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Production of Secondary Metabolites as Antioxidants from Marine-Derived Fungi and Bacteria

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Abstract: Forty and fifty three different isolates of marine fungi and bacteria, respectively were isolated from Egyptian environment. All fungal isolates showed antioxidant activities, isolate No. 37F,*Circinella muscae* (Sorokine) Berlese & De Toni showed strongest antioxidant activity (95.46%).Only 8 isolates from marine bacteria isolates showed antioxidant activity, isolate No. 20B *Bacillus brevis* showed strongest antioxidant activity (30.25%). The obtained results showed that marine fungal extracts have higher antioxidant activities comparing with marine bacterial extracts, indicating that marine fungal extracts can be considered as promising tool in antioxidant drug industries.
Key words: Secondary metabolites, marine bacteria and fungi, antioxidants.

Introduction

Reactive oxygen species produced by different ways such as ultraviolet light, ionizing radiation and chemical reactions have different pathological effects causing DNA damage, carcinogenesis and cellular degeneration related to aging[1]. Reactive oxygen species (ROS) is responsible for many diseases such as cancer, Alzheimer's disease, Parkinson's disease, epilepsy, inflammation, retrolental fibroplasias, atherosclerosis, lung injury, ischemia-reperfusion injury and other disorders[2].Although almost all organisms possess antioxidant defense and repair systems that protect them against oxidative damage, these systems are insufficient to prevent the damage. Natural antioxidants play an important role in the prevention of these diseases[3]. Antioxidant components are micro-constituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the initiation or propagation steps of oxidizing chain reactions, so involved in scavenging free radicals[4]. They are generally used for the protection of foods from oxidative damage by inhibiting the generation of reactive oxygen species (ROS) or by scavenging the performed free radicals. Synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are the most widely used and they are suspected to have toxic effect. It is important to find an effective antioxidant from natural origin such as edible plants, spices and herbs because these natural substances have been eaten safely for long time and also microbes in general are considered a cheap source for antioxidants[5-7].The marine environment comprises nearly three quarters of the earth's surface, and can be considered a soup of essentially all imaginable types of microbes[8].Marine floras include microflora (bacteria, actinobacteria, cyanobacteria and fungi), microalgae, macroalgae (seaweeds), and flowering plants (mangroves and other halophytes). Marine microorganisms are a source of new genes, and exploitation of which is likely to lead to the discovery of new drugs and targets. Secondary metabolites produced by marine bacteria have yielded pharmaceutical products[9,10],and also Filamentous fungi are attractive organisms for production of useful biological secondary metabolites[11].In this

study, free radical scavenging activities among ninety three strains of marinebacteria and fungi which isolated from Egyptian environment was investigated.

Experimental

Collection and isolation of bacterial and fungal isolates

The present study was carried out on 93 isolates from different marine sources such as Seedy Basherbeach at Alexandria (Mediterranean Sea), the rhizosphere around the mangrove trees areas, and El-Ein El-Sokhna beach (Red Sea). Sediment samples were collected in sterile tubes and kept in refrigerator until processed in laboratory. The isolates were obtained using standard serial dilution technique from the original samples. Each individual bacterial isolate was cultivated on medium which composed of the following ingredients (g/l): glucose (20.0),$CaCO_3$ (1.0), NH_4NO_3 (0.8), K_2HPO_4 (0.6), KH_2PO_4 (0.05), $MgSO_4.7H_2O$ (0.05), $MnSO_4.4H_2O$ (0.1), yeast extract (0.1) and agar (16.0), and the plates were incubated at 37ºC for 24 h[12].While each individual fungal isolate was cultivated on Czapek-Dox medium and the plates were incubated at 28ºC for 7 days[13].The ingredients were dissolved in 750 ml seawater. The final volume was completed up to one liter with distilled water. Media were sterilized by autoclaving at 121°C for 15 min.

