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Evaluating methods for the isolation of marine-derived fungal strains and production of bioactive secondary metabolites

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Abstract: In the present investigation we evaluate methods for the isolation and growth of marine-derived fungal strains in artificial media for the production of secondary metabolites. Inoculation of marine macroorganisms fragments in Petri dishes proved to be the most convenient procedure for the isolation of the largest number of strains. Among the growth media used, 3% malt extract showed the best result for strains isolation and growth, and yielded the largest number of strains from marine macroorganisms. The percentage of strains isolated using each of the growth media which yielded cytotoxic and/or antibiotic extracts was in the range of 23-35%, regardless of the growth media used. Further investigation of extracts obtained from different marine-derived fungal strains yielded several bioactive secondary metabolites, among which (*E*)-4-methoxy-5-(3-methoxybut-1-enyl)-6-methyl-2*H*-pyran-2-one is a new metabolite isolated from the *Penicillium paxilli* strain Ma(G)K.

Article

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Introduction

Since the early 1990's the oceans have been recognized as one of the latest untapped environments of unknown microorganisms. Up to then, the occurrence of true marine strains of bacteria and fungi has been only occasionally reported. With the advent of molecular biology tools, it has been possible to isolate and identify obligate marine bacteria and fungi present in the oceans. Continuous investigations demonstrated that marine microorganisms are an unlimited source of novel biologically active secondary metabolites (Fenical, 1993; Gallagher et al., 2010; Hughes & Fenical, 2010; Jensen & Fenical, 1994; Kelecom, 2002; Newman & Hill, 2006).

Marine-derived fungi, in particular, have yielded an increasing number of biologically active natural products (Bugni & Ireland, 2004; Saleem et al., 2007; Rateb & Ebel, 2011). While the majority of compounds isolated from marine-derived fungal strains are polyketide-derived, alkaloids, terpenes, peptides and compounds of mixed biosynthesis are also representative groups of secondary metabolites produced by these fungi. The chemical diversity of the marine-derived fungi secondary metabolism, along with the strains novelty, points this group of microorganisms as of much interest for the isolation of unusual bioactive natural products.

We have already started the first research program in Brazil aiming at the isolation of bioactive natural products from marine-derived microorganisms. A number of new compounds have been previously reported by us

(Hernandez et al., 2000; Hernandez et al., 2004; Lira et al., 2006; Pimenta et al., 2010). We have subsequently developed a multi-screening approach in order to obtain and select bioactive extracts from marine-derived fungi culture media (Vita-Marques et al., 2008). While a number of active extracts producing strains have been detected, such extracts have been obtained in only very limited amounts. Hampered by such circumstances, we envisaged a culture media screening aiming to improve the isolation of fungal strains which provide bioactive extracts, as well as the amount of extracts produced by such marine-derived fungi. Herein we report the results of this screening approach that enabled us to obtain several natural products in sufficient quantities for the evaluation of their biological activities.

Material and Methods

Fungal strains identification

Fungal isolates were identified by conventional and molecular approaches. Fungal macromorphology was examined by colony observation with a stereomicroscope (Leica MZ6, Wetzlar, Germany) and micromorphology analysis using a light microscope (Leica DM LS, Wetzlar, Germany) by wet mounts stained with Cotton Blue. Molecular identification was carried out by sequencing the 28S rDNA (D1/D2 region) and/or ITS1-5.8S-ITS2 rDNA regions coupled with phylogenetic analyses as described in Menezes et al. (2010). Identification sequences for each fungal strain which was identified can be requested to Dr. Lara Sette (larasette@rc.unesp.br).

Samples of macroorganisms and marine-derived filamentous fungi

Samples of marine invertebrates and of an alga were collected at Toque-Toque Island, off São Paulo state north coastline, Brazil: three samples of ascidians, a white *Didemnum* species, *Didemnum granulatum* (Didemnidae, Tokioka, 1954) and *Didemnum ligulum* (Didemnidae, Monniot, 1983); four samples of marine sponges, *Axinella cf. corrugata* (Axinellidae, George & Wilson, 1919), *Dragmacidon reticulatum* (Axinellidae, Ridley & Dendy, 1886), *Geodia corticostylifera* (Astrophorida, Hajdu, Muricy, Custodio, Russo & Peixinho, 1992) and *Mycale angulosa* (Mycalidae, Duchassaing & Michelotti, 1864) and one marine alga, *Sargassum* sp. Vouchers of these species are deposited at Museu Nacional, Universidade Federal do Rio de Janeiro (sponges), at Departamento de Zoologia, Setor de Ciências Biológicas, Universidade Federal do Paraná (ascidians) and at CEBIMar (*Sargassum* sp.). All samples were collected in sterilized plastic bags and immediately transported to the Universidade de São Paulo Marine Station, CEBIMar. Marine sponges and alga have been identified by Dr. Marcio R. Custódio, while ascidians have been identified by Dr. Rosana M. Rocha. In the laboratory, the animals and the alga were cleaned from visible debris and subsequently washed with a solution of $HgCl_2$ in EtOH (1 mg/mL) during 1 min, followed by three washes with sterilized sea water (Newel, 1976).

