https://dx.doi.org/10.21577/0103-5053.20220069

J. Braz. Chem. Soc., Vol. 33, No. 12, 1375-1385, 2022

Article

Chromatographic Profiles of Ethyl Acetate Extracts Produced by *Bacillus* sp. Collected from the Mangroves in the Brazilian Northeast

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The genus *Bacillus* is Gram-positive, anaerobic or aerobic facultative bacteria, often described to be associated with the mangrove rhizosphere. Secondary products of their metabolism, coming from places affected by pollution and environmental stresses, such as mangroves, have been attracting interest from the biotechnological point of view. In view of this scenario, this work analyzed the ethyl acetate extracts of the cultured *Bacillus* species, previously isolated from mangroves located in the state of Sergipe, Brazil. Chromatographic profiles of seven bacterial isolates collected from two different mangroves were analyzed using gas chromatography/mass spectrometry (GC-MS) and many important compounds of biotechnological interests, mainly those derived from azo-aromatic compounds such as pyrazines, pyrrolo-pyrazines, and piperazinediones, were detected. This study is one of the first, in our knowledge, prospecting volatile or semi volatile organic compounds from ethyl acetate extracts produced by bacteria isolated from the mangroves, and intents to increase the interest in this coastal ecosystem as a source of compounds with biotechnological applications.

Keywords: mangrove, *Bacillus*, GC-MS, *N*-compounds, secondary metabolites

Introduction

Mangroves are coastal ecosystems located in the transition between terrestrial and marine environments. They are typical of tropical and subtropical regions, and reported to contain distinctive plant species, which are associated with other plant and animal components.[1,2] Asia (42%) has the largest share of mangroves followed by Africa (20%), Central and North America (15%), Oceania (12%) and South America (11%). Brazil ranks third together with Nigeria for the largest global mangrove area.[1] Mangroves are characterized by constant changes related to tidal floods, high salinity and varied nutrient availability.[2] Due to the need for adaptation, bacteria mainly associated with the rhizosphere of plants in this ecosystem, are connected with the expression of metabolites with varied biological activities.[2-4] The anthropogenic action and environmental stress associated with this environment, induce changes in the composition of the microbiota and their community behavior, generating changes in the metabolism and production of compounds with biotechnological applications.[5-7]

Microbiological analyzes of the rhizosphere have shown an association of bacterial species producing volatile organic compounds and soluble metabolites, with different biosynthesis pathways, enzymatic mechanisms, and specific chemical structures.[8] Among these are bacteria from the genus *Bacillus*, which are Gram-positive, anaerobic or facultative aerobic, spore-forming that are commonly associated to the soil. Secondary metabolites from *Bacillus* spp. attract biotechnological interest, especially with respect to their antibacterial, antifungal and antioxidant activities.[9-12]

Secondary metabolites produced during or at the end of the stationary phase of microbial development are related to the relationships between bacteria and the environment.[13-17] They can be classified according to their water solubility

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Editor handled this article: Paulo Cezar Vieira

in soluble and not-soluble compounds, being these last ones divided into volatile and semi-volatile compounds. The production of secondary metabolites is linked to factors such as nutrient availability, oxygenation, pH and temperature.[5,8,18-22]

Volatile bacterial compounds can be distributed in several distinct chemical classes, including carboxylic acids, esters, pyrazines, alcohols, and others.[8,22] Some classes are more known to be produced by Gram-positive bacteria, such as nitrogenous compounds (*N*-compounds), specifically the class of pyrrolo-pyrazines (described as having antitumor, antibacterial, antioxidant activities), pyrazines (known for antifungal activity) and piperazinediones (associated with antioxidant and antitumor activities).[23-27] In the pharmaceutical industry, beta-lactam and sulfonamide drugs, with antibiotic activities, represent the class of *N*-compounds.[28]

In this context, the present study aimed to analyze the chromatographic profile of the ethyl acetate extracts of the cultured *Bacillus* sp. isolated from the mangroves located in the Northeastern Brazil thus, describing the secondary metabolites of biotechnological interest produced by these bacteria.

Experimental

Solvents and bacterial strains

Ethyl acetate (97% of purity, Synth, Labsynth, Brazil) was used for the liquid-liquid extraction.

Seven bacterial strains were collected from two mangrove regions of the Sergipe state situated in the Northeast region of Brazil: Praia Formosa (Treze de Julho), named as "13" and Parque Eólico (Barra dos Coqueiros), named as "BA". Two soil samples were collected from BA and five soil samples from 13, at distinct geographical coordinates, as described in Table 1. For isolation, 4 g of each sample was diluted in 40 mL of saline solution (0.9% NaCl) and 1 mL was spread on nutrient agar plates which were incubated at 28 ºC for 1 to 14 days, after which the colonies with different morphological aspects were selected and further cultivated until pure colonies were obtained. The bacterial strains used in this study were identified as belonging to the genus *Bacillus* as shown in Table 1, using molecular and biochemical methods.

