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Bioconversion of acetophenones by marine fungi isolated from marine algae *Bostrychia radicans* and *Sargassum* sp.

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ABSTRACT

The bioconversion of acetophenone derivatives **1-6** was investigated using whole cells of marine fungi *Botryosphaeria* sp. Br-09, *Eutypella* sp. Br-023, *Hydropisphaera* sp. Br-27 and *Xylaria* sp. Br-61 isolated from the red alga *Bostrychia radicans* and *Arthopyrenia* sp. SGPY-41, *Penicillium* sp. SMA2-8, *Pestalotiopsis* sp. SMA2-C isolated from brown alga *Sargassum* sp. Asymmetric reduction produced the enantiopure (*R*)- or (*S*)-alcohols **7-12** with high enantiomeric excess (>99 % *ee*). This study describes the first investigation with marine-derived fungi recovered from algae for biocatalytic reduction. The fungus *Botryosphaeria* sp. Br-09 showed excellent reductions for *ortho*-acetophenone derivatives **1-6**.

KEYWORDS: *Bostrychia radicans*, *Sargassum* sp., marine fungi, reduction, ketones

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INTRODUCTION

Marine environment contain a total of approximately 3.67 x 10^{30} microorganisms and 71 percent of the earth's surface is covered by the ocean [1]. This enormous microbial biodiversity has been little explored to produce novel enzymes and metabolites with biotechnological applications. Therefore, the marine environment represents a significant source of enzymes to be explored in biocatalytic reactions. In this context, marine algae were used for the stereoselective reductions of carbonylic compounds [2-6]. Fluoro, chloro and bromo acetophenone derivatives were reduced with good enantioselectivity using red algae *Cyanidioschyzon merolae* and *Cyanidium caldarium* [7]. Acetophenone derivatives are interesting compounds for biotransformation and have been effectively used as a building block for the asymmetric synthesis of drugs [8].

The literature reports the reduction of the carbonyl groups using various biocatalysts such as terrestrial microorganisms [9] and plants [10]. However, the number of reduction process by marine microorganisms has been little explored in

biocatalysis [11-12]. Some studies have showed the potential for biotransformation using marine fungi, such as *Rhinocladiella atrovirens* and *Rhinocladiella* sp., isolated from the Okinawan brown alga *Stypopodium* zonale, which were able to produce bromosesquiterpenes [13].

The friendly organic preparation methodology for the production of chiral compounds using marine fungi has been applied in our laboratory, and interesting results concerning the use of ketone reductions by whole microbial cells were obtained [14-15]. Recently, we reported interesting results to the reduction of *ortho*-acetophenone derivatives using red marine algae *Bostrychia radicans* and *B. tenella* and their associated bacteria, producing (*S*)-phenyl ethanols with high enantiomeric excesses [16].

Continuing the studies using organisms from marine environment, here we describe the first application of marine fungi isolated from marine algae *Bostrychia radicans* and *Sargassum* sp. for the stereoselective reduction of acetophenone derivatives. The use of microorganisms offers advantages for biocatalytic reactions, as the fast growth, different enzymes are obtained from multienzymatic system, and the application of the new biocatalysts for reduction of organic compounds. Seven species of marine fungi are shown as new biocatalysts for bioconversion of ketones.

MATERIALS AND METHODS

General methods

The reagents *ortho*-iodoacetophenone (**1**), *para*-iodoacetophenone (**2**), *ortho*-fluoroacetophenone (**3**), *ortho*-chloroacetophenone (**4**), *ortho*-bromoacetophenone (**5**) and *ortho*-nitroacetophenone (**6**) were purchased from Sigma-Aldrich. The products derived synthetically or from enzymatic processes were purified by column chromatography (CC) over silica gel (230-400 mesh) eluted with mixtures of *n*-hexane:EtOAc. The eluent column was monitored by TLC on pre-coated silica gel 60 F_{254} layer (aluminum-backed: Sorbent) eluted with *n*-hexane/EtOAc. The compounds were analyzed using a Shimadzu model 2010 GC/FID equipped with an Auto-injector AOC20i and Varian CP-Chiralsil-DEX β-Cyclodextrin column (25 m x 0.25 mm i.d.; 0.39 μm). The injector and detector were maintained at 200°C, the split ratio of the injector was 1:20, and the carrier gas was N_2 at 60 kPa. The *ee* values of alcohols were determined by GC-FID analyses. The programs used by GC/FID analyses, the syntheses protocol of racemic alcohols **7-12** and spectroscopy data (^{1}H-NMR, MS and IR) are described in the literature [16]. All the manipulations involving marine fungi were carried out under sterile conditions in a Veco laminar flow cabinet. Technal TE-421 or Superohm G-25 orbital shakers were used in the biocatalysed experiments. The optical rotations of alcohols **7-12** obtained from the biocatalytic reductions were determined in a 1 dm cuvette using a Perkin-Elmer model 241 polarimeter and were referenced to the Na-D line [16].

