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SHORT COMMUNICATION

Diversity and biotechnological potential of culturable bacteria from Brazilian mangrove sediment

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Abstract Mangrove ecosystems are environments subject to substantial degradation by anthropogenic activities. Its location, in coastal area, interfacing the continents and the oceans makes it substantially important in the prospection for biotechnological applications. In this study, we assessed the diversity of culturable bacteria present over the seasons at two depths (0–10 and 30–40 cm) in a mangrove sediment and in a transect area from the land to the sea. In total, 238 bacteria were isolated, characterized by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and further identified, by Fatty Acid Methyl Esther (FAME-MIDI), into the orders of *Vibrionales*, *Actinomycetales* and *Bacillales*. Also the ability of the isolates in producing economically important enzymes (amylases, proteases, esterases and lipases) was evaluated and the order *Vibrionales* was the main enzymatic source.

Keywords Environment · Enzymes · ARDRA · FAME-MIDI

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Introduction

The mangrove is a tropical coastal biome, located in the transition zone between land and sea, where the vegetation is dominated by a particular group of plant species (Zhou et al. 2006). This ecosystem is characterized by periodic tidal flooding which makes environmental factors such as salinity and nutrient availability highly variable, resulting in unique and specific characteristics (Holguin et al. 2006).

A phylogenetic and functional description of microbial diversity in the mangrove ecosystem has not been addressed to the same extent as that of other environments (Zhou et al. 2006). A more thorough description of the bacterial diversity and distribution in a mangrove would improve our understanding of bacterial functionality and microbial interactions found in that ecosystem (Kathiresan and Selvam 2006). Given the particular conditions of a mangrove and the adaptation of bacterial species to such conditions, the microbiota represents an important potential source of biotechnological resources to be exploited (Sivaramakrishnan et al. 2006). This biotechnological potential includes characterization of novel enzymes from previously uncharacterized bacterial species, with useful applications to many aspects of human life, from agriculture to medicine (Lageiro et al. 2007). The present work aimed to elucidate the bacterial diversity and the potential usefulness of microbiota inhabiting sediments of a well preserved mangrove in *Ilha do Cardoso* (Cananéia, Brazil).

Following the determination of the main culturable bacterial groups, an enzyme production analysis was conducted to address this diversity as a potential source for biotechnology.

Materials and methods

Mangrove and sampling points

The study was developed in a transect area, 340 m in length, along the flood gradient of the mangrove from *Ilha do Cardoso* (Cananéia—SP, Brazil). Five samples were collected from each of five different points (P1–P5) distributed over a transect. P1 had the coordinates 25°05′1, 87″S 47°57′41, 70″W and P5 was located at 25°05′12,61″S 47°57′41,21″W. The remaining points, P2, P3 and P4, were equidistantly distributed over the transect and separated from one another by 70 m. Samples were collected in stainless steel sampler cylinder (100 cm long and 7 cm in diameter), transferred to labeled plastic bags and stored in boxes. They were collected from depths of 0 to 10 and 30 to 40 cm during two different seasons; winter (12th July 2005) and summer (19th March 2006).

Bacterial isolation from mangrove sediment

Five sub-samples of sediment were collected from each point and were mixed into a composite sample. Aliquots of 10 g from each sediment mixture were added into 90 ml sterile water and shaken for 1 h at 150 rev/min. From the resulting suspensions, tenfold serial dilutions were prepared in sterilized water and 100 µl aliquots from dilutions 10^{-3}, 10^{-4} and 10^{-5} were plated onto a non-selective culture media containing Tryptone Soy Broth (TSB; Difco^{Tm}, Sparks, MD, USA) at 5% of the recommended amount (1.5 g l^{-1}), amended with 1.8% NaCl and supplemented with 50 mg Benomyl ml^{-1}. The plates were incubated at 28°C and the development of bacterial colonies was monitored for a 14-day period, with colony-forming unit (c.f.u.) estimation conducted per gram of mangrove sediment. The results were evaluated by ANOVA and average comparisons using Tukey's test at a 95% confidence level ($P < 0.05$).