Production of Secondary Metabolites

Each individual bacterial isolate was cultivated on a production medium (g/l): peptone 4.0, yeast extract 2.0 and sucrose 20.0 and inoculated with 2 ml of 24 h old cultures. The cultures were incubated at 37^{o}C on rotary shaker (150 rpm) for 3 days while the fungal isolates were cultivated on synthetic medium (malt – yeast – glucose – peptone medium;MYGP)[14]. The experimental cultures were grown in 250 ml Erlenmeyer flasks, each containing 50 ml of the synthetic medium and inoculated with 2 ml of 7-10 days old cultures. The cultures were incubated at 28^{o}C on rotary shaker (120 rpm) for 7 days.

Microbial Extraction

Ninety three isolates were screened for production of bioactive secondary metabolites. The solvent extraction was the first step in the whole separation process. At the end of fermentation period, the content of each flask (medium and mycelium) was extracted with two different solvents (ethyl acetate or chloroform) as described by Serizawa[15]. The combined solvent, dried over anhydrous sodium sulfate, filtered, then distilled to give a semisolid extract (test material) which used for further bioassay test (antioxidant bioassay).

Antioxidant activity

The stable free radical of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used to assay free radical scavenging activity[16]. The DPPH radical scavenging activity was measured according to Todaka[17]. Eight mg of DPPH were dissolved separately in 100 ml of chloroform and ethyl acetate and also samples (extracts)were dissolved separately in chloroform and ethyl acetate. Chloroform DPPH or ethyl acetate DPPH served as control. The tested material dissolved separately in ethyl acetate and chloroform (0.2 ml) was mixed vigorously with 3 ml of DPPH solution and kept in the dark for 30 min. The medium itself was used as control and was treated in the same manner as the culture broth. Absorbance at 517 nm was measured using spectrophotometer (UV/vis-2401 pc visible, Shimadzu, Kyoto, Japan) and the radical scavenging activity was quantified as units/ml according to the following formula;

Scavenging ability (%) = $(A_{517\ of\ control} - A_{517of\ sample} / A_{517\ of\ control}) \times 100$.

Microbial identification

Identification of fungi

Identification of the highest producer fungal isolate was carried out using the morphological characteristics and microscopic features were examined by optical light microscope (10×90) Olympus CH40 according to the following references;Ainsworth[18] as adictionary of the fungi, Zycha[19], for Mucorales group.

Identification of bacteria

Cell morphology

Bacterial cells were stained with Gram's stain according to the method described by Shaffer and Goldin[20]. After staining, the morphology of bacterial cells; including shape and staining features; was examined by optical light microscope (10×90, Olympus CH40).

Biochemical tests

The pure isolated strain (highest producer) was identified according to the methods of Sneath[21] as described in Bergey's Manual of Systematic Bacteriology to the genus level.

Results and discussion

Microorganisms isolation

Ninety three isolates were recovered from different marine samples, including 40 fungal isolates and 53 bacterial isolates. The number of isolates, rate of isolation obtained from Seedy basher was higher than those obtained from El-Ein El-Sokhna and El mangrove. The number of fungal isolates recovered from Seedy basher, El-Ein El-Sokhna and El mangrove were 18, 12 and 10, respectively. Whereas, the number of bacterial isolates From Seedy basher, El-Ein El-Sokhna and El mangrove were 31, 13 and 9, respectively (Table 1).

Table 1. Number of bacterial and fungal isolates from different marine sources.

Samples source	Bacteria	Fungi
Seedy basher	31	18
El-mangrove	9	10
El-Ein El-Sokhna	13	12
Total	53	40

Radical scavenging activity for bacterial and fungal extract (antioxidant bioassay).

