EXPLORING THE BIOTECHNOLOGICAL POTENTIAL OF BACTERIA ASSOCIATED WITH CAVE-DWELLING MARINE SPONGES

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ABSTRACT

This study aims to explore bacteria associated with cave-dwelling sponges from the Fernando de Noronha Archipelago (PE, Brazil) for biotechnologically relevant substances, including antimicrobials, biosurfactants, bioemulsifiers and enzymes. From thirty sponge specimens (Demospongiae and Calcarea) collected across different caves and grottoes, a total of 923 bacterial strains were isolated. Initially, 14.6% of these strains exhibited antibacterial activity against *Staphylococcus aureus* ATCC 29213, with thirty-six also showing activity against multidrug-resistant strains isolated from human and environmental sources. Additionally, 11.6% of the isolates produced stable emulsions in at least one oil tested (mineral oil, soybean frying oil waste, diesel oil and burnt engine oil). Among 331 marine bacteria evaluated for enzymatic production, 36.5% displayed alginate lyase activity, 51.6% agarase, 10.2% amylase, and 9.3% urease activity. *Vibrio* and *Pseudomonas* were the top identified genera among the bioactive isolates. Crude extracts of bioemulsifiers from two *Pseudomonas* strains showed significant inhibition of biofilm formation and dispersion of mature *S. aureus* biofilm. These findings highlight the potential of cave-dwelling sponge-associated bacteria as rich and yet untapped sources of biomolecules with diverse industrial and biomedical applications.

Keywords: Antibiofilm. Antimicrobials. Bioemulsifier. Cave dwelling sponges. Enzymes.

1 INTRODUCTION

The pursuit of novel biomolecules with unique biotechnological and biomedical applications has driven natural product research beyond conventional boundaries, delving into extreme environments[1]. Due to variations in luminosity, strong oligotrophy, low water circulation and low levels of nutrients, underwater caves have received increasing attention[2,3]. Organisms living in these harsh environments are expected to produce structurally unique metabolites, considering their high genetic diversity and adaptation mechanisms[3]. Nevertheless, Brazilian submarine caves, such as the ones found in Fernando de Noronha Archipelago, remain still unexplored for this purpose. One compelling aspect of these regions lies in their potential for unearthing rare and previously undocumented sponge taxa, including a significant number of species unique to these environments. This biodiversity stems from their volcanic origin, geographic isolation, and positioning[4]. Therefore, the present study aims to investigate the community of bacteria isolated from marine sponges sampled in the Fernando de Noronha Archipelago (PE, Brazil) regarding the investigation of substances with biotechnological potential, seeking to contribute to the research of new antimicrobials, enzymes, biosurfactants (BS) and bioemulsifiers (BE).

2 MATERIAL & METHODS

Thirty sponge samples were collected by SCUBA diving at depths ranging from 3 to 16 m, with seawater temperatures varying from 27 to 29 ºC, along three caves of the archipelago: Caverna da Sapata, Gruta da Ilha do Meio and Toca da Ressurreta. In the laboratory, the specimens were processed under aseptic conditions, and the sponge macerates were inoculated on the following culture media: BHI, BHI diluted tenfold (BHI 1:10), Marine, Marine diluted tenfold (Marine 1:10), minimal salt medium (MSM)[5], and MSM with mineral oil replacing glucose at 1.0% w/v. The plates were incubated at 28 °C (± 2 °C) and monitored daily for bacterial growth for at least 7 days. Two to three colonies of each morphotype were selected, purified from the primary culture, and stocked at -20 °C and -80 °C.

For the initial screening of antimicrobial activity in the cell-free supernatant (CFS) of the marine bacteria, *Staphylococcus aureus* ATCC 29213 was primarily used as the reference indicator strain[6]. Then, selected marine bacteria were tested against different (multi)drug-resistant bacterial strains isolated from human infections and from aquatic habitats, including *Acinetobacter baumannii*, *Aeromonas* sp., *Citrobacter freundii*, *Enterobacter* sp., *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Serratia* sp. *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*.