Table 1. Bacterial strains utilized in this study

Bacterial strains	Geo-localization	Isolate
Bacillus subtilis	10°49'01.09"S, 36°57'25.55"W	10F2-BA
Bacillus licheniformis	10°56'8.736"S, 37°2'47.676"W	7B4-13
	10°56'8.736"S, 37°2'47.676"W	7B3-13
Fictibacillus barbaricus	10°48'57.95"S, 36°57'30.59"W	6C2-BA
Bacillus megaterium	10°56'2.609"S, 37°2'46.728"W	5A1-13
Bacillus safensis	10°56'2.609"S, 37°2'46.728"W	5A3-13
Bacillus altitudinis	10°56'10.24"S, 37°2'50.18"W	8B1-13

Liquid-liquid extraction (LLE) of volatile compounds

For the preparation of organic extracts, the strains were cultivated in 25 mL of tryptic soy broth (TBS) under shaking (150 rpm) conditions at 37 °C for 24 h. After incubation for 24 h, the broth of each culture was centrifuged at 10.000 rpm for 20 min at 15 °C. Supernatant was sterilized by filtration using syringe filter (0.22 µm). The organic compounds were extracted with water/ethyl acetate (1:1). Approximately 75 mL of the sterilized supernatant were concentrated using the rotary evaporation at 45 °C to a final volume of 1 mL. A control using only the culture medium (without bacteria), treated in the same manner of samples, was also utilized. The experiment was carried out in triplicate.

Gas chromatographic and mas-spectrometry analysis of organic compounds produced by *Bacillus* spp.

The extracts were analyzed in a gas chromatograph coupled with a mass spectrometer (GC-MS, Shimadzu QP 2010-plus, Japan). An aliquot of 1.0 µL (at 1000 ppm), was injected in GC-MS in the spitless mode, using a capillary column DB-5 (50 m × 0.25 nm × 0.25 µm). Injector, detector and interface were maintained at 280 °C. The mass range varied from 45 to 450 Daltons and the energy used was 70 eV.

The oven heating program utilized was as follows: initial temperature of 40 °C for 2 min, followed by a heating rate of 3 °C min^{-1} until 280 °C, which was maintained for 10 min.

The compounds were identified using the LPTRI (linear program temperature retention indices) calculated according to the Van Den Dool and Kratz method.[29] LPTRI were automatically calculated by the software of the equipment, using a mixture of linear hydrocarbons (C_8 to C_{30}) as standards. The LPTRI were compared to those in the NIST library allowing the identification of peaks. The identification of compounds was considered positive

if the difference between experimental and theoretical indices was less than 10 units. A quantitative approach by normalized area was obtained by comparing the percentage areas [$(Area_{compound}/A_{total}) \times 100$], due to the lack of standards. Despite not allowing for absolute quantification, this method gives an approximated abundance of each compound in complex samples when commercial standards are not available.

Results

Approximately 100 compounds classified into *N*-compounds, alcohols, acids, aldehydes, ketones, esters, phenols and hydrocarbons (aliphatic and aromatic) were identified in this study. *N*-Compounds were the major chemical class obtained, formed mainly by aza-aromatic compounds (aromatic compounds where a carbon atom is replaced by a nitrogen atom in the ring), derived from pyrazine and hexahydro-pyrazine (or piperazine) and containing two N *per* molecule. Figure 1 shows the chromatographic profiles of the extracts from the seven bacterial samples and the chromatogram of the control (blank test with ethyl acetate), while Table 2 shows the identification of peaks and other details related to the chromatograms and data treatment. Table 2 is divided in three sections detailing all the oxygenated, hydrocarbons and *N*-compounds identified in this study. A similarity in the chromatographic profiles (including retention times and peak areas) of the seven samples can be observed in the Figure 1, probably because the studied bacteria belong to the same genus (*Bacillus*). In this initial analysis, a slight difference was observed between the products produced by *Fictibacillus barbaricus* (6C2-BA) and others. This sample (6C2-BA) showed two major peaks, which were found only in this sample (highlighted in Figure 1), corresponding to two high molecular weight alcohols: 1-hexadecanol and 1-octadecanol.

Chemical structures of the main *N*-compounds are shown in Figure 2, and as shown, they all seem to be originated from hexa-hydrogenated pyrazine, and can be divided into three main subclasses: piperazines, piperazinediones and pyrrolo-piperazinediones (Figure 2a).