ARDRA characterization of bacterial isolates

The genotypic characterization of the bacterial isolates, into ribotype groups, was based on the ARDRA patterns. Bacterial DNA was extracted from each isolate using the Wizard DNA extraction kit (Promega, Madison, USA), according to the manufacturer's instructions. The 16S rDNA gene was amplified from DNA extracted from each strain using the primers 27F and 1378R (Heuer et al. 1997). The PCR products were subjected to restriction analysis with 1 U of a 4 bp recognition site endonuclease *Hae*III and further separated by electrophoresis on a 2.4% agarose gel (stained with ethidium bromide and visualized by UV light). Distinct cleavage patterns were considered as different ribotypes identified by FAME.

Bacterial identification by MIDI-FAME

Isolates from each ribotype (10% of the total) were selected and each strain was inoculated on tryptone soy broth agar (TSBA; Difco^{Tm}, Sparks, MD, USA) for identification by MIDI-FAME. The FAME analysis was conducted by gas chromatography with automatic injector and Flame Ionization Detector (FID) (Agilent, 6850 and 7683). The output profile was organized into a chromatogram and a report generated by the Microbial Identification System software (Sherlock TSBA40 library; MIDI Inc., Newark, DE, USA). The final results, based on similarities found between the database and the nominated areas, identified the isolates.

Enzymatic production of isolates

The phenotypic characterization of isolates was conducted to evaluate the biotechnological potential of culturable bacteria found in mangroves. It was determined by a batch of assays, evaluating the production of amylases, proteases, esterases and lipases. In each of these analyses, the enzyme activities were measured by the value obtained subtracting the halo size (enzyme activity) by the diameter of the bacterial colony, generating a semi-quantitative approach.

Determination of amylolytic activity

Isolates were grown on 5% TSBA medium containing 1% starch, and incubated at 28°C for 72 h. After bacterial growth, 5 ml of 1% iodine solution was added to each plate and the presence of a stainless zone around the colony indicated amylase production (Hankin and Anagnostakis 1975).

Determination of proteolytic activity

Bacterial isolates were inoculated onto plates containing agar medium and incubated at 28°C for 5 days. After colony development, the medium was covered with Frasier solution for 2 min. Proteolytic activity was detected by the visualization of a transparent area around the colonies (Smibert and Krieg 1994).

Determination of esterase activity

Esterase activity was detected using a culture medium described by Sierra (1957). After sterilization of the culture medium, Tween 80 (previously sterilized) was added to a final aqueous concentration of 1% (v/v). After inoculation, bacterial growth was observed and the presence of degradation zone was considered indicative of esterase activity.

Determination of lipolytic activity

In order to detect lipolytic activity, a similar methodology was used as described above for esterase activity, with the exception that Tween 80 was replaced by Tween 20. The presence of a degradation zone was noted after bacterial growth (Sierra 1957).

Results and discussion

Isolation and characterization of the culturable bacterial community

Bacterial isolations were performed during two seasons and at different sediment depths, allowing a comprehensive description of the culturable microbiota found in this ecosystem. The results revealed a diverse and dense bacterial community inhabiting the mangrove sediment. The amendment of the medium with 1.8% of NaCl ensured the selection of bacteria typically found in mangrove ecosystems. The bacterial diversity found in the isolations from the two seasons revealed to be statistically different ($P < 0.05$). In the winter, the number of bacteria had a log value of 6.85 c.f.u. g^{-1} of sediment, while in the summer the value was 6.22 log c.f.u. g^{-1} of sediment. However, no significant differences were observed between samples collected from different depths. Almeida et al. (2007) found similar results when studying four estuaries during different seasons.

ARDRA typing and identification of isolates

A total of 238 colonies (159 from the winter and 79 from the summer) were chosen for further characterization with ARDRA analysis, generating ten distinct cleavage patterns (named ribotypes). Samples containing around 10% of isolates from each ribotype were used for bacterial identification by the FAME-MIDI. FAME identification is highly reliable for similarities higher than 0.70 at species level, while lower levels can affiliate isolates to higher taxonomic groups, like genus or families (Heyrman et al. 1999). Also, in some phylogenetic groups, this technique is less sensitive, like for *Vibrio*, where all isolates were classified only as *Vibrio* sp. Besides it, the identified groups were classified into three orders; *Actinomycetales* (ribotypes B, G, H, I and J), *Bacillales* (ribotypes C, D, E and F) and *Vibrionales* (ribotype A) (Fig. 1). The order *Actinomycetales* was the most diverse group, composed of seven different genera; *Brevibacterium, Dermabacter, Kocuria, Kytococcus, Microbacterium, Nesterenkonia* and *Rothia*. The order *Bacillales* was represented by four genera: *Bacillus, Kurthia, Paenibacillus* and *Staphylococcus*; while the order *Vibrionales* was represented by the genera *Vibrio* and *Listonella* (Table 1).