After surface sterilization, fragments of approx. 1 cm^3 from the inner part of each macroorganism specimen were inoculated on Petri dishes using three different procedures: a) spreading the fragments on different recipes of agar plates media; b) placing the fragments, without spreading, on agar plates containing the same media recipes; c) inoculation of homogenized fragments in agar plates with the same growth media. Homogenized fragments were placed in test tubes with 10 mL of sterilized sea water and homogenized using a sterile Ultra-Turrax®. Serial dilutions were obtained to 10^{-2} and 100 µL of each dilution was inoculated. Media used in Petri dishes are provided below. Growth in Petri dishes were performed at 25 °C.

All fungi growth media (pH 8.0) were prepared using artificial sea water (ASW: Vita-Marques et al., 2008): artificial sea water agar (ASW: 1 L of ASW; agar 15 g), glucose peptone yeast (GPY: glucose 1 g; soybean meat peptone 0.5 g; yeast extract 0.1 g; ASW 1L; agar 15 g), 2% malt (2%M: malt extract 20 g; ASW 1 L; agar 15 g); 3% malt (3%M: malt extract 30 g; mycological peptone 5 g; ASW 1 L; agar 15 g), Tubaki (TUB: glucose 30 g; yeast extract 1 g; peptone 1 g; K_2HPO_4 1 g; $MgSO_4$ 0.5 g; $FeSO_4$ 0.01 g, ASW 1L pH 8.0; agar 15 g), potato-carrot medium (PCM: Potatoes cooked and smashed 20 g; carrots cooked and smashed 20g; ASW 1 L; agar 20 g); corn meal medium (CMM: 42 g of corn flour were stirred overnight in 500 mL of distilled H2O at 60 °C, filtered and the filtered medium was diluted to 1 L with ASW; agar 15 g), oat meal medium (OMM: 30 g of oat flakes were boiled in 500 mL of distilled H_2O during one hour, filtrated and the filtered medium was diluted to 1 L with ASW; agar 20 g) and cellulose medium (CELM: cellulose 10 g, yeast extract 1 g; 1 L ASW; agar 15 g). Rifampicin was added in all media used for inoculation at a concentration of 300 mg/L in order to prevent bacterial contamination.

After inoculation, plates were regularly examined in order to verify the growth of filamentous fungi. Isolation of strains was performed as previously reported (Vita-Marques et al., 2008). Pure fungal colonies obtained by successive purification steps were photographed, morphologically described, subjected to microscopic analysis and deposited at the Microbiology Laboratory, Departamento de Ecologia e Biologia Evolutiva, Universidade Federal de São Carlos. Fungal strains have been preserved using three distinct procedures: a) in distilled water stored at 4 °C (Deshmukh, 2003); b) in glycerol 10% stored at -18 °C (Deshmukh, 2003) and c) in microbiological tubes containing agar slants, maintained at

2-4 °C, periodically transferred each three months. Media used for preservation were respectively the same used for strains isolation and growth.

Small scale fungi cultivation

All fungal samples were firstly grown in Petri dishes using the same media employed for isolation. After growth, 2 mm surface circles of mycelia were obtained using sterilized Pasteur pipettes and inoculated in 250 mL of culture broth of identical media formulation in which each strain was isolated. Cultures were cultivated at 25 °C, in still mode. The time of incubation varied according the each strain growth rate. Rapidly growing strains were incubated during seven days, while slowly growing strains were incubated up to 28 days.

Crude extracts from culture media

After growth in liquid media, fungal cultures were processed as follows. The media of strains grown in clarified media (GPY, 2%M, 3%M and TUB) were filtered through a Celite bed under vacuum. The mycelia of each culture were extracted with MeOH during 1 min in an ultrasonic bath and filtered. The filtered liquid media were adsorbed onto C_{18} reversed-phase cartridges. The columns were washed with 100% H_2O, and this fraction was discarded in order to eliminate salts and media nutrients. Then the columns were eluted with 35 mL 1:1 H_2O/MeOH (F1) followed by 35 mL 100% MeOH (F2). The mycelia MeOH extracts (MyE) and the two fractions (F1 and F2) obtained from the solid-phase extraction were evaporated to dryness *in vacuo* (speedvac®).

Strains grown in non-clarified media (PCM, CMM, OMM, CELM) yielded crude extracts obtained as follows. EtOAc (250 mL) was added to each flask containing the growth media+mycelia. The mixture was blended and left in a shaker (200 rpm) overnight, filtered through a Celite bed under vacuum and transferred to a separatory funnel. The EtOAc was collected, evaporated in vacuo, transferred to labeled vials with MeOH and evaporated to dryness *in vacuo* (speedvac®).

Antimicrobial and cytotoxic assays of crude extracts

Procedures for antimicrobial and cytotoxic assays have been previously reported in Kossuga et al. (2009) and in Seleghim et al. (2007), respectively.

HPLC-UV-MS analyses

HPLC-UV-MS analyses were performed using a Waters Alliance 2695 coupled on-line with a Waters 2996 photodiode array detector, followed by a Micromass ZQ2000 MS detector with an electrospray interface. The photodiode array scanned the samples at λ_{max} 200-400 nm. The MS detector was optimized to the following conditions-capillary voltage: 3.00 kV, source block temperature: 100 °C, desolvation temperature: 350 °C, operating in electrospray positive mode, detection range: 200-800 Da with total ion count extracting acquisition. The cone and desolvation gas flow were 50 and 350 L/h, respectively, and were obtained from a Nitrogen Peak Scientific N110DR nitrogen source. Data acquisition and processing were performed using Empower 2.0.