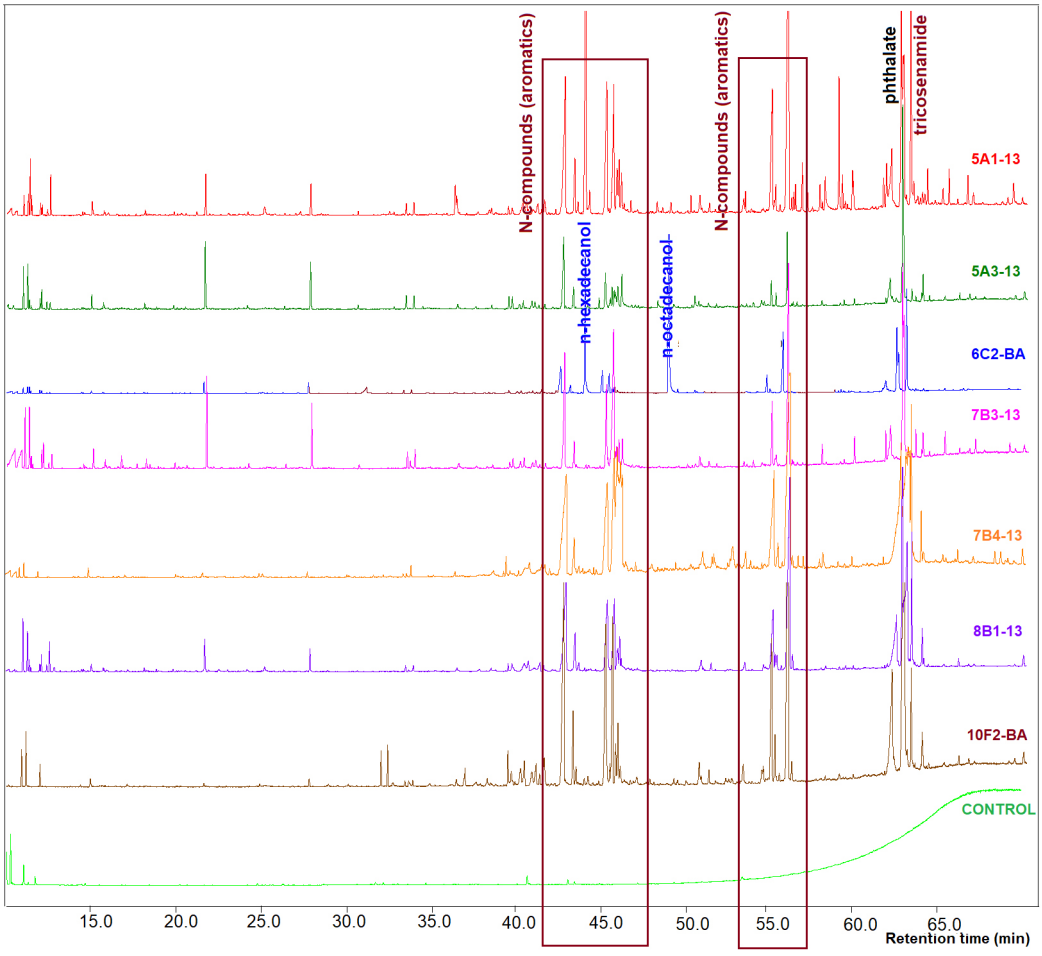

Figure 1. Chromatographic profiles (GC-MS) of organic extracts highlighting the presence of *n*-hexadecanol and *n*-octadecanol, as well as the main chemical classes of *N*-compounds: pyrrolo-pyrazinediones. Chromatographic conditions described in the Experimental section.

Table 2. Relative peak area, retention time and LPTRI of compounds detected in the organic extracts, analyzed by GC-MS (oxygenated compounds, hydrocarbons and *N*-compounds)

Distribution of the oxygenated compounds				LPTRI		Relative area / %							Reference
Class		t_R / min	Name	Experimental	NIST	10F2-BA	8B1-13	7B4-13	7B3-13	6C2-BA	5A3-13	5A1-13	
Oxygenated compounds	acids	10.41	2-methyl butanoic	851	852	n.d.	n.d.	0.65	5.89	n.d.	n.d.	n.d.	1
		10.78	3-methyl butanoic	862	862	n.d.	n.d.	0.30	3.73	n.d.	n.d.	n.d.	2
		31.25	*trans*-cinnamic	1428	1435	n.d.	n.d.	n.d.	n.d.	1.37	n.d.	n.d.	3
		45.68	palmitoleic	1941	1941	n.d.	n.d.	n.d.	0.68	n.d.	2.93	n.d.	4
		46.11	palmitic	1959	1968	0.99	0.39	n.d.	1.34	n.d.	5.81	1.27	5
		50.40	oleic	2141	2141	n.d.	n.d.	n.d.	0.24	n.d.	2.06	n.d.	4
			subtotal acids			0.99	0.39	0.95	11.87	1.37	10.80	1.27	
	alcohols	12.35	2-heptanol	908	904	n.d.	0.21	n.d.	0.26	n.d.	0.77	n.d.	6
		41.62	*n*-pentadecanol	1782	1772	1.86	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7
		44.18	1-hexadecanol	1880	1875	n.d.	n.d.	n.d.	n.d.	15.99	n.d.	n.d.	8
		49.17	1-octadecanol	2086	2081	0.19	n.d.	n.d.	n.d.	36.63	0.83	0.41	9
		50.85	1-nonadecanol	2161	2176	n.d.	n.d.	0.16	n.d.	n.d.	n.d.	0.75	10
		56.16	ethoxyphenyl-propanol	2427	n.f.	0.99	n.d.	n.d.	n.d.	n.d.	n.d.	1.16	n.f.
			subtotal alcohols			3.04	0.21	0.16	0.26	52.62	1.61	2.32	
	aldehydes	14.41	benzaldehyde	962	962	n.d.	n.d.	n.d.	0.23	n.d.	n.d.	n.d.	11
		17.55	benzeneacetaldehyde	1044	1046	n.d.	n.d.	n.d.	0.20	n.d.	n.d.	n.d.	5
		24.13	benzaldehyde, 2,4-dimethyl-	1215	1206	n.d.	n.d.	n.d.	0.39	n.d.	n.d.	n.d.	11
		30.62	dodecanal	1408	1411	n.d.	n.d.	n.d.	0.30	n.d.	n.d.	n.d.	12
			subtotal aldehydes			n.d.	n.d.	n.d.	1.12	n.d.	n.d.	n.d.	
	esters	8.99	butanoic acid, ethyl ester	809	806	n.d.	0.24	n.d.	0.44	n.d.	0.66	n.d.	13
		9.35	acetic acid, butyl ester	820	815	0.18	0.89	n.d.	1.23	0.25	2.20	0.53	14
		11.32	1-butanol, 3-methyl-, acetate	878	878	n.d.	0.32	n.d.	0.45	0.41	0.87	1.09	15
		11.41	1-butanol, 2-methyl-, acetate	881	880	n.d.	0.11	n.d.	0.16	n.d.	n.d.	0.38	16
		44.28	salicylic acid, benzyl ester	1884	1881	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.73	17
		57.86	monopalmitin	2513	2520	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.19	5
		62.05	sebacic acid, 2,6-dimethoxyphenyl ethyl ester	2721	2732	12.50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	18
		62.32	glutaric acid, methylbutenyl-dimethoxyphenyl ester (isomer 1)	2737	n.f.	n.d.	6.66	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
		62.95	glutaric acid, methylbutenyl-dimethoxyphenyl ester (isomer 2)	2772	n.f.	n.d.	16.52	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
			subtotal esters			12.68	24.73	0.00	2.28	0.66	3.73	3.92	
Oxygenated compounds	phenols	14.98	phenol	977	979	0.30	0.21	0.16	1.02	n.d.	1.81	0.40	19
		33.91	2,4-di-*tert*-butylphenol	1512	1512	0.20	0.20	0.21	1.08	0.33	1.91	0.31	20
		40.22	phenol, 2-(1-phenylethyl)-	1730	1721	n.d.	n.d.	0.11	n.d.	n.d.	n.d.	n.d.	n.f.
			subtotal phenols			0.50	0.41	0.49	2.10	0.33	3.72	0.71	
			total of oxygenated compounds			17.21	25.74	1.59	17.64	54.98	19.86	8.21	