By analysing the ribotype frequencies and associating them with the identities determined by FAME-MIDI, it was possible to make predictions about the population fluctuation of each order over the year and at different depths (Fig. 1). A higher frequency of isolates from the order *Vibrionales* was clearly visible in winter samples, while isolates from orders *Bacillales* appeared to be more abundant in summer and at depth of 0–10 cm. The order *Actinomycetales* was more constant through the considered changes (Fig. 1).

The bacterial groups that were detected in the mangrove sediment are commonly found in marine and estuarine environments (Holguin and Bashan 1996; Thompson et al. 2004; Sousa et al. 2006; Stevens et al. 2007). The order *Vibrionales* was previously described from marine environments responding to variations in weather or to environmental factors such as salinity (Thompson et al. 2004; Sousa et al. 2006). The order *Actinomycetales* is widely found in soils, where it shows little response to abiotic factors (Tiago et al. 2004). Considering the species occurrence within the *Actinomycetales* order, although the genera *Nesterenkonia*, *Kytococcus*, *Kocuria* and *Rothia* were isolated at low densities, these groups were expected to be residents of mangrove sediments, according to previously described results (Tiago et al. 2004). Concerning the order *Bacillales*, representatives of this group were

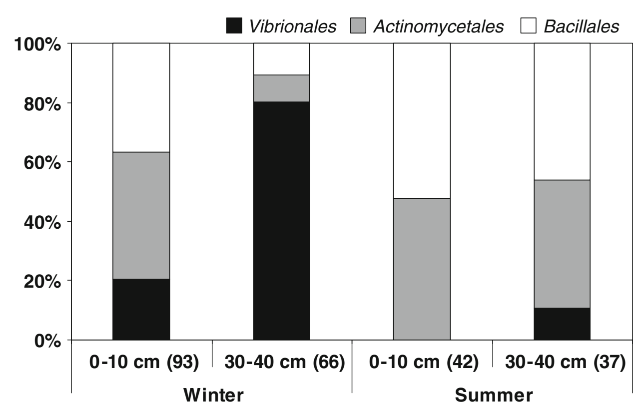

Fig. 1 Relative abundance of the three orders represented by the ten ribotypes found among the bacterial isolates from the mangrove sediments sampled in different seasons and at two depths. The number after the depth, in parentheses, represents the number of analyzed isolates

Table 1 Identification by FAME-MIDI of ribotypes constituted by isolates from mangrove sediments

