicrobials tested.

2.6. Sequencing

Some strains that presented positive results for any of the tests performed and showed resistance to multiple antibiotics were selected 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 27 and 532FB and 907 and 1492RAB. The polymerase chain reaction was performed containing Taq polymerase 5U, dNTP (25 mM), 10 × buffer, $MgCl_2$ (50 mM), 1 μl of each primer to 100 pmol, 1 μl of DNA and sterile MilliQ water to complete to 50 μl. The temperature cycling protocol was set as follows: initial holding, 5 min at 94°C, followed by 35 cycles of: denaturation, 1 min 30 s at 94°C; annealing, 1 min 30 s at 52°C and extension, 2 min at 72°C. Final holding, 15 min at 72°C to assure that all the products were annealed to their double-stranded form. 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 reaction mixture for sequencing consisted of Big Dye Terminator v 3.1 sequencing buffer, 5 × sequencing buffer, primers 27 and 532FB and 907 and 1492RAB (3.2 pmol/μl), 150 ng of DNA and sterile MilliQ water to complete to 10 μl. This mix was forwarded to the sequencer 3500 Genetic Analyser (Applied Biosystems™, Thermoscientific) programmed with 28 cycles of denaturing temperature at 95° C for 1 min, annealing temperature at 95° C for 15 s, 50° C extension temperature for 15 s and a final soaking at 60° C for 2 min.

3. Results

3.1. Production of antimicrobial substances

In our work, seventy-six strains were isolated from marine and estuarine environments. Seven of them showed antimicrobial potential, with four strains presenting inhibition zone against *Esherichia coli* and five against *Staphylococcus aureus*. Among these, two had a broad antimicrobial potential, inhibiting both *E. coli* and *S. aureus*. Strains 15, 20 and 75 showed strong suppression to *S. aureus* with inhibition zones greater than 20 mm (Table 1).

3.2. Enzymatic activity detection

In this study, from a total of 76 strains isolated from marine and estuarine environments, 63.2% had enzymatic activity against at least one of the three substrates tested: starch, cellulose, and casein. Fourteen colonies showed the ability to hydrolyze the starch available in the medium and of these, four showed a degradation halo equal to or greater than 20 mm. Thirty-one strains showed cellulolytic activity with 24 having displayed enzymatic index greater than or equal to 20 mm and 38 strains showed proteolytic activity on casein, with 10 showing an index equal to or greater than 20 mm (Table 2).

Of all the 31 strains that have a high enzyme potential to at least one of the substrates tested (halo greater than 20 mm), 25 (1, 6, 7, 8, 9, 12, 13, 15, 16, 18, 21, 22, 24, 30, 31, 37, 42, 43, 47, 49, 52, 53, 62, 63 and 71) were isolated from the waters of Guanabara bay beaches while only 6 (28, 45, 46, 66, 67 and 73) were isolated from the waters of oceanic beaches.

Table 1
Spectrum of action against the two types of strains tested.

Positive strains	*Escherichia coli*	*Staphylococcus aureus*
15	++[c]	+++[d]
16	–[a]	+[b]
20	–	+++
46	++	–
70	+	++
71	++	–
75	–	+++

The inhibition halos were measured in millimeters and classified as: [a]: no suppression (no inhibition zone); [b]: weak suppression (inhibition zone between 0–10 mm); [c]: mediam suppression (inhibition zone between 11–20 mm); [d]: strong suppression (inhibition zone above 20 mm).

Table 2
Enzymatic index $(I)^2$ of the isolated strains on different substrates.

Positives strains	Amylolytic	Cellulolytic	Proteolytic in casein
1	2.000	–	2.080
4	–	–	1.333
5	–	–	1.878
6	–	4.142	1.509
7	2.250	2.000	2.111
8	–	2.250	2.857
9	–	2.660	–
10	–	–	1.800
12	–	3.250	–
13	–	3.625	1.130
15	1.108	–	2.216
16	2.291	3.000	1.363
17	–	1.900	1.323
18	–	4.125	1.720
21	–	2.636	2.526
22	–	4.142	2.437
23	–	1.928	1.125
24	–	4.833	1.470
28	–	3.750	0.180
30	1.681	3.000	1.500
31	–	–	2.250
35	–	–	1.476
37	1.312	2.150	1.222
38	–	–	1.850
40	1.360	1.055	1.055
42	–	3.660	–
43	–	3.660	–
44	–	1.570	1.140
45	2.000	1.687	1.226
46	1.700	1.684	2.419
47	–	–	2.710
49	–	2.000	–
52	–	–	2.600
53	–	2.666	–
54	–	–	1.300
59	1.700	–	–
58	–	1.666	–
61	–	–	1.130
62	–	3.000	1.269
63	–	4.857	1.411
64	1.125	–	1.760
66	1.155	3.243	–
67	–	3.333	1.205
69	1.036	–	1.396
70	1.326	–	–
71	–	4.142	1.080
73	–	3.538	1.065
75	–	–	1.750

The calculations of enzyme levels were made in centimeters. $(I)^2 \geq 2$ cm: high enzymatic activity; $(I)^2 < 2$ cm: low enzymatic activity; $(I)^2 = -$: No enzymatic activity.