Table 2. Relative peak area, retention time and LPTRI of compounds detected in the organic extracts, analyzed by GC-MS (oxygenated compounds**,** hydrocarbons and *N*-compounds) (cont.)

Distribution of hydrocarbons				LPTRI		Relative area / %							Reference
Class		t_R / min	Name	Experimental	NIST	10F2-BA	8B1-13	7B4-13	7B3-13	6C2-BA	5A3-13	5A1-13	
Hydrocarbons	aromatics	10.95	ethylbenzene	867	868	1.10	1.54	0.14	2.76	0.39	4.45	0.41	16
		11.21	*m*-xylene	875	874	1.95	1.38	0.24	3.22	0.54	5.57	0.51	21
		11.94	styrene	897	895	n.d.	0.21	n.d.	0.85	0.21	1.14	0.24	22
		12.03	*o*-xylene	900	898	0.66	0.51	0.08	1.14	0.18	2.01	0.21	23
		15.74	1,3,5-trimethyl benzene (mesithylene)	997	996	n.d.	n.d.	n.d.	0.13	n.d.	n.d.	n.d.	12
			aromatics			3.72	3.64	0.46	8.10	1.32	13.18	1.37	
	aliphatics	18.10	5-methyl decane	1058	1.056	n.d.	n.d.	n.d.	0.45	n.d.	n.d.	n.d.	4
		18.31	2-methyl decane	1064	1.063	n.d.	n.d.	n.d.	0.18	n.d.	n.d.	n.d.	24
		19.79	*n*-undecane	1102	1.100	n.d.	n.d.	n.d.	0.26	n.d.	n.d.	n.d.	standard, MS
		26.31	3-methyl-dodecane	1281	1.274	n.d.	n.d.	n.d.	0.23	n.d.	n.d.	n.d.	25
		36.49	*n*-hexadecane	1598	1.600	n.d.	n.d.	0.11	n.d.	n.d.	n.d.	n.d.	standard, MS
		39.34	*n*-heptadecane	1698	1.700	n.d.	n.d.	0.08	n.d.	n.d.	n.d.	n.d.	standard, MS
		44.62	*n*-nonadecane	1898	1.900	n.d.	n.d.	0.11	n.d.	n.d.	n.d.	n.d.	n.f.
		47.08	*n*-eicosane	1998	2.000	n.d.	n.d.	0.12	n.d.	n.d.	n.d.	n.d.	n.f.
		47.91	5-ethyl-nonadecane	2033	2.034	n.d.	n.d.	0.09	n.d.	n.d.	n.d.	n.d.	26
		49.43	*n*-heneicosane	2098	2.100	n.d.	n.d.	0.17	n.d.	n.d.	n.d.	n.d.	standard, MS
		51.67	*n*-docosane	2198	2.200	n.d.	n.d.	0.32	0.19	n.d.	0.44	n.d.	standard, MS
		52.48	5-methyl-docosane	2235	2.252	n.d.	n.d.	0.15	n.d.	n.d.	n.d.	n.d.	24
		53.82	*n*-tricosane	2297	2.300	n.d.	n.d.	0.15	0.38	n.d.	0.75	n.d.	standard, MS
		57.51	*n*-pentacosane	2497	2.500	n.d.	n.d.	0.12	1.15	n.d.	n.d.	0.77	standard, MS
		59.23	*n*-hexacosane	2597	2.600	n.d.	n.d.	0.13	1.50	n.d.	n.d.	1.23	standard, MS
		61.15	*n*-heptacosane	2673	2.700	n.d.	n.d.	0.11	1.61	n.d.	0.75	0.74	standard, MS
		63.39	*n*-octacosane	2797	2.800	n.d.	n.d.	n.d.	1.45	n.d.	0.54	0.75	standard, MS
		64.04	squalene	2817	2.814	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.29	27
		65.10	2-methyl-octacosane	2852	2.861	n.d.	n.d.	0.11	1.21	n.d.	n.d.	0.50	5
		66.89	*n*-nonacosane	2898	2.900	n.d.	n.d.	0.14	0.92	n.d.	n.d.	0.48	standard, MS
		68.90	*n*-triacontane	2996	3.000	n.d.	n.d.	0.11	0.56	n.d.	n.d.	n.d.	standard, MS
			aliphatics			n.d.	n.d.	2.01	10.09	n.d.	2.48	4.75	
			total of hydrocarbons			3.72	3.64	2.47	18.19	1.32	15.66	6.13	

Table 2. Relative peak area, retention time and LPTRI of compounds detected in the organic extracts, analyzed by GC-MS (oxygenated compounds, hydrocarbons and *N*-compounds) (cont.)