Isolate	Ribotype	Order	Best match	Similarity (%)
3B31	A	Vibrionales	*Listonella* sp.	0.852
5B324	A	Vibrionales	*Listonella* sp.	0.739
5B338	A	Vibrionales	*Vibrio aestuarianus*	0.852
1B318	A	Vibrionales	*Vibrio fluvialis*	0.884
1A312	A	Vibrionales	*Vibrio* sp.	0.914
1B321	A	Vibrionales	*Vibrio* sp.	0.866
1B35	A	Vibrionales	*Vibrio* sp.	0.853
3B34	A	Vibrionales	*Vibrio* sp.	0.867
5A36	A	Vibrionales	*Vibrio* sp.	0.929
5B321	A	Vibrionales	*Vibrio* sp.	0.807
5B330	A	Vibrionales	*Vibrio* sp.	0.920
5B337	A	Vibrionales	*Vibrio* sp.	0.848
5B350	A	Vibrionales	*Vibrio* sp.	0.778
1B2S	A	Vibrionales	*Vibrio* sp.	0.882
1B3S	A	Vibrionales	*Vibrio* sp.	0.816
2A33	A	Vibrionales	*Vibrio* sp.	0.782
3A372	B	Actinomycetales	*Brevibacterium mcbrellneri*	0.628
4A11S	B	Actinomycetales	*Dermabacter* sp.	0.497
5A1S	B	Actinomycetales	*Dermabacter* sp.	0.446
1A362	B	Actinomycetales	*Microbacterium* sp.	0.600
3A357	B	Actinomycetales	*Microbacterium* sp.	0.533
3B311	B	Actinomycetales	*Microbacterium testaceum*	0.705
1A362	B	Actinomycetales	*Microbacterium imperiale*	0.793
2A17S	B	Actinomycetales	*Microbacterium imperiale*	0.700
3A35	B	Actinomycetales	*Microbacterium imperiale*	0.740
1A364	C	Bacillales	*Bacillus cereus*	0.635
5A328	C	Bacillales	*Bacillus cereus*	0.671
2B35	C	Bacillales	*Bacillus mycoides*	0.559
5A312	C	Bacillales	*Bacillus pumilus*	0.630
1A339	C	Bacillales	*Bacillus* sp.	0.523
4B2S	C	Bacillales	*Bacillus* sp.	0.325
1B39	C	Bacillales	*Bacillus subtilis*	0.550
1A33	C	Bacillales	*Bacillus* sp.	0.570
1B313	C	Bacillales	*Bacillus* sp.	0.690
3B39	C	Bacillales	*Paenibacillus* sp.	0.480
4A16S	D	Bacillales	*Staphylococcus* sp.	0.611
4A1S	D	Bacillales	*Staphylococcus* sp.	0.778
1A321	E	Bacillales	*Paenibacillus pabuli*	0.704
1A310	E	Bacillales	*Paenibacillus* sp.	0.533
1A328	E	Bacillales	*Paenibacillus* sp.	0.618
1A365	F	Bacillales	*Kurthia gibsonni*	0.554
5A310	F	Bacillales	*Kurthia sibirica*	0.429
1A322	G	Actinomycetales	*Nesterenkonia* sp.	0.590
3A33	G	Actinomycetales	*Nesterenkonia halobia*	0.721
3A11S	H	Actinomycetales	*Kytococcus sedentarius*	0.676
3B14S	H	Actinomycetales	*Kytococcus sedentarius*	0.660
5A9S	H	Actinomycetales	*Kytococcus sedentarius*	0.555

Table 1 continued

Isolate	Ribotype	Order	Best match	Similarity (%)
3B8S	I	Actinomycetales	*Kocuria* sp.	0.647
3B35	J	Actinomycetales	*Rothia* sp.	0.605

The isolates codes are formed by the point of sampling (1–5), followed by the layer sampled (A and B indicate 0–10 and 30–40 cm, respectively) and the number of the isolate. In addition, those isolates with the letter S in the end were obtained by sampling at summer

previously reported in estuarine and mangrove environments (Holguin and Bashan 1996).

It is remarkable that, although culture-independent approaches did not commonly corroborate with culture-based analysis, the identified groups are also described in other studies based on culture-independent analysis in similar environments. In Canada, the bacterial community associated with several fish, mollusks, sediments and marine waters were analysed, revealing the presence of several genera that were also isolated in the present work. Such genera included *Microbacterium* spp., *Kocuria* spp., *Bacillus* spp. and *Vibrio* spp., as the dominant organisms in this environment (Schulze et al. 2006).

Enzyme production by isolated bacteria

The detection of these groups in marine and estuarine environments can be further explored as a source for

Table 2 Enzyme production by the isolates found in the mangrove sediment and classified into the three bacterial orders