To assess the BS- and BE-producing potentials, the CFS of bacteria isolated from marine sponges were subjected to two screening strategies for these biomolecules, namely, emulsification test (E_{24})[7] and drop collapse assay[8]. In all tests, the controls used were 10% SDS (positive) and MSM (negative). The emulsification index (%E_{24}) was calculated by the measurement of the emulsion height divided by the total liquid height.

For the screening regarding the production of enzymes, the bacteria were cultured on different agar media for the detection of agarase[9], alginate lyase[10], amylase[11] and urease[12] production. The resulting halos were measured, and the Enzymatic Index (EI) was calculated. A positive result was determined for those exhibiting an EI ≥ 2.0.

All bioactive marine strains were identified by MALDI-TOF MS and/or 16S rRNA gene sequencing[13].

Remarkably, among the marine bacteria studied, two strains of *Pseudomonas* genus, both isolated from the sponge *Dercitus (Stoeba) latex*, were selected for BE extraction via acetone precipitation of the CFS. The crude BE extracts (named BE23 and BE44) were tested for antiadhesive[14] and antibiofilm[15] activities against *S. aureus* ATCC 29213.

3 RESULTS & DISCUSSION

A total of 923 bacterial strains were isolated from thirty Demospongiae and Calcarea specimens sampled in different caves and grottoes of the Fernando de Noronha Archipelago.

When tested for antibacterial activity, 14.6% of the isolates presented positive results against the reference strain *S. aureus* ATCC 29213. Among these, thirty-six also showed bioactivity against at least one of the (multi)drug-resistant strains isolated from human and environmental sources tested (Fig. 1A). Remarkedly, three sponge-isolated strains excelled in terms of their antibacterial activity against more than twelve of the pathogens tested. Sponge microbiomes are widely acknowledged as rich reservoirs of potentially novel antimicrobial agents[16], a critical resource amidst the growing concern of antimicrobial resistance[17].

Of the 331 marine bacteria evaluated for enzymatic production, 36.5% (121) were positive for alginate lyase, 51.6% (171) for agarase, 10.2% (34) for amylase and 9.3% (31) for urease activity (Fig. 1B). Marine-derived enzymes are capable of exhibiting remarkable properties due to their unique composition, including thermostability, salt tolerance, biocompatibility and high effectiveness[18]. As a result, they are excellent candidates for a variety of biotechnology applications, in food, detergent, livestock, cosmetics, pharmaceutical, and medical sectors[19].

Regarding the E_{24} test, 107 (11,6%) marine bacteria were positive, forming stable emulsions in mineral oil and, among these, 61 (57%) exhibited strong emulsifying activity (%E_{24} > 50). Moreover, nine strains also presented strong emulsions in soybean frying oil waste, three in diesel oil, and seven in burnt engine oil (Fig 1C). In addition to the high %E_{24} values, the emulsion layers of these strains also remained stable for more than a month. As no strain was able to drop collapse, they were all exclusively classified as BE producers[20]. Emulsion layer formation and stabilization are key traits of BE, important for industrial applications in the food, petroleum, pharmaceutical, cosmetics, painting and coating sectors[21].