Distribution of *N*-compounds				LPTRI		Relative area / %							Reference
Class		t_R / min	Name	Experimental	NIST	10F2-BA	8B1-13	7B4-13	7B3-13	6C2-BA	5A3-13	5A1-13	
N-compounds	amides	16.63	acetamide, *N*-(2-methylpropyl)	1020	1.027	n.d.	n.d.	n.d.	0.64	n.d.	n.d.	n.d.	28
		47.82	pentadecanamide	2029	2.025	0.20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29
		55.23	9-octadecenamide	2366	2.375	0.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	30
		59.84	heneicosanamide	2697	n.f.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.96	n.f.
		63.19	tricosanamide	2786	n.f.	3.65	4.80	26.21	0.18	11.27	1.45	9.74	n.f.
			subtotal amides			4.00	4.80	26.21	0.82	11.27	1.45	10.70	
	amines	33.63	1-dodecanamine, *N*,*N*-dimethyl	1503	1509	n.d.	n.d.	0.08	0.44	n.d.	n.d.	n.d.	31
		39.48	1-tetradecanamine, *N*,*N*-dimethyl	1703	1694	1.60	0.30	0.37	0.32	n.d.	1.55	n.d.	29
		44.78	1-hexadecanamine, *N*,*N*-dimethyl	1904	1894	n.d.	n.d.	n.d.	n.d.	n.d.	1.23	n.d.	29
			subtotal amines			1.60	0.30	0.45	0.77	0.00	2.78	0.00	
	piperazinediones	40.11	alkyl-C5-piperazine-2,5-dione	1726	n.f.	n.d.	n.d.	n.d.	0.56	n.d.	0.91	n.d.	n.f.
		40.24	alkyl-C5-piperazine-2,5-dione	1731	n.f.	1.59	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
		40.33	alkyl-C5-piperazine-2,5-dione	1735	n.f.	n.d.	n.d.	n.d.	0.69	n.d.	1.46	n.d.	n.f.
		40.45	alkyl-C5-piperazine-2,5-dione	1739	n.f.	1.63	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
		41.36	hydroxymethyl-piperazine-2,5-dione	1773	1.772	0.42	0.64	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
		43.54	alkyl-C6-piperazine-2,5-dione	1857	n.f.	0.83	0.40	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
		43.77	alkyl-C6-piperazine-2,5-dione	1865	n.f.	n.d.	n.d.	0.10	n.d.	n.d.	n.d.	n.d.	n.f.
		45.50	alkyl-C7-piperazine-2,5-dione	1934	n.f.	0.83	n.d.	n.d.	16.87	n.d.	1.68	n.d.	n.f.
		46.30	alkyl-C7-piperazine-2,5-dione	1966	n.f.	n.d.	n.d.	4.83	n.d.	n.d.	n.d.	n.d.	n.f.
		46.37	alkyl-C7-piperazine-2,5-dione	1969	n.f.	n.d.	n.d.	2.28	n.d.	n.d.	n.d.	n.d.	n.f.
		50.71	C2-phenyl-piperazine-2,5-dione	2155	n.f.	1.40	0.71	n.d.	0.61	n.d.	1.36	n.d.	n.f.
		51.03	C2-phenyl-piperazine-2,5-dione	2169	n.f.	n.d.	n.d.	0.65	n.d.	n.d.	n.d.	n.d.	n.f.
		51.27	C2-phenyl-piperazine-2,5-dione	2179	n.f.	0.58	n.d.	n.d.	0.36	n.d.	0.60	0.30	n.f.
		51.50	C2-phenyl-piperazine-2,5-dione	2190	n.f.	n.d.	0.33	0.26	n.d.	n.d.	n.d.	n.d.	n.f.
		53.36	C3-phenyl-piperazine-2,5-dione	2293	n.f.	0.96	0.61	0.51	0.37	n.d.	n.d.	6.97	n.f.
		55.33	C4-phenyl-piperazine-2,5-dione	2383	n.f.	n.d.	0.67	0.69	n.d.	n.d.	n.d.	21.07	n.f.
		56.18	C4-phenyl-piperazine-2,5-dione	2412	n.f.	n.d.	0.58	4.53	n.d.	n.d.	n.d.	n.d.	n.f.
		61.62	toluyl-hydroxymethyl-piperazine-2,5-dione	2724	n.f.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.11	n.f.
		63.93	bis(toluyl)-piperazine-2,5-dione	2814	n.f.	n.d.	0.23	0.19	n.d.	n.d.	n.d.	0.31	n.f.
			subtotal piperazinediones			8.23	4.17	14.04	19.46	n.d.	6.01	32.75	

Table 2. Relative peak area, retention time and LPTRI of compounds detected in the organic extracts, analyzed by GC-MS (oxygenated compounds, hydrocarbons and *N*-compounds) (cont.)