One mg of each sample was diluted in appropriate vials containing MeOH (1 mL). Analyses were performed using a C_{18} reversed-phase Inertsil® column (4.6×250 mm, 5 µm), using a gradient of 1:1 MeCN/MeOH in H_2O+0.1% formic acid at 1 mL/min, starting at 10% organic eluent and ended at 100% organic eluent after 40 min.

Isolation of secondary metabolites from the culture media of selected fungal strains

Strain AC(M2)14A, isolated from the sponge *Axinella* cf. *corrugata* and identified as *Penicilium raistrickii,* was inoculated in four 500 mL Erlenmeyer flasks containing 250 mL of 2% malt extract medium each. The strain was grown in static mode during 14 days at 25 °C. After growth, the culture medium was filtered over Celite. The filtered culture medium was adsorbed onto a C_{18} reversed-phase column cartridge (10 g), which was washed with H_2O (25 mL), then eluted with 1:1 H_2O/MeOH (25 mL) and 100% MeOH (25 mL). HPLC/UV/MS analysis of the MeOH fraction indicated a major peak with λ_{max} 240 and 310 nm in the UV spectrum and a peak with *m/z* 259 in the mass spectrum, corresponding to the $[M+H]^+$ ion of **1**. HPLC purification (column: Inertsil Phenyl, 4.6 x 250 mm, 5 µm, 150 Å; eluent: 7:3 MeOH/H_2O) of the major compound led to the isolation of 1,3,6-trihydroxy-8-methyl-9*H*-xanthen-9-one, or norliquexanthone (**1**), the identity of which was established based on comparison of its spectral properties with published data (Sundholm, 1978).

Strain AS(F)39, isolated from the alga of the genus *Sargassum* and identified as *P. steckii*, was inoculated in a 500 mL Schott flask containing 250 mL of corn meal broth. The strain was grown in still mode during 28 days at 25 °C. After growth, 250 mL of EtOAc was added and the mixture was shaken overnight before filtration over Celite and subsequent liquid-liquid partition. The organic phase was evaporated to dryness, to give 57 mg of a crude extract which displayed antibiotic activity against *Candida albicans* ATCC36801 and *Streptoccocus mutans* UA159 and presented a ^1H NMR spectrum of a single aromatic compound contaminated with fatty acids. HPLC purification of this crude extract (column: C_{18} Inertsil ODS-3, 4.6 x 250 mm, 5 µm; eluent: 75:25 MeOH/H_2O; flow: 1 mL/min) led to the isolation of (*S*)-8-methoxy-3,5-

dimethylisochroman-6-ol (2) (Lai et al., 1990; Masuma et al., 1994; He et al., 2004; Kerti et al., 2007). The comparison of ^{1}H, ^{13}C NMR and MS data of **2** with literature values (Masuma et al., 1994; Cox et al., 1979) did not allow us to distinguish it from 6-methoxy-3,7-dimethylisochroman-8-ol (**3**) (Cox et al., 1979). Therefore, compound **2** was identified by analysis of COSY, HSQC and HMBC NMR spectra. The absolute configuration of **2** was established by comparison of its $[\alpha]_D^{25}$+142 (*c* 0.1, MeOH) with the literature values (Lai et al., 1990; Masuma et al., 1994; He et al., 2004; Kerti et al., 2007).

Strain DG(M3)6'C, isolated from the ascidian *Didemnum granulatum* and identified as *Penicillium* sp., was inoculated in two 500 mL Schott flasks containing 250 mL of 3% malt broth each. The strain was grown in static mode during 14 days at 25 °C. After growth, 250 mL of EtOAc was added to each Erlenmmeyer, the mixture was blended and left shaking overnight before filtration over Celite and subsequent liquid-liquid partitioning. The organic phase was evaporated to dryness, to give a cytotoxic crude extract. The whole EtOAc extract was subjected to a solid-phase extraction on a C_{18} reversed-phase cartridge (10 g), using 100% H_2O, 1:1 H_2O/MeOH and 100% MeOH as eluents. The 1:1 H_2O/MeOH and 100% MeOH fractions were pooled, evaporated to dryness to give 25.7 mg. This fraction was purified by HPLC, using a Waters DeltaPak column (300×7.8 mm, 15 µm, 100 Å) using 37.5/37.5/25 MeOH/MeCN/H_2O as eluent+0.1% of trifluoroacetic acid, at a flow rate of 2.0 mL/min. The purification yielded 2.2 mg of a single major metabolite, identified as 13-desoxy-phomenone (**4**), identified by analysis of spectroscopic data (UV, MS, ^{1}H , ^{13}C, COSY, HSQC and HMBC NMR data) and comparison with literature data (Tirilly et al., 1983).