Collect and identification of marine algae

The red alga *Bostrychia radicans* and brown alga *Sargassum* sp. were collected in the South Atlantic Ocean off the northern coast of the State of São Paulo, Brazil, by group researches of HM Debonsi and RGS Berlinck, respectively. *Bostrychia radicans* was identified using conventional taxonomy methods by N. S. Yokoya from the Botanic Institute of São Paulo, Brazil (http://www.ibot.sp.gov.br/).

Isolation of marine fungi associated with red alga *B. radicans*

The isolation of marine fungi associated with *B. radicans* was performed by ALL De Oliveira and HM Debonsi. *B. radicans* was cleaned from epiphytes and stored in flasks containing sterilized artificial sea water (ASW) supplemented with chloramphenical (0.2 g/l), and then immersed in ethanol 70 % for 15 s and washed three times with ASW. The disinfection of fresh alga was performed by three different methodologies. Initially the alga was immersed in ethanol 70 % for 15 s and washed three times with ASW. In addition, the alga was washed with ethanol 70 % for 10 s, immersed in HClO 0.01 % for 5 s, and washed three times with ASW. Than, the algal biomass was washed with ethanol 70 % for 5 s, immersed in HClO 0.01 % for 5 s, and washed three times with ASW. Next, the algal biomass was cut in small slices using a scalpel, and the fragments were transferred to Petri dishes containing PDA medium (potato-dextrose-agar).

The Petri dishes were incubated in an oven at 32°C for 8 days. The microorganisms grown on the plates containing PDA were isolated and purified using conventional procedure.

In another procedure, the biomass of *B. radicans* (20.0 g) was triturated in a blender with ASW (0.2 l). Then, chloramphenical (0.2 g) and agar-agar (15.0 g) was added to the cell culture medium, and the volume was completed to 1 l. The algal culture medium was harvested in plates and incubated in an oven at 32°C for 8 days. The different colonies of microorganisms grown on the plates were isolated and purified.

Isolation of marine fungi associated with brown alga *Sargassum* sp.

The isolation of the marine fungi associated with the brown alga *Sargassum* sp. was performed by MHR Seleghim and S Romminger. The biomass of alga *Sargassum* sp. was sterilized with $HgCl_2$ solution (0.001 g/l) in ethanol 5 % for 60 s and washed three times with sterilized ASW. The algal biomass was used in different conditions for the isolation of associated microorganisms:

a) The algal biomass was cut into small slices (1 cm^2) using a scalpel, and the fragments were transferred to Petri dishes containing malt extract 2 % (MA2) and glucose-potato-yeast (GPY) media.

b) The alga *Sargassum* sp. was rubbed onto the agar surface.

All the Petri dishes were incubated in an oven at 25°C for 7 days. The microorganisms grown on the plates containing MA2 [malt extract 2 % (20.0 g), agar (15.0 g), artificial sea water (1 l)] and GPY [glucose (1.0 g), peptone soybean (0.5 g), yeast extract (0.1 g), agar (15.0 g)] were isolated, purified and identified. Artificial sea water was used to prepare the solid and liquid culture media [14].

Identification of marine fungi associated with marine algae *B. radicans* and *Sargassum* sp.

Marine fungi were identified by LD Sette. Morphological characterization was carried out by colony observation through a stereoscope (Leica MZ6, Wetzlar, Germany) and by squash mounts stained with Lactophenol and Cotton Blue using a light microscope (Leica DM LS, Wetzlar, Germany) [17]. Molecular identification was performed by ITS1-5,8S-ITS2 (strains Br-09, Br-023, SMA2-C, SMA2-8 and SGPY-41) 28S rDNA D1/D2 (strains Br-27 and Br-61) sequencing as described by Sette *et al.* [18]. Using conventional and molecular approaches fungi isolated from *B. radicans* were identified as *Botryosphaeria* sp. Br-09, *Eutypella* sp. Br-023, *Hydropisphaera* sp. Br-27 and *Xylaria* sp. Br-61. While fungi derived from *Sargassum* sp. were identified as *Pestalotiopsis* sp. SMA2-C, *Penicillium* sp. SMA2-8 and *Arthopyrenia* sp. SGPY-41.