Order	Isolate	Enzymatic activity			
		Amilase	*Protease*	*Esterase*	*Lipase*
Vibrionales	1A311	ND	4.24 ± 0.12 b	ND	ND
	1B32	5.13 ± 0.07 a–e	ND	**2.97 ± 0.28 ab**	3.27 ± 0.08 bc
	1B35	6.44 ± 0.75 a–c	2.31 ± 0.22 cd	ND	ND
	2A33	ND	**8.96 ± 0.77 a**	2.28 ± 0.14 c–f	2.74 ± 0.11 b–g
	2B324	1.78 ± 0.06 fg	0.00	2.53 ± 0.27 a–d	**4.80 ± 0.11 a**
	2B326	5.46 ± 0.30 a–d	0.00	1.57 ± 0.26 i–k	1.91 ± 0.08 h–k
	2B327	0.00	4.36 ± 0.22 b	1.89 ± 0.11 e–j	2.31 ± 0.09 e–k
	2B331	**6.65 ± 1.04 ab**	ND	2.24 ± 0.46 c–g	2.51 ± 0.14 c–i
	2B38	ND	0.00	2.40 ± 0.10 b–e	2.00 ± 0.23 g–k
	3B33	0.00	**9.48 ± 0.64 a**	2.19 ± 0.17 c–h	2.33 ± 0.16 e–k
	4A312	ND	3.35 ± 0.53 bc	2.43 ± 0.14 b–e	**3.15 ± 0.22 b–d**
	4A313	6.13 ± 0.70 a–d	ND	0.00	1.91 ± 0.12 h–k
	4A35	5.00 ± 0.58 a–e	ND	1.58 ± 0.11 h–k	2.79 ± 0.18 b-f
	4B312	3.42 ± 0.48 d–f	3.35 ± 0.53 bc	2.43 ± 0.14 b–e	**3.33 ± 0.00 b**
	4B314	**7.26 ± 0.90 a**	ND	1.08 ± 0.12 jk	1.83 ± 0.04 i–k
	4B37	4.17 ± 0.48 b–f	ND	1.72 ± 0.06 f–j	2.87 ± 0.07 b–e
	4B38	6.22 ± 0.95 a–c	2.5 ± 0.28 cd	1.67 ± 0.00 g–k	2.40 ± 0.05 d–k
	4B39	0.00	7.93 ± 0.86 a	1.89 ± 0.00 e–j	1.67 ± 0.00 j–k
	5A32	4.86 ± 0.22 a–e	2.06 ± 0.48 cd	2.16 ± 0.08 e–i	2.71 ± 0.35 b–h
	5A36	ND	0.00	2.52 ± 0.29 b–d	2.5 ± 0.00 c–i
	5B31	4.7 ± 0.22 a–e	ND	2.16 ± 0.18 e–i	**3.38 ± 0.09 b**
	5B312	6.16 ± 0.71 a–c	0.00	ND	ND
	5B313	5.40 ± 0.00 a–e	0.00	**3.14 ± 0.14 a**	2.46 ± 0.18 c–j
	5B323	6.24 ± 0.88 a–c	ND	1.66 ± 0.11 g–k	2.05 ± 0.10 f–k
	5B328	5.92 ± 0.38 a–d	3.03 ± 0.25 b–d	0.00	0.00
	5B329	ND	**8.21 ± 0.74 a**	2.00 ± 0.08 d–j	1.61 ± 0.04 kl
	5B33	4.44 ± 0.38 b–f	ND	2.16 ± 0.08 e–i	2.71 ± 0.20 b–h
	5B336	ND	ND	**2.78 ± 0.19 a–c**	2.33 ± 0.16 e–k
	5B337	3.77 ± 0.37 c–f	ND	2.50 ± 0.00 b–e	2.42 ± 0.07 d–k
	5B340	**6.60 ± 0.40 ab**	ND	0.00	1.86 ± 0.02 i–k