Strain Ma(T)H, isolated from the sponge *M. angulosa* and identified as *Trichoderma* sp., was inoculated in one 500 mL Schott flask containing 250 mL of Tubaki medium. The strain was grown in static mode during 28 days. After growth, the culture medium was filtered over Celite. The filtered culture medium was adsorbed into a C_{18} reversed-phase cartridge (10 g), which was washed with H_2O (25 mL), then eluted with 1:1 H_2O/MeOH (25 mL) and 100% MeOH (25 mL). The 1:1 H_2O/MeOH fraction (11.5 mg) displayed cytotoxic activity against colon, breast and human leukemia cancer cell lines. This fraction was separated by HPLC, using a C_{18} reversed-phase Phenomenex column (4.6×250 mm, 4 µm), using 1:1 H_2O/MeOH as eluent. Five fractions were obtained, of which Ma(T)HF2-P3 (0.6 mg), Ma(T)HF2-P4 (1.9 mg) and Ma(T)HF2-P5 (0.1 mg) were considered as pure metabolites, related to tricothecenes. However, only the structure of compound Ma(T)HF2-P4 could be established as roridin A (**5**) by analysis of its spectroscopic data (Steinmetz et al., 2008). Only the relative configuration of **5** has been established by us.

Strain Ma(G)K, also obtained from the sponge *M. angulosa* and identified as *P. paxilli*, was inoculated in ten 500 mL Schott flasks containing 200 mL of 2%M medium each, in a rotator shaker (100 rpm) at 25 °C during seven days. Then, 200 mL of EtOAc was added to each Schott flask, and left shaking at 100 rpm during 24 h at 25 °C. The mixture of the culture media and EtOAc was filtered through a Celite bed and the organic phase was separated by liquid-liquid partitioning. After evaporation, the EtOAc extract (400 mg) was separated by chromatography on a cyanopropyl-bonded sílica-gel cartridge (2 g), eluted first with 100% CH_2Cl_2 (fraction Ma(G)K-A, 130.0 mg), then 100% EtOAc (fraction Ma(G)K-B, 68.2 mg) then 100% MeOH (fraction Ma(G)K-C, 155.0 mg). Analysis by LC/UV/MS indicated the presence of peaks with UV absorptions at 220-230 and 280-300 nm, in the mass range between 240 and 260 Da in fraction Ma(G)K-A. This fraction was separated by HPLC using a C_{18} reversed-phase column Inertsil ODS-3 (4.6× 250 mm; 5µm) and a gradient of 1:1 MeOH/MeCN in H_2O+0,1% formic acid, with a 1.0 mL/min flow rate and monitoring at λ=254 nm. This separation yielded 6.0 mg of 5-(3-hydroxybutanoyl)-4-methoxy-6-methyl-2*H*-pyran-2-one (pyrenocine B, **6**), 42.7 mg of (*E*)-5-but-2-enoyl-4-methoxy-6-methyl-2*H*-pyran-2-one (pyrenocine A, **7**) and 0.8 mg of (*E*)-4-methoxy-5-(3-methoxybut-1-enyl)-6-methyl-2*H*-pyran-2-one (**8**), representing a new natural product for which the name pyrenocine J is suggested. All compounds were identified by analysis of spectroscopic data and comparison with literature data.

Pyrenocine J (**8**): yellowish white amorphous solid. UV λ_{max} nm (ε.) MeOH: 228 (49012), 285 (14646). FT-IR (cm^{-1}): 2926, 1709, 1558, 1251. ^{1}H NMR (400 MHz, TMS, DMSO-d_6): δ(ppm); 1.20 (3H, d, *J*=6.3 Hz, H3-10), 2.28 (3H, s, H3-12), 3.20 (3H, s, H3-13), 3.83 (overlapping m, H3-11, H-9), 5.61 (1H, s, H-3), 5.81 (1H, dd, *J*=7.2 and 16.0 Hz, H-8), 6.20 (1H, d, *J*=16.0 Hz, H-7). ^{13}C NMR (100 MHz, TMS, DMSO-d_6): δ (ppm); 18.1 (C-12), 20.9 (C-10), 55.2 (C-13), 56.6 (C-11), 77.2 (C-9), 87.4 (C-3), 109.1 (C-5), 119.9 (C-7), 137.4 (C-8), 159.4 (C-2), 162.2 (C-4), 169.7 (C-6). ESI$^+$-MS: *m/z* 247.0960 $[M+Na]^+$(calcd for $C_{12}H_{16}O_4Na$, 247.0941). $[\alpha]_D^{25}$ +34.4 (c 0.08, MeOH).

Results and Discussion

Table 1 indicate that the number of fungal strains (688) we recovered from eight species of macroorganisms is in agreement with the results of previous investigations that marine invertebrates, in particular filter-feeding animals, host a large number of microorganisms (Friedrich et al., 2001; Lee et al., 2001; Piel, 2009; Bugni & Ireland, 2004; Fenical, 1993; Kobayashi & Ishibashi, 1993; Pietra, 1997). The three Didemnidae ascidian species we collected yielded a slightly smaller number of associated

fungi than sponges. A similar result was obtained from a single specimen of marine alga, *Sargassum* sp. Two distinct collections of the ascidian *Didemnum ligulum* and of the sponge *Axinella* cf. *corrugata* were made. We have observed that the number of fungal strains associated with these animals varied in each collection (data not shown), but we cannot ascertain the reason why we observed such a variation in the number of strains isolated, in each case.

Table 1. Number of strains isolated from each marine macroorganism.

	Macroorganism	Strains	Total
Ascidians	white Didemnidae	55	227
	Didemnum granulatum	26	
	Didemnum ligulum	146*	
Sponges	*Axinella cf. corrugata*	111*	410
	Dragmacidon reticulata	40	
	Geodia corticostyllifera	80	
	Mycale angulosa	179	
Alga	*Sargassum* sp.	51	51
Total			688

*Strains obtained from two distinct collections.