The antioxidant activity of each bacterial and fungal extracts act as free radical scavengers or hydrogen donors, the free radical scavenging activity assay was carried out**.** When the free radical have been scavenged, will convert its color to yellow because as odd electron of the radical becomes paired off in the presence of a hydrogen donor, the absorption intensity will be decreased and resulting discoloration with respect to the number of electrons captured**.** The reduction in the number of DPPH molecules can be correlated with the number of available hydroxyl group. Marine organisms are expected to have high levels of the scavenging of reactive oxygen species (ROS) through a combination of photosynthesis, symbiont oxygen production, and intense sunlight intensities leading to UV induced free radical production. So it could be expected that organisms which highly exposed to ROS should have effective antioxidant mechanisms. Many of them contain powerful or completely novel- antioxidant compounds. So that, marine organisms could be expected to be an interesting source of antioxidant compounds[22].The antioxidant activities of different bacterial extracts were recorded at different times. It was clear that the antioxidant activities were higher at 120 min than at fewer times (30, 60, and 90 min).Fifty three bacterial strains were screened for antioxidant activity by using ethyl acetate and chloroform, the antioxidant activities of ethyl acetate extracts were slightly greater than that of chloroform extracts. Only five isolates from Seedy basher samples have antioxidant activities in the case of using ethyl acetate as a solvent, isolates No. 4B, 14B, 15B, 19B and 20B showed antioxidant activitiesof3.5%, 9.89%, 4.25%, 2.41% and30.25%, respectively, while the other isolates showed no antioxidant activities. Also, all chloroform extracts failed to show any antioxidant activity. Each of ethyl acetate and chloroform extracts of El-mangrove failed to show any antioxidant activity. The different extracts of El-Ein El-Sokhna showed varieties in their antioxidant activities, ethyl acetate extract of isolates No. 42B, 43B and53B showed antioxidant activities of 26.2%, 22.9% and 22.9%, respectively and only one isolate from chloroform extract (isolate No. 53B) showed antioxidant activity of 17.5% (Table 2).

Table 2.Antioxidant activity of active bacterial extracts.

Bacterial code number	Scavenging ability (%)							
	Ethyl acetate extract				Chloroform extract			
	Time (min)				Time (min)			
	30	60	90	120	30	60	90	120
4B	0.8210	1.9400	3.0400	3.5200	0.0	0.0	0.0	0.0
14B	2.3700	5.4700	9.2000	9.8900	0.0	0.0	0.0	0.0
15B	0.0	1.0500	3.30000	4.2500	0.0	0.0	0.0	0.0
19B	0.0	0.6130	1.5700	2.4100	0.0	0.0	0.0	0.0
20 B	17.9500	23.1600	29.4500	30.2500	0.0	0.0	0.0	0.0
42B	0.0	0.0	0.0	0.0	24.311	24.779	25.853	26.605
43B	0.0	0.0	0.0	0.0	8.981	15.128	18.782	22.935
53B	7.431	15.761	15.788	17.522	9.165	15.782	19.532	22.935

Marine bacteria produce active secondary metabolites to protect themselves from activated oxygen produced by sunlight; therefore, their potent antioxidant activities were expected. In present study, bacterial isolates failed to show expected strong antioxidant activities and this may be due to the inadequate amount of extracted bioactive substances isolated from examined marine bacteria. Obtained results were in agreement with Proksch[23].While, Selim[10] reported high antioxidant activity from exopolysaccharides isolated from marine bacteria. Forty fungal strains were screened for antioxidant activity by using ethyl acetate and chloroform. All eighteen isolates from Seedy basher showed antioxidant activities and the antioxidant activities of ethyl acetate extracts were slightly greater than that of chloroform extracts. Four samples from Seedy basher showed high antioxidant activities (more than 85% of the DPPH free radical scavenging activity after 120 min), the four samples including; isolates No. 1F and 14F (chloroform extract) have antioxidant activities of 88.3% and 85.19%, respectively and isolates No. 2F and 15F (ethyl acetate extract)have antioxidant activities of 85.45% and 91.20%, respectively (Table 3).

Table 3.Antioxidant activity of fungal strain isolated from Seedy basher.