Biocatalytic reduction of acetophenone derivatives by marine fungi from algae *B. radicans* and *Sargassum* sp.

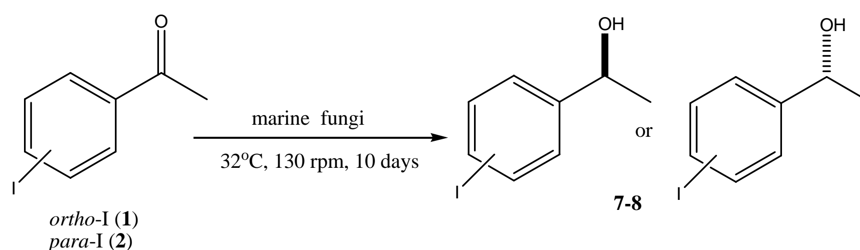
Marine fungi were cultivated in 250 ml Erlenmeyer flasks containing malt extract 2 % (100 ml) for 3 days, and then the ketones **1-6** (0.50 g) solubilized in dimethylsulfoxide (300 µl) were added. The Erlenmeyer flasks were stirred at 32°C for 10 days on an orbital shaker (150 rpm). Next, the mycelia were harvested by filtration and extracted with EtOAc (3 x 30 ml). The organic phases were filtered, dried over Na_2SO_4 and evaporated under vacuum. The biocatalytic reactions were analyzed by GC/FID, and the products were purified by CC over silica gel to yield alcohols **7-12** (Tables 1-3).

RESULTS AND DISCUSSION

The first investigation to reduction of the iodoacetophenones **1-2** was performed using two marine fungi isolated from alga *Bostrychia radicans*. The experiments were carried out with growing cells in order to determine the conversion and selectivity of products (Table 1). In these experiments, the reduction of the iodoacetophenones **1-2** with *Botryosphaeria* sp. Br-09 produced alcohols **7-8** with high optical purities (>99 % *ee*) and excellent conversions (>98 %).

Nevertheless, when the reactions were performed with *Xylaria* sp. Br-61, the production of iodophenylethanols **7-8** yielded minor improvements in conversion. The *ortho*-iodophenylethanol (**7**) was obtained with high enantiomeric excess (>99 % *ee*), and *para*-iodophenylethanol (**8**) showed modest optical purity (42 % *ee*, Entry 4). The selectivity of (*S*)-*para*-iodophenylethanol (**8**), obtained by *Botryosphaeria* sp. Br-09 and *Xylaria* sp.

Table 1. Bioconversion of the iodoacetophenones **1** and **2** by marine fungi isolated from the red alga *Bostrychia radicans*.



Entry	Ketones	c (%) ketones	c (%) [*] alcohols	*ee* (%) alcohols	ac
			Botryosphaeria sp. Br-09		
1	*o*-iodo **1**	0	100 [76]	99	*R*
2	*p*-iodo **2**	2	98 [74]	99	*S*
			Xylaria sp. Br-61		
3	*o*-iodo **1**	32	68	99	*R*
4	*p*-iodo **2**	70	30	42	*S*

c (%): conversion determined by GC/FID analyses; *ee* (%): enantiomeric excess; ac: absolute configuration; *Yield isolated.

Br-61, was in accordance with Prelog rule. While (*R*)-*ortho*-iodophenylethanol (**7**) was formed by *Botryosphaeria* sp. Br-09 and *Xylaria* sp. Br-61. *R*- and *S*-Alcohols were obtained in accordance to type of marine fungi and substrates used. In the literature it is common to obtain enzymatic reduction of ketones with Prelog selectivity.

To extend our investigation, other acetophenone derivatives **3-6** were used and different results were obtained according to the fungi employed. The *Botryosphaeria* sp. Br-09 presented excellent conversion for the reduction of *ortho*-ketones **4-6** and (*S*)-alcohols **10-12** with high selectivities (>99 % *ee*) were obtained (Table 2, Entries 2-4). However, the (*S*)-*ortho*-fluorophenylethanol (**9**) showed low selectivity (42 % *ee*).