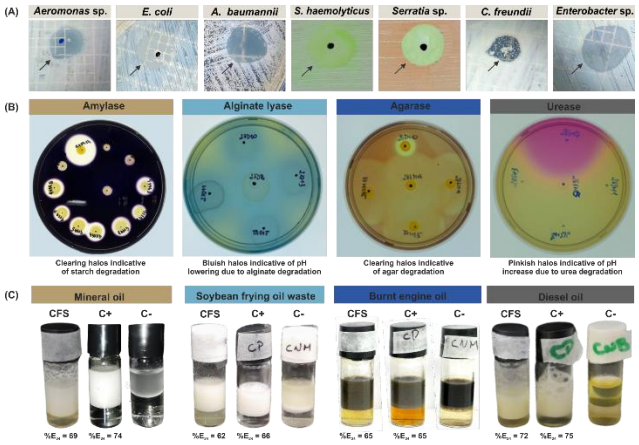

Figure 1 An overview of the main results obtained in the biotechnological prospect of bacteria associated with marine sponges sampled in the Fernando de Noronha Archipelago. (A) Antibacterial activity against different pathogens. Indicator bacteria are shown above the figures, and arrows indicate inhibition halos. (B) Enzymatic activity, including Amylase; Alginate lyase; Agarase; and Urease. (C) Emulsifying activity in different oils. CFS: cell-free supernatant of sponge-associated bacteria. Controls, C+: positive (SDS 10%) and C-: negative (MSM).

Both BE extracts, BE44 and BE23, inhibited biofilm formation and were able to disrupt the mature biofilm of *S. aureus* ATCC 29213. BE44 (12.5 mg/mL) exhibited a reduction in biofilm formation, reaching up to 71% ($p < 0.001$) at the highest concentration tested (50 mg/mL) (Figure 2A). After treating the mature staphylococcal biofilm with the same extract at concentrations of 25 and 50 mg/mL, dispersions of 52% ($p < 0.01$) and 67.2% ($p < 0.01$) were observed, respectively (Figure 2B). In parallel, the effects of reducing biofilm formation and dispersion of mature biofilm in the presence of BE23 were significant ($p < 0.05$) only at the highest concentration of the extract tested (50 mg/mL). Natural-based antibiofilm agents are structurally and functionally more diverse than synthetic ones[22]. Added to this, their biocompatibility and biodegradability contribute to a reduction in side effects[22]. Consequently, BS and BE represent suitable solutions to address the challenges posed by biofilm formation and biofouling across various industrial sectors[23].

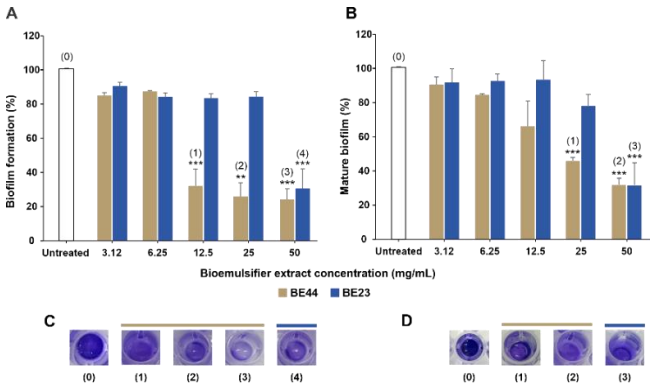

Figure 2 Antibiofilm and antiadhesive activities on biofilm-producing *S. aureus* ATCC 29213 from the bioemulsifying extracts BE44 and BE23. Activity when: (A) co-incubated *S. aureus* cells with forming biofilm and (B) in mature *S. aureus* biofilms. The wells of the 96-well plate showing biofilms in the absence or in the presence of concentrations of BEs that demonstrated significant activity, according to the numbering, are shown in (C), for anti-adhesive activity, and (D) for anti-biofilm activity. Error bars represent standard deviation. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to culture without treatment (blank, unfilled columns).

4 CONCLUSION

These findings revealed the unexplored biotechnological potential of bacteria associated with cave-dwelling sponges, particularly from the Fernando de Noronha Archipelago. The discovery of antibacterial properties of these microorganisms against pathogens of clinical relevance opens new perspectives for future investigations in biodiscovery. Also, the promising bioemulsifying and enzymatic activities demonstrated by these bacteria hold promise for diverse applications in the search for new sustainable alternatives. Especially, the antibiofilm potential observed can contribute to the development of therapeutic strategies for the control and eradication of biofilms, whether in medicine or in industrial contexts.