The number of fungal isolates recovered from each macroorganism depended on the inoculation method and growth media used for the isolation of strains. Figure 1 shows that the spread of diluted homogenates on Petri dishes yielded 144 strains, while the spread of marine organism fragments on Petri dishes led us to obtain 197 strains. The isolation of marine-derived fungi by simply inoculating 1 cm^3 fragments of marine organisms on Petri dished yielded 347 strains. This last procedure enabled us to obtain pure strains faster than using the fragment or homogenate spread. The same procedure was used by Höller et al. (2000), who were able to isolate 681 strains of marine-derived fungi from sixteen species of marine sponges, while we isolated 410 strains from four species of marine sponges. A quite similar protocol was reported by Kjer et al. (2010) as very appropriate for the isolation of marine-derived fungal strains. Therefore, this method appears to be highly suitable for the isolation of marine-derived fungal strains associated to marine organisms. We subsequently used only the deposition of marine macroorganism fragments on the agar of Petri dishes for the recovering of pure fungal strains.

When considering the culture media used for the isolation and purification of marine-derived fungal strains, we observed a large variation in the number of strains obtained, depending on the media used for such purpose (Figure 2), in agreement with Bredholt et al. (2008) for the isolation of Streptomycetes and Sangnoi et al. (2009) for the isolation of marine gliding bacteria. The use of artificial sea water (ASW) as a growth medium provided the smallest number of strains (seven). This result is not surprising when one considers that this is a selective and a nutritionally very depleted medium. Only strains which need almost no nutrients for growth will be isolated on ASW agar. Furthermore, such strains usually grow more slowly. Thus, it is possible that some strains we have not obtained in nutritionally depleted media may have been overlooked, because they were left to grow for only 28 days. Some fungal strains may require up to three months of incubation for growth, such as *Penicillium tropicoides* (Houbraken et al., 2010). The marine fungus *Plectosporium oratosquillae*, for example, requires 21 days to grow 15-16 mm in potato-carrot agar (Duc et al., 2009). In our experiments, other nutritionally depleted media, such as cellulose and Tubaki, yielded a considerable number of marine-derived fungi. Similar results were obtained by Menezes et al. (2010). These results confirmed the fact that the isolation of fungal strains from the marine environment is highly dependent on the media used for isolation (Bugni & Ireland, 2004). When nutritionally richer media have been used, such as oat meal, potato and carrot, 2% malt and 3% malt, a much larger number of strains were obtained (Figure 2). Furthermore, 3% malt is a medium free of complex fat mixtures, a suitable feature for obtaining crude extracts from microbial growth media. We observed that oat meal, potato-carrot and corn meal media yielded more lipid-rich extracts. Fremlin et al. (2009) and Kjer et al. (2010) also reported the advantages of using malt-based media for the isolation and growth of marine-derived fungal strains for the production of bioactive extracts. The use of different media is useful to enhance the isolation of taxonomically unrelated strains. For example, in the present investigation nine different media yielded 410 fungal strains from four marine sponge species, while Höller et al. (2000) obtained 681 strains from sixteen species of marine sponges using six different media. It is desirable to use media with drastically distinct composition for such purposes, but the growth of fungal strains must be adequately performed in each media. A careful choice of media and growth conditions must be considered depending on the purposes aiming the isolation of marine fungal strains.

In the case of isolation and growth of marine-derived fungal strains, the effect of seawater concentration in the media is also significantly relevant, as showed by Bugni & Ireland (2004), Yang et al. (2007) as well as by our previous investigations (Vita-Marques et al., 2008; Pimenta et al., 2010). In general, high sea water concentration inhibits rather than promotes the growth and secondary metabolites production.

The percentage of strains isolated which yielded bioactive crude extracts varied consistently between 20 and 35% for each growth medium (Table 2 and Figure 3). Interestingly, we did not observe any correlation between a particular medium feature, such as being nutritionally rich or depleted, and the isolation of strains which yielded

bioactive crude extracts. For example, 34% of the strains isolated in Tubaki (a nutritionally depleted medium) gave active extracts, while over 29% of strains obtained in the nutrient rich corn meal medium produced active crude extracts (Figure 3). These results show that a considerable percentage of marine-derived microbial strains produce bioactive secondary metabolites, regardless of the media used for their isolation. Exception made to the strains isolated from the marine sponges *Axinella* cf. *corrugata* and *Geodia corticostyllifera*, and from the alga *Sargassum* sp., the majority of strains isolated from the remaining marine macroorganisms yielded cytotoxic rather than antibiotically active crude extracts. Among 435 extracts tested in cytotoxicity assays, 23% displayed moderate or potent cytotoxic activity (Table 3 and Figures 4 and 5). Additionally, strains yielded an almost comparable number of potently and moderately cytotoxic crude extracts, in all four cancer cell lines on which the extracts have been tested. Considering the antibiotic activity displayed by the crude extracts obtained from the marine fungal strains isolated, the largest number of extracts were active against *Staphylococcus aureus*, followed by extracts active against *Streptococcus sanguinis* and *Streptococcus mutans* (two strains), but only a small number of crude extracts displayed a significant activity in antimicrobial assays.