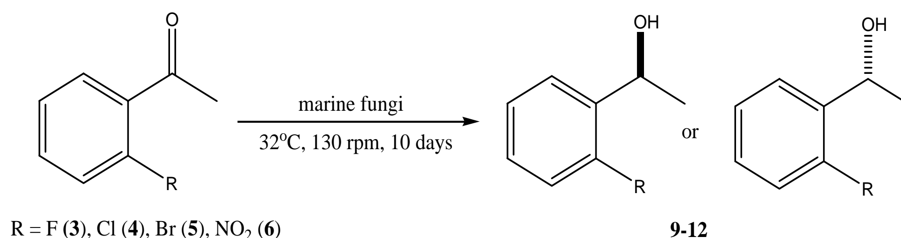
To the fungus *Eutypella* sp. Br-023, the better results were obtained for the *ortho*-nitrophenyl-ethanol (**12**), (98 % *ee*, c 92 %, Table 2, Entry 8) and *ortho*-bromophenyl-ethanol (**11**) (90 % *ee*, c 96 %, Table 2, Entry 7). The *ortho*-fluoroacetophenone (**3**) was reduced with minor conversion and a decrease of enantiomeric excess was observed (c 77 %, 90 % *ee*). For chloroacetophenone (**4**) the reduction with *Eutypella* sp. Br-023 was unsuccessful (Table 2, Entry 6).

The fungus *Hydropisphaera* sp. Br-27 catalyzed the bioreduction of ketones **5-6** with high enantiomeric excess (>99 % *ee*) and good conversion (Table 2, Entries 11-12). On the other hand, the bioreduction of ketone **4** showed unsatisfactory performance until 10 days of incubation with *Hydropisphaera* sp. Br-27. While the reduction of *ortho*-fluoro-ketone **3** was achieved with excellent conversion and good enantiomeric excess (Table 2, Entry 9).

When we investigated the bioreduction of *ortho*-ketones **4-5** with *Xylaria* sp. Br-61, the fungus promoted poor results in comparison with other fungi strains isolated from the alga *B. radicans* (Table 2, Entries 14-15). The ketones **4-5** were not reduced to alcohols **10-11** using fungus *Xyalria* sp. Br-61. However, the fungus *Xylaria* sp. Br-61 afforded the best results for the bioreduction of *ortho*-fluoroketone **3**, producing the alcohol **9** with high selectivity (>99 % *ee*) and good conversion (c 83 %), (Table 2, Entry 13).

The second investigation was conducted with three marine fungi isolated from brown marine alga *Sargassum* sp. The reactions using *Pestalotiopsis* sp. SMA2-C and *Arthopyrenia* sp. SGPY-41 presented similar results for the

Table 2. Bioconversion of the *ortho*-acetophenones **7-10** by marine fungi isolated from the red alga *Bostrychia radicans*.



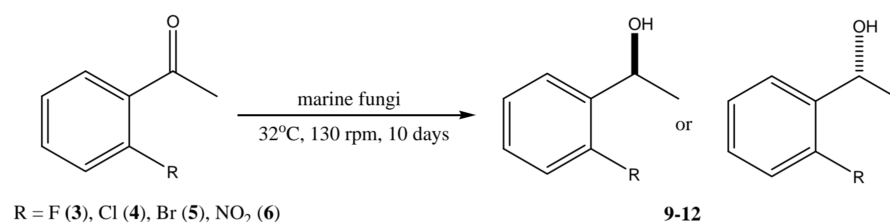
Entry	Ketones	c (%) ketones	c (%) [*] alcohols	*ee* (%) alcohols	ac
			Botryosphaeria sp. Br-09		
1	*o*-fluoro **3**	1	99 [81]	42	*S*
2	*o*-chloro **4**	0	100 [85]	99	*S*
3	*o*-bromo **5**	0	100 [78]	99	*S*
4	*o*-nitro **6**	10	90 [70]	99	*S*
			Eutypella sp. Br-023		
5	*o*-fluoro **3**	23	77	90	*S*
6	*o*-chloro **4**	92	8	99	*S*
7	*o*-bromo **5**	4	96	90	*R*
8	*o*-nitro **6**	8	92	98	*S*
			Hydropisphaera sp. Br-27		
9	*o*-fluoro **3**	1	99	80	*S*
10	*o*-chloro **4**	96	4	99	*S*
11	*o*-bromo **5**	24	76	99	*R*
12	*o*-nitro **6**	30	70	99	*S*
			Xylaria sp. Br-61		
13	*o*-fluoro **3**	17	83	99	*S*
14	*o*-chloro **4**	100	0	-	-
15	*o*-bromo **5**	100	0	-	-
16	*o*-nitro **6**	70	30	99	*S*

c (%): conversion determined by GC/FID analyses; *ee* (%): enantiomeric excess; ac: absolute configuration; *Yield isolated.