Distribution of *N*-compounds				LPTRI		Relative area / %							Reference
Class		t_R / min	Name	Experimental	NIST	10F2-BA	8B1-13	7B4-13	7B3-13	6C2-BA	5A3-13	5A1-13	
N-compounds	pirazines	12.52	pyrazine-2,5-dimethyl-	913	915	n.d.	0.99	n.d.	0.70	n.d.	0.68	0.94	32
		41.14	C6-piperazine	1764	n.f.	1.52	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.f.
		43.74	C7-piperazine	1864	n.f.	n.d.	n.d.	0.07	n.d.	n.d.	n.d.	n.d.	n.f.
		46.49	C8-piperazine	1974	n.f.	n.d.	n.d.	0.50	n.d.	n.d.	n.d.	n.d.	n.f.
			subtotal piperazines			1.52	0.99	0.58	0.70	0.00	0.68	0.94	
	pirrolopyrazine	40.86	hexahydro-pyrrolo-pyrazinedione	1754	n.f.	1.34	n.d.	0.39	0.58	n.d.	1.69	n.d.	n.f.
		41.58	hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione	1788	1795	n.d.	0.22	n.d.	n.d.	n.d.	0.73	n.d.	33
		42.81	hexahydro-alkyl-pyrrolopyrazinedione	1828	n.f.	19.05	11.80	8.34	12.14	4.85	16.50	9.77	n.f.
		43.35	hexahydro-alkyl-pyrrolopyrazinedione	1849	n.f.	3.88	2.41	1.18	1.94	1.02	3.39	2.08	n.f.
		45.25	3-(2-methylpropyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione	1923	1908	13.94	7.44	5.49	7.18	3.16	7.30	7.58	34
		45.57	hexahydro-alkyl-pyrrolopyrazinedione	1936	n.f.	n.d.	n.d.	n.d.	n.d.	2.32	2.97	n.d.	n.f.
		45.70	3-(2-methylphenyl)-hexahydro-pyrrolo[1,2-*a*]pyrazine-1,4-dione	1941	1935	12.16	5.60	5.90	1.21	0.41	2.70	6.12	35
		45.92	hexahydro-alkyl-pyrrolopyrazinedione	1950	n.f.	2.92	1.76	7.85	1.52	0.72	4.24	3.84	n.f.
		53.07	hexahydro-alkyl-pyrrolopyrazinedione	2263	n.f.	n.d.	n.d.	1.14	n.d.	n.d.	n.d.	0.64	n.f.
		54.44	hexahydro-alkylphenyl-pyrrolopyrazinedione	2328	n.f.	0.96	0.33	0.09	n.d.	n.d.	n.d.	n.d.	n.f.
		54.96	hexahydro-alkylphenyl-pyrrolopyrazinedione	2352	n.f.	9.47	5.67	5.05	4.67	2.73	3.91	0.80	n.f.
		56.05	hexahydro-alkylphenyl-pyrrolopyrazinedione	2403	n.f.	n.d.	25.13	19.01	12.44	17.22	10.12	10.44	n.f.
			subtotal pyrrolopyrazinediones			63.72	60.36	54.44	41.68	32.43	53.56	41.27	
	others	41.01	C2-hydroxypiperidine	1760	n.f.	n.d.	n.d.	n.d.	0.53	n.d.	n.d.	n.d.	n.f.
		43.52	morpholinocarbonyl-imidazolidinone	1855	n.f.	n.d.	n.d.	0.22	n.d.	n.d.	n.d.	n.d.	n.f.
		44.12	cyanoacetyl-piperidine	1878	n.f.	n.d.	n.d.	n.d.	0.23	n.d.	n.d.	n.d.	n.f.
			subtotal others *N*-compounds			n.d.	n.d.	0.22	0.75	n.d.	n.d.	n.d.	
			total *N*-compounds			79.08	70.62	95.93	64.17	43.70	64.48	85.66	

LPTRI: linear program temperature retention indices; n.f.: LPTRI not found in the literature, in this case, it was used the comparison with NIST library and a similarity higher than 80; n.d.: compound not detected in the sample; MS: mass spectrometry.

In Figure 2b, one can observe two important derivatives of pyrrolo-piperazinediones, one with an isobutyl group and the other with a toluyl group. Besides these compounds, other *N*-compounds such as amines and amides were also detected. Table 3 shows the total number of compounds identified for each of the tested microorganisms.

Chemical classes such as ketones, esters, ethers and phenols were present only as trace compounds in all the samples. Sample 5A3-13 had phenols in its composition, more specifically phenol and 2,4-bis-1,10-dimethyl-phenol. Among the acids, those with low molecular weight were more prominent (methyl butanoic) for 7B3-13 and 7B4-13, while fatty acids with 16 carbon atoms, typical of vegetable fats, were predominant in samples 5A3-13 and 7B4-13, with emphasis on palmitic acid (*n*-hexadecanoic). *trans*-Cinnamic acid was found only in sample 5A3-13.

Table 2 shows the distribution of hydrocarbons in the different samples and demonstrates the diversity related to the number of compounds identified. Hydrocarbons were not found in large concentrations but were present only at trace levels.

N-Compounds presented in Table 2 were found in all the samples, at high levels. Samples 5A1-13 and 7B4-13 stood out with respect to amides, with an unsaturated amide with 23 carbon atoms (tricosenamide) showing approximately 11 and 29% of the relative area, respectively. Most of the identified *N*-compounds belong to the pyrrole-pyrazine class, and only two compounds could be confirmed by the mass spectra and the LPTRI. Their concentrations (measured by the relative peak area) are 10.28% for pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(isobutyl) (PPDHI) in 10F2-BA and 3.44% for 6C2-BA at 45.25 min, and pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(toluyl) (PPDHT), percentual area was 8.97% for 10F2-BA and 0.45% for 6C2-BA at 45.70 min.