Table 2 continued

Order	Isolate	Enzymatic activity			
		Amilase	*Protease*	*Esterase*	*Lipase*
Actinomicetales	1A358	**3.35 ± 0.34 ab**	0.00	0.00	0.00
	1A373	0.00	0.00	0.00	4.07 ± 0.18 ab
	1B18S	2.80 ± 0.14 b–f	0.00	0.00	**4.25 ± 1.25 ab**
	1B5S	2.2 8 ± 0.25 e–h	2.43 ± 0.20 ef	**2.77 ± 0.15 b**	2.29 ± 0.17 c–e
	1B7S	3.25 ± 0.05 a–c	0.00	0.00	4.00 ± 0.00 ab
	2A13S	**3.26 ± 0.08 a–c**	3.71 ± 0.29 a–d	0.00	**4.83 ± 0.16 a**
	2A1S	0.00	3.08 ± 0.14 a–e	0.00	0.00
	2A21S	0.00	3.71 ± 0.29 a–d	0.00	3.95 ± 0.12 ab
	2A23S	3.22 ± 0.10 a–d	0.00	0.00	1.94 ± 0.22 c–e
	2A324	3.05 ± 0.05 a–e	0.00	0.00	3.83 ± 0.23 ab
	2A370	3.20 ± 0.14 a–d	0.00	0.00	**4.25 ± 0.25 ab**
	3A1S	0.00	3.23 ± 0.22 a–e	0.00	0.00
	3A35	2.28 ± 0.14 e–h	3.00 ± 0.09 b–e	0.00	0.00
	3B2S	1.92 ± 0.04 gh	**4.02 ± 0.18 ab**	0.00	0.00
	3B311	3.25 ± 0.09 a–c	0.00	0.00	3.08 ± 0.08 b–d
	3B320	0.00	0.00	0.00	3.84 ± 0.27 ab
	4A11S	0.00	**4.28 ± 0.46 a**	**3.37 ± 0.34 a**	0.00
	4A353	2.79 ± 0.18 b–f	0.00	0.00	4.22 ± 0.25 ab
	5A2S	0.00	3.71 ± 0.41 a–d	**3.02 ± 0.23 ab**	0.00
	5A4S	**3.64 ± 0.14 a**	0.00	0.00	0.00
	5A5S	0.00	3.79 ± 0.12 a–c	0.00	0.00
	5B2S	0.00	3.28 ± 0.27 a–e	0.00	0.00
	5B3S	2.44 ± 0.23 d–g	**4.07 ± 0.34 ab**	0.00	0.00
	5B5S	3.11 ± 0.35 a–d	0.00	0.00	3.30 ± 0.12 bc
Bacillales	1A33	**5.06 ± 0.33 a**	4.67 ± 0.18 ab	4.67 ± 0.18 ab	1.48 ± 0.05 d
	1A337	2.86 ± 0.33 ab	**5.53 ± 0.36 a**	0.00	0.00
	1A339	3.75 ± 0.66 ab	3.74 ± 0.06 bc	1.16 ± 0.03 d	**3.86 ± 0.18 a**
	1A364	0.00	0.00	0.00	0.00 ± 0.00 e
	1B39	**4.83 ± 0.73 a**	**5.36 ± 0.13 a**	1.18 ± 0.23 cd	1.73 ± 0.24 cd
	2A349	3.62 ± 0.53 ab	4.04 ± 0.35 a–c	1.50 ± 0.17 b–d	1.47 ± 0.14 d
	2B35	3.20 ± 0.33 ab	2.84 ± 0.04 c	1.15 ± 0.03 d	1.97 ± 0.03 cd
	3A312	**4.09 ± 0.86 ab**	**5.25 ± 0.36 ab**	1.32 ± 0.08 cd	1.45 ± 0.13 d
	4B2S	2.33 ± 0.59 b	5.11 ± 0.33 ab	**2.34 ± 0.08 a**	**2.68 ± 0.20 b**
	5A1S	0.00	0.00	0.00	0.00
	5A312	0.00	4.63 ± 0.67 ab	**1.80 ± 0.04 a–c**	**1.33 ± 0.01 d**
	5B312	0.00	0.00	**1.97 ± 0.04 ab**	2.33 ± 0.16 bc

The isolates classified as the ten higher producers are shown in bold; *ND* means not determined

industrial and biotechnological applications. The availability of nutrients and content of organic matter are variable in the mangrove. In marine sediments, *Microbacterium* spp. and *Vibrio* spp. have been found to be associated with marine animals where they contribute to the decomposition of biological molecules (Yu et al. 2004).

Isolates affiliated to *Vibrionales* appeared to be the predominant enzyme-producing group within the community when compared with other groups (*Actinomycetales* and *Bacillales*), mainly for the production of amylase and protease. The other enzymes could be produced in equal amounts by the three groups (Table 2). The order *Vibrionales* revealed to be metabolically versatile, with a high production of enzymes. Previous studies also indicated that *Vibrionales* was an enzyme-producing group, and an isolate of *Vibrio fluvialis* from mangrove sediments was used to produce an alkaline extracellular protease with high efficiency for use in industrial detergents (Venugopal and

Saramma 2006). Although the *Vibrionales* were the major enzymatic producers, other isolated bacteria (*Actinomycetales* and *Bacillales*) also demonstrated the ability to produce targeted enzymes, albeit at lower levels.

An extremely high production of the enzymes by the isolates was not observed. Hence, the data present isolates with enzymatic production in the mangrove, where exceptional environmental conditions occur, like the higher salinity and the low availability of oxygen. Considering that, these enzymes could be further explored in their differential functionality, and for genetic breeding leading to higher production levels.

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