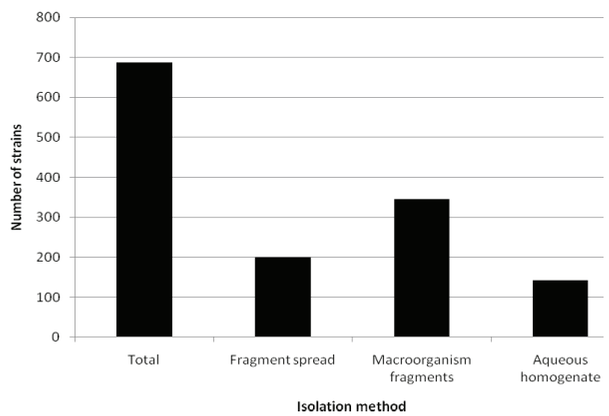


Figure 1. Number of strains isolated from marine macroorganisms using different inoculation methods.

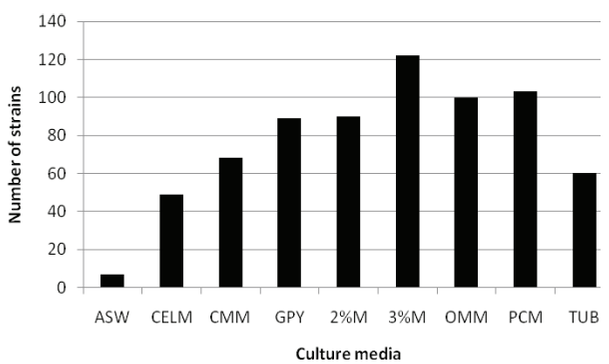


Figure 2. Number of marine-derived fungal strains isolated using different culture media. Growth media: see experimental section.

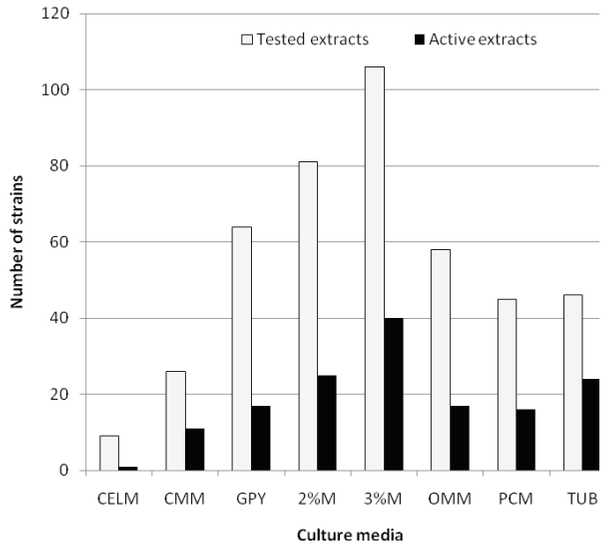


Figure 3. Tested fungal extracts and active fungal extracts per culture medium. See the experimental section for growth media abbreviations.

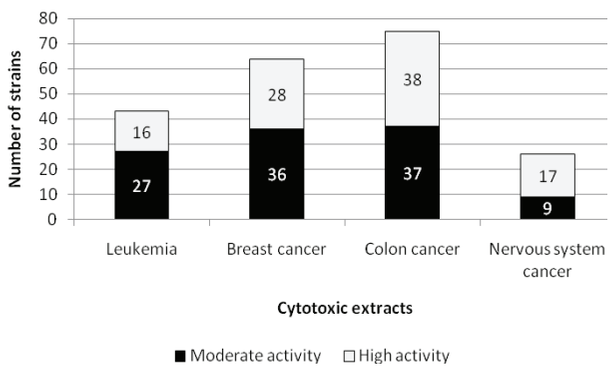


Figure 4. Cytotoxic activity of extracts obtained from marine-derived fungi. Cancer cell lines: Leukemia (HL60 human cancer cells), Breast (MDA-MB435 human mammalian breast cancer cells), Colon (HCT8 human colon cancer cells), Nervous system cancer (CNS 295 cancer cells). Potency: moderate cytotoxic activity=50% to 75% inhibition of cancer cell growth; high cytotoxic activity=>75% inhibition of cancer cell growth.

The evaluation of methods used for the isolation of marine-derived fungal strains has been occasionally addressed. Höller et al. (2000) used a single procedure for the incubation of marine sponge fragments in Petri dishes, using six different media for the isolation of marine-derived fungi, and isolated 681 fungal strains from sixteen species of sponges, but did not mention the effect of distinct growth media on the number of strains recovered. Jensen et al. (2005) were able to obtain 6,425 actinomycetes strains from 288 marine sediment and marine macroorganism samples. Actinomycetes were obtained from 77% of all samples inoculated in growth media. The authors showed the significant influence of isolation methods on the recovery of actinomycete strains and indicated the importance on

Table 2. Number of strains isolated from marine macroorganisms which yielded bioactive crude extracts*

Macroorganism	# strains which yielded crude extracts	# tested crude extracts	# strains which yielded active crude extracts
White *Didemnum*	55	40	13
D. granulatum	26	26	5
D. ligulum	146	44	18
Axinella cf. *corrugata*	111	67	15
D. reticulata	40	40	26
G. corticostilifera	80	56	32
M. angulosa	179	127	37
Sargassum sp.	51	35	5
Total	688	435	151

*Only extracts obtained in >20 mg were subjected to bioassays.