In addition to these, some probable isomers of these compounds were observed in the samples, at relatively high concentrations, (Table 2) as the isomers of pyrrolo[1,2-*a*] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) PPDHMP at retention times of 42.81; 43.35; 45.57; 45.92 and 53.07 min, and the isomers of PPDHMPH in retention times of 54.44; 54.96 and 56.05 min.

The unequivocal identification of these compounds was not possible due to the lack of retention indices in the

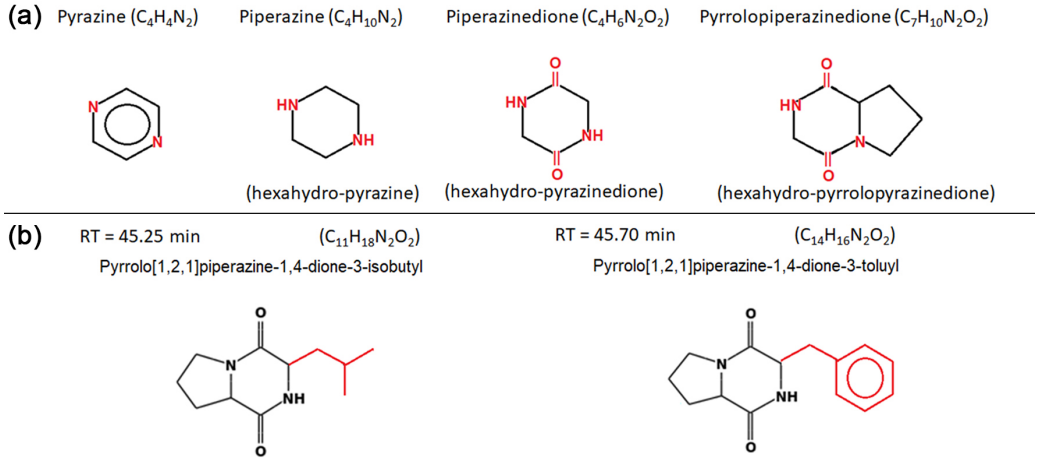

Figure 2. Chemical structure of the main *N*-compounds found in the extracts. (a) Basic structures of *N*-compounds found, and (b) *N*-compounds derived from the above structures, and that were identified in samples using LPTRI.

Table 3. Number of compounds identified from each organic extract

Species	Sample	Number of compounds			
		Hydrocarbons	*N*-Compounds	*O*-Compounds	Total
B. megaterium	5A1-13	11	16	11	38
B. safensis	5A3-13	8	19	10	37
Fictibacillus barbaricus	6C2-BA	4	9	6	19
Bacillus licheniformis	7B3-13	18	21	16	55
	7B4-13	18	25	6	49
B. altitudinis	8B1-13	4	20	10	34
B. subtilis	10F2-BA	3	21	8	32

literature. Future studies should confirm this identification using compound isolation tools and MS and nuclear magnetic resonance (NMR) analysis. It should be noted that these compounds were found in the largest concentration, not only in the nitrogenous class, but among all identified classes (among 10.6 and 25.4% of the relative area).

Besides *N*-compounds, for *B. licheniformis* (7B3-13, 7B4-13) the presence of hydrocarbons, mainly saturated, also stands out, while for *B. safensis* (5A3-13) the emphasis is on oxygenates, especially esters. Also, for the sample 7B3-13 (*B. licheniformis*), for which the distribution of classes was more uniform, presence of oxygenated compounds (acids, aldehydes and esters) was more notable.

Figure 3 shows the distribution of chemical classes according to the relative area of each compound. This approach, considering the relative area (%) of the compounds, can be considered a semi-quantitative analysis, since these are directly related to the mass concentrations of the compounds. The relationship between concentration and area is defined by a constant (response factor) that was not considered here due to the absence of commercial standards that would allow the development of the method and the construction of calibration curves. Therefore, this comparison is restricted only to this analysis and has a comparative focus, that is, it allows to compare the samples and determine which sample has the highest concentration of each compound, but the actual concentration is not defined. In this analysis, the class with the largest areas was also the *N*-compounds, confirming the previous qualitative analysis, with the exception of *F. barbaricus* (6C2-BA) which presented alcohols as the majority compounds, with 57.23% of relative area.

In Figure 3a, it is observed that, with the exception of sample 6C2-BA, for all other samples there is a clear predominance of *N*-compounds. It is important to highlight that these bacteria were isolated from mangrove but were cultivated in laboratory with a specific cultivation medium. As shown in Figure 3a, all samples other than 6C2-BA present *N*-compounds as the predominant class. The distribution of *N*-compounds can be seen in Figure 3b, where the emphasis is first on the pyrrole-pyrazinediones and, then, on the amides.