Fungal code number	Scavenging ability (%)							
	Ethyl acetate extract				Chloroform extract			
	Time (min)				Time (min)			
	30	60	90	120	30	60	90	120
1F	41.82	52.82	67.8	77.16	59.23	77.15	80.09	88.31
2F	40.96	66.14	80.45	85.45	36.71	42.10	45.66	52.67
3F	33.15	47.40	60.11	77.00	20.99	42.26	44.44	69.64
4F	5.49	59.77	60.07	75.01	49.01	60.12	70.77	73.78
5F	11.75	39.31	45.67	55.11	55.00	55.62	60.00	72.32
6F	10.43	38.16	44.25	52.20	24.88	28.76	34.45	48.86
7F	17.41	33.42	45.45	59.26	27.15	37.84	40.44	48.17
8F	12.85	17.01	22.65	39.61	15.75	17.60	21.34	39.91
9F	32.34	43.66	56.33	68.92	24.81	38.03	55.09	63.57
10F	20.76	38.50	56.98	72.20	24.68	30.00	60.09	73.03
11F	16.71	22.50	40.98	60.53	17.15	25.83	30.76	59.28
12F	28.11	43.33	56.45	80.03	24.06	59.31	60.46	83.11
13F	10.00	24.02	45.05	63.01	20.43	31.16	45.22	83.11
14F	33.55	43.68	67.90	84.11	36.04	54.71	77.98	85.19
15F	51.68	55.26	78.22	91.20	42.15	63.17	80.78	80.51
16F	34.17	50.73	60.08	65.07	43.17	45.78	55.90	73.07
17F	12.50	36.16	45.09	60.06	25.00	43.12	51.11	53.10
18F	26.87	46.16	58.09	68.14	26.80	47.10	50.19	56.12

Ten isolates from El-mangrove showed less antioxidant activities in comparison with Seedy basher and El-Ein El-Sokhna. Chloroform extracts showed antioxidant activities slightly greater than that of ethyl acetate extracts. All fungal extracts showed antioxidant activities less than 50% except isolate No.22F showed antioxidant activity of 75.78% (Table 4).

Table 4.Antioxidant activity of fungal strains isolated from El-mangrove.

Fungal Code number	Scavenging ability (%)							
	Ethyl acetate extract				Chloroform extract			
	Time (min)				Time (min)			
	30	60	90	120	30	60	90	120
19F	11.02	22.05	37.09	47.06	19.03	34.15	40.09	48.31
20F	10.96	16.04	20.15	25.15	06.71	12.10	15.66	22.17
21F	03.15	07.44	10.01	17.08	10.11	12.16	14.04	19.14
22F	5.49	59.77	60.07	73.01	49.01	60.12	70.77	75.78
23F	10.05	19.01	25.17	35.76	15.03	15.22	30.01	32.12
24F	10.43	18.06	24.45	42.10	14.88	18.16	24.05	44.06
25F	11.11	13.22	15.15	29.16	07.15	12.84	30.04	38.07
26F	10.15	16.01	20.34	29.01	13.15	15.60	20.14	29.81
27F	12.04	23.06	26.30	33.12	14.01	18.13	25.59	38.45
28F	10.06	18.40	26.18	32.20	14.38	20.06	30.19	33.87

El-Ein El-Sokhna samples were represented by twelve fungal isolates. All fungal isolates showed antioxidant activities and the antioxidant activities of ethyl acetate extracts were slightly greater than that of chloroform extracts. Eight samples from El-Ein El-Sokhna showed high antioxidant activities (more than 85% of the DPPH free radical scavenging activity after 120 min). Two chloroform extracts of isolates No. 34F and 35F showed antioxidant activities of 83.45% and 83.90%, respectively and six ethyl acetate extracts of isolates No. 31F, 33F, 36F, 37F, 38F and 39F showed high antioxidant activities of 93.32%, 85.11%, 89.99%, 95.46%, 84.91% and 90.08%, respectively (Table 5).

Table 5.Antioxidant activity of fungal strains isolated from El-Ein El-Sokhna.

Fungal code number	Scavenging ability (%)							
	Ethyl acetate extract				Chloroform extract			
	Time (min)				Time (min)			
	30	60	90	120	30	60	90	120
29F	26.77	33.53	49.51	66.77	13.20	20.16	36.58	65.34
30F	16.15	22.11	34.11	36.15	15.12	15.79	21.39	31.32
31F	22.54	42.60	56.36	93.32	28.43	57.00	73.05	83.07
32F	20.11	27.14	30.17	72.01	18.97	28.92	69.91	79.99
33F	20.21	36.66	66.65	85.11	28.33	48.76	68.11	83.98
34F	31.81	54.66	69.28	82.81	53.83	55.08	73.01	83.45
35F	49.13	57.01	63.16	79.13	30.65	41.60	75.61	83.90
36F	38.15	63.33	76.02	89.99	57.14	68.04	71.11	88.34
37F	45.46	51.66	72.85	95.46	44.20	60.02	77.02	86.45
38F	56.01	60.12	74.50	84.91	57.09	67.91	72.16	83.65
39F	40.08	60.05	84.44	90.08	36.56	53.03	62.18	89.65
40F	3.37	4.69	10.96	11.37	07.96	28.13	32.85	33.65