3.3. Antimicrobial resistance profile

Twelve strains showed a multidrug resistance profile (Table 3). Nine (30, 35, 37, 38, 40, 44, 56, 62 and 69) were isolated from the beaches located in Guanabara bay and three (20, 64 and 66) isolated from the oceanic beaches, two of them from Itaipu.

3.4. Sequencing

Fourteen strains were selected for sequencing. Five showed multidrug resistance profile and enzymatic activities and were identified as *Pseudomonas stutzeri*

Table 3
Resistance profile to antimicrobials.

Profile	Strains resistant
1–CTX	8, 13
2–CLO + CFL	59, 70
3–CLO + CTX	6, 22
4–CFO + CTX	24
5–AMP + CTX	28
6–AMP + CFL + TET	30
7–AMP + CFL + CLO	15
8–CLO + CFO + CFL	31
9–AMP + CFL + CLO + CFO	56
10–AMP + CTX + CFO + CFL	35, 37, 40, 44, 64, 69
11–AMP + CTX + CFO + CFL + TET	66
12–AMP + CTX + CFO + CLO + CFL + TET	62

AMP: ampicillin; CFL: cephalothin; CFO: cefoxitin; CTX: cefotaxime; CLO: chloramphenicol; TET: tetracycline.

(strain 30), *Bacillus cereus* (strains 40 and 66) and *Bacillus thuringiensis* (strains 64 and 69). Strain 56 only showed multidrug resistance profile and was identified as *Pseudomonas poae*. Two strains (15 and 70) showed capacity to produce antimicrobial substances and enzymatic activities and were characterized as *Bacillus* sp. and *Pseudomonas stutzeri*, respectively. Six strains (6, 13, 24, 28, 63 and 71) showed an enzymatic production profile being identified as *Kocuria rhizophila*, *Bacillus megaterium*, *Bacillus pumilus*, *Paenibacillus* sp., *Bacillus licheniformis*, *Bacillus* sp. and *Pseudomonas stutzeri*, respectively.

4. Discussion

Marine ecosystems have been investigated for the search for new compounds susceptible of biotechnological applications, as enzymes and antimicrobial substances due to their well-known habitat-related features such as salt tolerance, hyperthermostability, barophilicity, and cold adaptivity. A review written by Satheesh et al. [13] show marine microorganisms as producers of inhibitors substances of other microorganisms and macroorganisms. Comparing fresh waters as bromeliad tank water ecosystems studied by Carmo et al. [14] and marine and estuarine environments, studied by us, the latter showed to be a more efficient source of strains producing antimicrobial compounds (5% and 9.2%, respectively). According to Avendaño-Herrera et al. [15], this higher percentage may be due to the fact that bacteria from marine environments may produce antibacterial substances that contribute to maintain ecological stability, as the interrelationships between microorganisms and macroorganisms in the various marine ecosystems.

The largest number of isolates with potential production of antimicrobial substances (AS) may be related to the environment. Of the seven strains with potential production of AS, four (15, 16, 71 and 75) were isolated from beaches located in the Guanabara bay region, while three (20, 46 and 70) were isolated from ocean beaches. The production of antimicrobial substances can be an advantage to microorganisms in the competition by nutrients in waters that present high bacterial counts like the Guanabara bay waters [5]. Despite three strains had been isolated from ocean beaches, two (20 and 46) were isolated from Itaipu, which has highest bacterial counts between the ocean beaches studied, probably influenced by the great input of organic matter received by the Itaipu lagoon that ends on this beach.

Many studies have been reporting the potential and applicability of marine amylolytic, cellulolytic and proteolytic enzymes in the pharmaceutical, textile, leather, paper, mineral, and food industries [16–20].