Discussion

This study elaborates a detailed chromatographic profile of the bacterial ethyl acetate extracts by GC-MS. *N*-Compounds were found to be produced by all the isolates at high levels which could be related to the growth media used for their cultivation. Many studies[5,16,29,30] have reported the importance of growth conditions, in particular the cultivation media for the production of secondary metabolites. TSB (containing pancreatic digest of casein and soy peptone) was utilized in this study to cultivate the bacteria isolated from the mangroves, probably responsible for the increased expression of nitrogen compounds. Amino acids present in culture media, for example, were attributed as the main precursor for the generation of *N*-compounds, as well as benzoic acids and phenolic compounds in the study by Sun *et al.*[15] and Tyc *et al.*[16] In another study, Kim *et al.*[31] associated the production of hydrocarbons with the presence of phenylalanine in the culture medium. TSB used in this study also provides phenylalanine during cultivation.

Many volatiles identified in this study have been associated with important biological activities. *Streptomyces* sp. isolated from mangroves (producers of metabolites such as pyrazines, pyrrolo-pyrazines and phenolic compounds) have been previously reported with antioxidant, metal chelating and superoxide radicals eliminating activity.[32]

Volatile and semi-volatile amides such as 9-octadecenamide (oleamide) were identified by Donio *et al.*[33,34] as constituents of a biosurfactant purified from *Bacillus* sp.; presenting antibacterial, antifungal

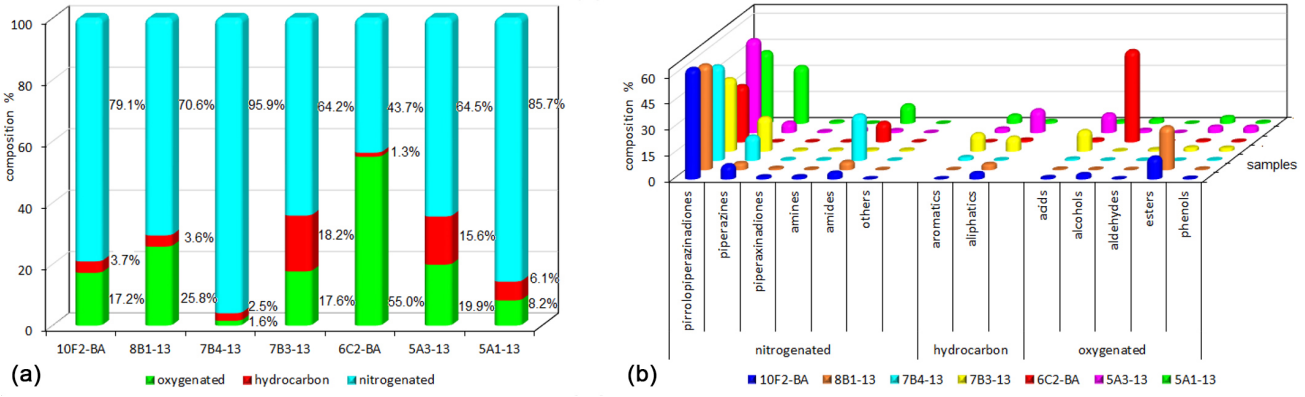

Figure 3. Quantitative approach by normalized area for organic bacterial extracts by chemical classes (a) and subclasses (b) expressed as percentage of peak areas.

and anticancer activities against epithelial carcinoma of mammary cells. Other authors[23,24,26,27] have also reported the identification of pyrrole [1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) produced by bacteria such as *S. xylosus*, correlating these compounds with antimicrobial and anticancer activities.

Alcohols with high molecular weight, like *n*-hexadecanol and *n*-octadecanol, (produced by sample 6C2-BA in this study), have been associated with antibacterial activity against *S. aureus*.[35] Venugopal *et al.*[36] identified *n*-pentadecanol in actinomycetes extracts and related it with the antibacterial activity of the extract. This compound was detected at high relative area in the sample 10F2-BA.

Fatty acids such as palmitic acid, can be related to anti-inflammatory, antibacterial and antifungal activities[37-39] while *trans*-cinnamic acid is recognized with antifungal, antimicrobial and antioxidant properties.[40-42]

The production of aromatic aldehydes such as cinnamaldehyde, benzene acetaldehyde and benzaldehyde have been related to biological activities such as inhibition of biofilm formation and antimicrobial activity, respectively.[18,42-45] Calvo *et al.*[11] also identified the presence of benzaldehyde in extracts of *B. velezensis*, which demonstrated antifungal potential *in vitro* and *in vivo*. Trihydroxy benzaldehyde has been related to apoptotic induction ability and antitumor potential, as well as antibacterial activity.[46,47] Kim *et al.*[48] associated this same compound with hypoglycemic potential, hypocholesterolemic *in vivo* as well as lipid cell reducer, with nutraceutical potential potentially applicable in the treatment of obesity.

Phenolic compounds such as phenol and 2,4-bis(1,1-dimehtyl)-phenol, produced by *B. safensis* in this work, were reported by other authors[49-54] for their anti-biofilm, antibacterial, antifungal, antioxidant and anticancer activities.

Conclusions

This study highlights the use of GC-MS for detailed chemical profiling of the cultured bacterial ethyl acetate extracts. A number of biological activities could be associated with the main compounds detected in this study, emphasizing the potential of coastal ecosystem as a source of compounds with biotechnological application. Among the most interesting compounds from the point of view of potential biological activities, were the *N*-compounds, especially those derived from azo-aromatic compounds such as pyrazines, pyrrolo-pyrazines, and piperazinediones. As mentioned before, to the best of our knowledge, this is one of the first study presenting a detailed chromatographic profile of the secondary metabolites from bacteria isolated from the mangroves.

Acknowledgments

We would like to thank CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior) for the master scholarship that supported the first author.

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Submitted: October 22, 2021
Published online: April 29, 2022