reduction of *ortho*-ketones **3-6** and alcohols **10-12** were obtained with good selectivities (Entries 2-4 and 10-12, Table 3) and minor selectivity for fluoro-alcohol **9** (Table 3, Entries 1 and 9). Acetophenones **3-4** were biotransformed with high conversion and ketones **5-6** with poor conversion by whole cells of *Pestalotiopsis* sp. SMA2-C and *Arthopyrenia* sp. SGPY-41. These studies clearly show that the long period of reaction (10 days) used was important. In some cases, according to the type of ketone, the reduction occurred and did not promote the degradation of alcohol produced (Table 3, Entries 1-2 and 9-10). On the other hand, the reduction of ketones **5-6** was not satisfactorily obtained with longer reaction time (Table 3, Entries 3-4 and 11-12).

The fungus *Penicillium* sp. SMA2-8 from *Sargassum* sp. catalyzed efficiently the reduction of *ortho*-acetophenones **3-5** in providing alcohols **9-11** with high enantiomeric excesses and good concentrations. In these reactions, only the *ortho*-ketone **6** was reduced with low

Table 3. Bioconversion of the *ortho*-acetophenones **7-10** by marine fungi isolated from the brown alga *Sargassum* sp.



Entry	Ketones	c (%) ketones	c (%) alcohols	*ee* (%) alcohols	Ac
		Pestalotiopsis sp. SMA2-C			
1	*o*-fluoro **3**	2	98	80	*R*
2	*o*-chloro **4**	22	78	99	*S*
3	*o*-bromo **5**	93	7	99	*S*
4	*o*-nitro **6**	95	5	99	*S*
		Penicillium sp. SMA2-8			
5	*o*-fluoro **3**	15	85	94	*R*
6	*o*-chloro **4**	2	98	99	*S*
7	*o*-bromo **5**	54	46	99	*S*
8	*o*-nitro **6**	85	15	98	*S*
		Arthopyrenia sp. SGPY-41			
9	*o*-fluoro **3**	2	98	80	*S*
10	*o*-chloro **4**	22	78	99	*S*
11	*o*-bromo **5**	93	7	99	*S*
12	*o*-nitro **6**	95	5	98	*S*

c (%): concentration determined by GC/FID analyses; *ee* (%): enantiomeric excess; ac: absolute configuration.

concentration by *Penicillium* sp. SMA2-8 (Table 3, Entries 5-8).

On the basis of these results, the fungus *Botryosphaeria* sp. Br-09 was used to obtain the yields and absolute configurations of the alcohols **7-12** (Tables 1-2). The absolute configurations of alcohols **7-12** were determined by comparing the specific rotation signs measured with those reported in the literature [16, 19]. The alcohols **7-12** were obtained in accordance with Prelog´s or anti-Prelog´s rule [20]. In conclusion, the use of fungi isolated from marine algae showed that they are efficient biocatalysts for the reduction of aromatic ketones. Interesting reactions of reduction of acetophenone derivatives were obtained by marine fungi isolated from marine algae, which are unusual microorganisms to biotransformations.

Endophytic fungi *Xylaria* sp. and *Penicillium citrinum* were used in biotransformation of ketones [21-22]. From 522 microorganisms, *Penicillium citrinum* catalyzes the reduction of 4-bromo-3-oxo 4-bromo-3-oxobutyrate (BAM) to optically active (*S*)-BHBM with 98.1% *ee* [21]. Secondary metabolites (cytosporins) were produced in the culture broth of the marine fungus *Eutypella scoparia*, which was isolated from the external part of the mollusk *Onchidium* sp. [23]. Filamentous fungus *Pestalotiopsis palmarum* from crude-polluted soil metabolized polycyclic aromatic hydrocarbons and extra-heavy crude oil [24].

CONCLUSIONS

In conclusion, the new biocatalysts for the reduction of acetophenones were obtained using

whole fungal cells of seven filamentous fungi isolated from marine algae. These studies describe the first isolation of fungi associated with the red marine algae *B. radicans* and brown alga *Sargassum* sp. for biocatalytic applications. Enantiopure alcohols **7-12** were formed with fungal biomass (up to >99 % *ee*) in good concentrations. The use of new marine microorganisms from different sources is interesting for biocatalytic reduction of aromatic ketones.

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