Marine fungi are considered as an important source of biologically active secondary metabolites with a broad range of biological activities due to the marine environment have special ecological niche in terms of its specific composition in both organic and inorganic substances, as well as temperature ranges, and pressure

conditions[24].Also, we can attribute the antioxidant activity of different fungal extracts, this may be due to the marine chemodiversty, which is also heightened by their composition of sea water itself[25].

Microbial identification

Fungal identification

According to the obtained results, the fungal isolate No 37Fshowed highest antioxidant activity. The fungal colony characteristically by forming sporangia globose, columellate, borne terminally on strongly hooked, circinate, often umbellate, branches that are borne along the length of sporangiophores; sporangiophores nonapophysate; heterothallic; smooth-walled zygosporangia borne between opposed or apposed suspensors. Sporangiophores up to 15 mm in height, 18 µm in diam., sympodially branched; fertile branches circinate, bearing a single sporangium, two sporangia, or a single sporangium and a sterile spine; many shorter sporangiophores with alternate arrangement of sporangia and without spines; sporangia globose to slightly dorsiventrally flattened, variable in shape; sporangiospores globose, sometimes short oval, variable in diam., smooth, hyaline, black in mass. These characteristics indicate that strain No. 37F is *Circinella muscae* (Sorokine) Berlese & De Toni based on the description byZycha[19].According to the obtained data, marine isolate No.37F, *Circinella muscae* which have strong antioxidative activity and proved to be pioneer isolate for antioxidant bioassay but our result was in agreement with El-Sayed[6],they reported that *Circinella muscae* No.171 which isolated from soil have strong antioxidant activities in the case of using ethyl acetate and also chloroform.

Bacterial identification

Bacterial isolate No. 20B which showed the highest antioxidant activity was subjected for identification. The pure isolated strain was identified according to the methods of Sneath[21] as described in Bergey's Manual of Systematic Bacteriology to the genus level. Morphological and biochemical identification results were summarized in Table (6).

Table 6. Identification of bacterial isolate No.20B.

Characteristics	***B. brevis***
Physiological	
Gram stain reaction	+
Capsule	-
Motility	+
Catalase	+
Anaerobic growth	
Voges-Proskauer test	-
Acid from	
D-Glucose	-
D-Mannitol	+
L-Arabinose	-
Xylose	-
Sucrose	-
Trehalose	-
Sorbitol	-
Lactose	-
Mannitol	-
D- Xylose	-
Utilization of citrate and propionate	-
Reduction of nitrate to nitrite	-
Production of indole	-
Growth at 7.5% NaCl	+
Starch hydrolysis	+

B. brevis is a novel applicant biocontrol agent and has antifungal activity. *B. brevis* has been shown to control *Botrytis cinereainin vitro* and *in vivo* on tomato and lettuce[26].*B. brevis* produced different antibiotics responsible for disease suppression[27,28]. It produces a single cyclic antibiotic, gramicidin S, which has fungicidal effect[29,30] and this may be due to an extracellular adversarial substance that impels the swelling of the pathogen's hyphaltips, and the mold cells were bulbous and swollen with contracted and granulated cytoplasm[31].*B. brevis* No.G1 produces a highly stable chitinase that has been applied to vegetables to combat mold diseases with remarkable efficacy[32].*B. brevis* strain FJAT-0809-GLX, an isolate that inhibits the growth of a number of pathogenic bacteria and fungi, such as *Fusarium oxysporum*, *Escherichia coli* and *Ralstoniasolanacearum*[33,34], suggesting that it has great potential as an agent for the biological control of many diseases. Cell-free culture broth of *B. brevis* strain, and twenty-four compounds were found in the crude extract by GC/MSD[34], suggesting that it could be a rich source of biologically active compounds. According to our obtained results, the marine bacteria *Bacillus brevis* isolate No.20B which showed antioxidative activity and proved to be first recorded for antioxidant bioassay.

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