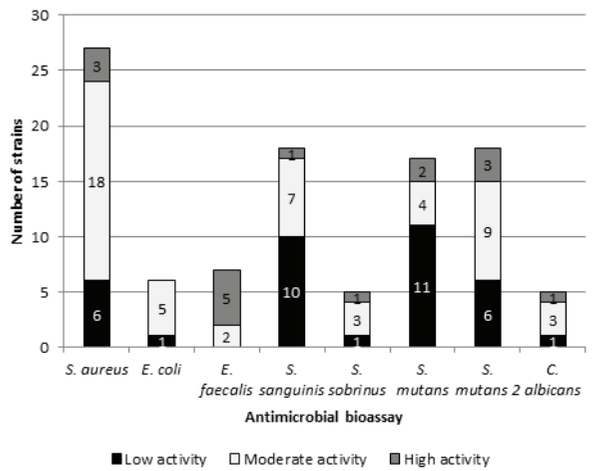


Figure 5. Antimicrobial activity of extracts obtained from marine-derived fungi. MIC = minimal inhibitory concentration. Potency: low antimicrobial activity = MIC≥250 µg/mL; moderate antimicrobial activity = MIC at 125 µg/mL; high antimicrobial activity = MIC≤67.5 µg/mL.

the improvement of culture methods for the isolation and growth of obligate marine actinomycetes. Romanenko et al. (2008) used diluted homogenates of the mollusk *Anadara broughtoni* for the isolation of 149 strains of marine bacteria. Only eight of the 149 strains isolated displayed antibiotic activity on human pathogenic bacteria. Bredholt et al. (2008) used four different media for the isolation of 3200 actinomycetes from Norwegian marine sediments. Extracts of all strains were obtained in three different growth media, and tested against *Micrococcus luteus* and *Candida albicans*. The percentage of active extracts varied depending on the site of sediment collection, ranging from 13% to 58% of strains which produced active extracts. The importance of media used for extracts production was not discussed. Hong et al. (2009) used eleven different media for the isolation of actinomycetes from mangrove samples, aiming to test crude extracts produced by these bacterial strains in antibiotic, cytotoxic and "biochemical" assays. However, the authors did not discuss any aspect related to the importance of media used for the isolation or growth of the strains obtained, or even related to the activity of the extracts obtained. A single growth medium was used by Dharmaraj & Sumantha (2009) for the isolation of 94 streptomycetes strains from four species of marine sponges. More than 28% of the strains isolated produced extracts that displayed broad antibacterial activities. Antibacterial activity was observed for extracts of 58 of the strains isolated, while 63 of the strains isolated produced antifungal extracts. Zhang et al. (2009) reported the isolation of 43 fungal strains obtained from eight species of marine algae and three species of marine invertebrates using a single growth medium. Over 84% of the strains isolated displayed antibiotic activity against at least one human microbial pathogen, and over 23% inhibited at least four of such pathogenic strains. Sangnoi et al. (2009) also used a single medium for the isolation of 84 strains of marine gliding bacteria from biofilms, invertebrates and algae. Among the strains isolated, 27 were selected based on their morphological features for growth in four different media for the production of extracts. After growth, extracts obtained from these media were tested for cytotoxic activity against four cancer cell lines. The results obtained showed that strains grew faster in a casitone, malt extract plus yeast extract based medium than in a baker's yeast paste medium, or in a peptone plus yeast extract based medium, or even in skim milk plus yeast extract based medium, and produced the largest number of cytotoxic extracts in the same medium as well.

The above mentioned studies indicate the importance of a careful choice on the conditions used for strains isolation and growth for secondary metabolites production, in order to obtain a significant number of strains which produce bioactive compounds.

In the present investigation, a few strains were

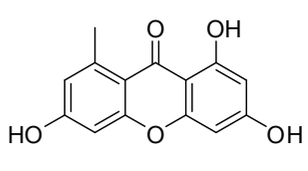
selected based on their bioactive and chemical (HPLC/UV/MS) profiles, as well as considering the generation of suitable amounts of extracts from the culture media, aiming at the isolation of pure secondary metabolites. These strains were also submitted to taxonomic identification.

Norliquexanthone (**1**), also known as 1,3,6-trihydroxy-8-methyl-9H-xanthen-9-one, was isolated from the fungus *P. raistrikii* obtained from the sponge *Axinella* cf. *corrugata* and identified by analysis of spectroscopic data along with comparison with literature data (Sundholm, 1978). The structure and absolute stereochemistry of (*S*)-8-methoxy-3,5-dimethylisochroman-6-ol (**2**), isolated from *P. steckii* obtained from an alga belonging to the genus *Sargassum*, could be established by analysis of spectroscopic data and also by comparison with literature data (Lai et al., 1990; Masuma et al., 1994; He et al., 2004; Kerti et al., 2007). A *Penicillium* sp. strain DG(M3)6'C, isolated from the ascidian *Didemnum granulatum*, yielded 13-desoxy-phomenone (**4**), identified by analysis of spectroscopic data and comparison with literature data (Tirilly et al., 1983). Roridin A (**5**) was isolated from *Trichoderma* sp. obtained from the sponge *M. angulos*a and also identified by analysis of spectroscopic data and comparison with literature data (Steinmetz et al., 2008). All known compounds isolated were reportedly bioactive as antimicrobial and/or cytotoxic agents.