REFERENCES

1 YADAV, A. N. 2021. J. Appl. Biol. Biotechnol. 9 (3). 1-6.
2 GIUDICE, A. L. RIZZO, C. 2022. Mar. Drugs. 20. 617.
3 GEROVASILEIOU, V. BIANCHI, C. N. 2021. *In* Oceanography and Marine Biology: An Annual Review. Hawkins, S. J. (ed). 1nd ed. Editors Taylor & Francis. 59. 1-88.
4 MURICY, G. LAGE, A. SANDES, J. KLAUTAU, M. PINHEIRO, U. LAPORT, M. S. *et al.* 2024. J. Mar. Sci. Eng. 12. 657.
5 GUIMARÃES, C. R. PASQUALINO, I. P. DE SOUSA, J. S. NOGUEIRA, F. C. S. SELDIN, L. DE CASTILHO, L. V. A. *et al*. 2021. Colloid Surface B 208. 112072.
6 MARINHO, P. R. MOREIRA, A. P. PELLEGRINO, F. L. MURICY, G. BASTOS, M. C. SANTOS, K. R. *et al*. 2009. Mem. Inst. Oswaldo Cruz. 104. 678–682.
7 COOPER, D. G. GOLDENBERG, B. G. 1987. Appl. Environ. Microbiol. 53 (2). 224-229.
8 JAIN, D. K. COLLINS-THOMPSON, D. L. LEE, H. TREVORS, J. T. 1991. J. Microbiol. Methods. 13. 271-279.
9 HU, Z. LIN, B.-K. XU, Y. ZHONG, M.Q. LIU, G.-M. 2009. J. Appl. Microbiol., 106 (1). 181-190.
10 NAKAMURA, L. K. 1987. Int. J. Syst. Evol. Microbiol. 284-286.
11 ATLAS, R. M. 2010. Handbook of Microbiological Media. (ed). 4th ed. Taylor and Francis Group, LLC. Washington, D.C.
12 CHRISTENSEN, W. B. 1946. J. Bacteriol. 52 (4).
13 LAPORT, M. S. BAUWENS, M. DE OLIVEIRA NUNES, S. WILLENZ, P. GEORGE, I. MURICY, G. 2017. Antonie van Leeuwenhoek, 110 (4). 489–499.
14 MERGHNI, A. DALLEL, I. NOUMI, E. KADMI, Y. HENTATI, H. TOBJI, *et al*. 2017. Microb. Pathog. 104. 84–89.
15 ROSSI, C. C. SANTOS-GANDELMAN, J. F. BARROS, E. M. ALVAREZ, V. M. LAPORT, M. S. GIAMBIAGI-DEMARVAL, M. 2016. Lett. Appl. Microbiol. 63 (3). 215–221.
16 DE OLIVEIRA, B. F. R., FREITAS-SILVA, J., CANELLAS, A. L. B., COSTA, W. F., LAPORT, M. S. 2023. Curr. Pharm. Biotechnol. 24 (4). 471–485.
17 DE LA FUENTE-NUNEZ, C., CESARO, A., HANCOCK, R. E. W. 2023. Drug. Resist. Updat. 71. 101012.
18 ZHANG, J. JIANG, L. CHEN, X. LV, K. BASIONY, M. ZHU, G. KARTHIK, L. *et al.* 2021. Curr. Opin. Biotechnol. 69. 308–315.
19 GHATTAVI, S. HOMAEI, A. 2023. Int. J. Biol. Macromol. 230. 123136.
20 UZOIGWE, C. BURGESS, J. G. ENNIS, C. J. 2015. Front. Microbiol. 6. 245.
21 SAŁEK, K. GUTIERREZ, T. 2016. AIMS Microbiol. 2. 92-107.
22 MISHRA, R. PANDA, A. K. DE MANDAL, S. SHAKEEL, M. BISHT, S. S. KHAN, J. 2020. Front. Microbiol. 11. 566325.
23 JIMOH, A. A. BOOYSEN, E. VAN ZYL, L. TRINDADE, M. 2023. Front. bioeng. biotechnol. 11. 1244595.

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