In the study by Lee et al. [21] bacteria isolated from the coastal waters of Peninsular Malaysia showed a large enzyme potential, commonly producing amylolytic and proteolytic enzymes (56% and 36%, respectively) which coincides with the results found in our study for proteases (50%), however in contrast with amylase results that were less frequent in this work (18.4%). The cellulolytic activities presented in our work obtained the largest halos of hydrolysis when compared to the other substrates tested. This result may be related to the decomposition of marine plants and algae as a source of carbon for bacterial metabolism. Nunes et al. (2011) [22] describes the action of cellulases and xilases during the decomposition process of aquatic macrophytes quoting the highest levels during the beginning of the process.

The high rate of strains with potential enzymatic degradation of different substrates tested from Guanabara bay waters may be related to the high concentration of these substrates in this environment, once Guanabara bay is highly impacted by urban and industrial effluents [23].

The environment can influence the bacterial community composition. A high incidence of heterotrophic bacteria in marine estuaries resistant mainly to antimicrobials used for treating infectious diseases in humans (especially ampicillin, cephalosporins and chloramphenicol), as found in this study, may indicate contamination by domestic wastewater according to Salloto et al. [3]. Our results showed that of twelve strains with multidrug resistance profile (Table 3), nine (30, 35, 37, 38, 40, 44, 56, 62 and 69) were isolated from Guanabara bay beaches and of three (20, 64 and 66) isolated from the ocean beaches, two were isolated from Itaipu, regions that present the most impacted values, corroborating Salloto et al. [3] and our enzymatic activities results.

Schneider et al. [24], analyzing the resistance profile of bacteria isolated from surface water samples, observed a variation in the resistance frequency between one to seven antimicrobial agents, with the largest number of isolates showing resistance to an only antimicrobial type; different of our results, where the multidrug resistance was extremely frequent, appearing in approximately 91% of resistant isolates. It was also observed that the profile 10 (AMP + CTX + CFO + CFL) showed the highest prevalence (five isolates) compared to other profiles, which may be related to dissemination of resistance genes to beta-lactam. There was only one resistance profile to one antimicrobial, the profile 1 (CTX) that appears in two resistant isolates. Of the nine antimicrobials used in the test, six had resistant isolates; where one of these isolates were resistant to six antimicrobials, profile 12 (AMP + CTX + CFO + CFL + CLO + TET). The resistance against the beta-lactam was more prevalent among the

isolates. Pitout [25] mentions that the high rate of resistance to beta-lactams is based on plasmids carriers of genes encoding beta-lactamases, enzymes responsible for breaking the beta-lactam ring present in these antimicrobials. The resistance to quinolone may be associated with the plasmid-mediated quinolone resistance that was reported in 1998 by Martinez-Martinez et al. [26] and since then has been commonly found in strains spread by the environment [27]. The sensitivity percentage (73%) of the strains against the CTX is satisfactory, since this is a choice in the treatment of infections caused by Gram-negative bacteria resistant to other drugs [28]. The sensitivity to aminoglycosides (amikacin and gentamicin) and fluoroquinolones (ciprofloxaxin) also appear in works with strains isolated from seafood, showing up as viable treatment options in case of infection too [29,30]. These results may suggest the presence of mobile genetic elements, because many antimicrobial resistance genes seen in Gram-negative bacteria are part of gene cassettes inserted into the cell [31].

All strains identified are widely distributed in many different types of environments, all having been described previously in marine environment. *Pseudomonas stutzeri*, *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus thuringiensis*, *Bacillus pumilus* and *Paenibacillus* sp. are identified as producers of enzymes, for example, amylase and protease. *Paenibacillus* is also used in studies for its use in bioremediation of impacted environments, as well as *Pseudomonas poae* and *Bacillus megaterium* that are capable of degrading hydrocarbon and benzene, respectively and *Kocuria rhizophila* which is producing biosurfactant [32–42]. The presence of this species showing these features suggests an influence of the environment in bacterioplankton community composition.

The highest number of strains resistant to antimicrobials found in Guanabara bay waters also shows the impact of anthropogenic influence in this region. At the same time, we demonstrate that pollution may influence the characteristics and composition of bacterioplankton; it was also observed that the study of heterotrophic bacteria present in polluted marine and estuarine environments is of utmost significance, since a large percentage of the microorganisms had some antimicrobial potential which in turn can be used to fetch a new line of drugs in order to combat microbial resistance to existing drugs. Further studies, including socioeconomic order, should be performed to monitor the quality of the studied ecosystem. The monitoring of coastal waters is critical to establish risks that may affect the health of the environment and the local biota, including bacteria, preserving the richness of the ecosystem, as well as the health of the population. In addition, marine environment seems to be an excellent area for enzyme bioprospecting activity once habitat-related characteristics are desirable features recognized from a general biotechnological perspective.

Disclosure of interest

The authors declare that they have no competing interest.

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