The fungal strain Ma(G)K, obtained from the sponge *M. angulosa* and identified as *P. paxilli*, gave an extract which was cytotoxic against MDA-MB435 (human mammalian cancer cells), HCT8 (human colon), CNS 295 (central nervous system cancer cells) and HL60 (leukemia) cells. Fractionation of this crude extract yielded three 2-pyrones, belonging to the class of pyrenocines, of which two were known (**6** and **7**), and one was a new natural product (**8**). Pyrenocines B (**6**) and A (**7**) were first isolated from *Pyrenochaeta terrestris* and identified by spectroscopic and X-ray diffraction analysis (Sato et al., 1979; Sato et al., 1981). Compounds **6** and **7** isolated by us from *P. paxilli* growth medium presented ^{1}H and ^{13}C chemical shifts virtually identical to those reported in the literature (Sato et al., 1981).

The new compound **8** presented a $[M+Na]^{+}$ ion at m/z 247.0960, corresponding to the formula $C_{12}H_{16}O_4$. Typical pyrenocines ^{1}H NMR signals observed at δ 3.83 (s, OMe-11), 2.28 (s, Me-12) and 5.61 (s, H-3), as well as ^{13}C signals at δ 159.2 (C-2), 87.4 (C-3), 162.2 (C-4), 109.1 (C-5), 169.7 (C-6), 56.6 (C-11) and 18.1 (C-12) were in agreement with literature values for the 2-pyrone moiety in **8**. The presence of a 1-(*E*)-3-methoxybut-1-enyl chain in **8** could be ascertained by analysis of COSY and HMBC spectra. In the COSY spectrum, the terminal methyl group at δ 1.20 showed a vicinal correlation to the oxymethine proton at δ 3.8 (m, H-9), which was sequentially coupled to the vinylic proton at δ 5.81 (dd, 7.2 and 16 Hz, H-8), which in turn was coupled to another vinylic proton at δ 6.20 (d, 16 Hz, H-7). In the HMBC spectrum, one of the two methoxyl groups (δ_H 3.2; δ_C 55.2) showed a long range coupling to the carbon at δ 77.1 (C-9), thus establishing its connection to C-9. Finally, the vinylic protons at δ 5.81 and 6.20 showed long range couplings to the carbons C-4, C-5 and C-6, enabling us to completely establish the planar structure of **8** as (*E*)-4-methoxy-5-(3-methoxybut-1-enyl)-6-methyl-2*H*-pyran-2-one. Although **8** is optically active, with $[\alpha]_D^{25}$ +34.4 (c 0.08, MeOH), it did not present a Cotton effect in circular dichroism analysis. Due to the small amount of **8** available, we did not attempt to establish its absolute configuration. On this basis, **8** was identified as a new natural product for which we propose the name pyrenocin J. Pyrenocines have been reported as being cytotoxic and antibiotic (Sparace et al., 1987; Amagata et al., 1998; Rukachaisirikul et al., 2007).

In conclusion, in the present investigation we



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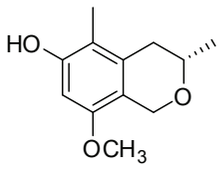

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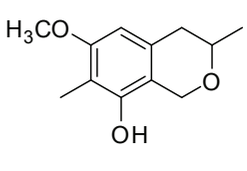

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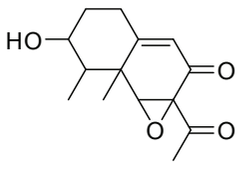

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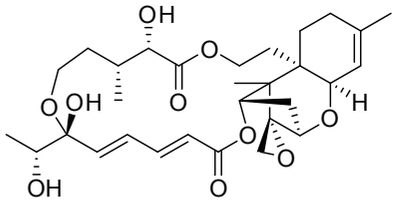

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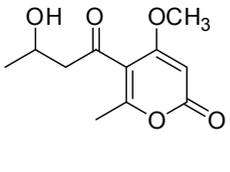

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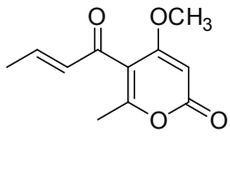

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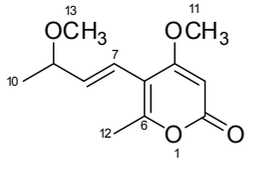

8

observed that adequate procedures for the isolation, growth and secondary metabolite production by marine-derived fungi should be carefully evaluated. Deposition of marine macroorganisms fragments on Petri dishes appears to be the most suitable inoculation method for the isolation of marine-derived fungal strains. While nutrient-rich media are suitable for the isolation of a large number of marine-derived fungal strains that produce bioactive extracts, nutrient-depleted media may yield taxonomically distinct strains which also present active extracts. Growth conditions must be adjusted depending on the media used for the isolation, and secondary metabolites production marine-derived fungi. The use of 3% malt medium proved to be the most suitable for the isolation of a significant number of bioactive secondary metabolite producing fungal strains from marine macroorganisms. The use of distinct isolation conditions enabled us to obtain a wide variety of marine-derived fungal strains, which yielded several active secondary metabolites after fractionation of selected bioactive crude extracts. Among the compounds isolated, pyrenocine J (**8**) is a new natural product isolated from the marine-derived fungus *P. paxilli* obtained from the sponge *M. angulosa.*

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