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Screening of bacterial antioxidant exopolysaccharides isolated from Egyptian habitats

Selim M. S.*, Mohamed S. S., Shimaa R. H., El Awady M. E. and El Sayed O. H.

Microbial Biotechnology Department, National Research Centre, El-Bohos Street, Cairo, Egypt

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ABSTRACT

The present study was carried out on 83 isolates from marine (Mediterranean and Red Seas) and soil (seawedge from El-Fayoum and El-kanater) habitats. After three days of incubation the crude exopolysaccharides were separated and examined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical. Seven isolates from soil and 22 isolates from marine habitats showed antioxidant activity. The highest nine isolate showed antioxidant activities were identified as follow M7 (Bacillus circulans) (98.10%) M8 (Bacillus licheniformis) (97.34%), S1 (Bacillus alvei) (95.83%), M3 (Bacillus insolitus) (85.01%), S22 (Bacillus polymyxa) (84.31%), M9 (Bacillus marinus) (83.26 %), M6 (Bacillus anthracis) (81.50), M5 (Staphylococcus sp.) (80.42%) and M4 (Bacillus brevis) (76.90%) isolates.

Keywords: bacteria, exopolysaccharides, identification of bacteria, antioxidant

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INTRODUCTION

One of the most apparent influences of modern times have had on people is their desire to go back to the basics, to the natural and to the organic. The use of plants, microorganism, algae and animals for treating human disease arises from their innate biosynthetic capacity to produce a broad spectrum of powerful natural bioactive molecules. These bioactive phyto-molecules can be used as human drugs or can be chemically modified to modulate therapeutic activities [1]. The antioxidant potency enables the scavenging of reactive oxygen species (ROS). Because of the possible toxicities of the synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hyroxytoluene (BHT), there is an increasing attention towards natural antioxidants [2].

Organisms are well known to have an abundance of antioxidant compounds that have been shown to be effective at removing ROS such as superoxide, anions, hydrogen peroxide, and hydroxyl radicals from the body. ROS are strongly associated with cardiovascular disease, cancer and various neurodegenerative disorders. The main sources of free radicals are oxidation reactions of polyenoic fatty acids [3]. Antioxidants have become one among the most important topics in human nutrition because of high concentrations of free lipid radicals, both in food and *in vivo* after food ingestion [4].

Now in the 21st century, many investigators are exploring or re-evaluating the wealth of compounds with potential therapeutic benefits in organisms. Interest in polymeric materials increases year by year.

Polysaccharides are among the most plentiful biomaterials [5], and have attracted much attention in the field of biochemistry and pharmacology [6]. Polysaccharides play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms [7, 8].

The aim of present work is to isolate and identify bacterial strains that produce exopolysaccharides as antioxidant agents.

EXPERIMENTAL SECTION

1. Collection of samples

Five samples were collected from soils and marine. Two soil samples were taken from the seawedge at El-Fayoum and El-kanater, Egypt. Three marine samples were taken from Sidi Bisher beach at Alexandria (Mediterranean Sea), Marsa-Alam (the rhizosphere around the mangrove tree) areas, and El-Ein El-Sokhna beach (Red Sea). Sediment samples were collected in sterile tubes and kept in refrigerator until processed in laboratory.

2. Isolation of bacteria

A known weight (10 g) of each soil sample was suspended into 90 ml of sterilized NaCl saline aqueous solution (0.85 % w/v) using the serial dilution method [9]. Obtained suspensions were plated for isolation of bacteria and the plates were incubated at 37ºC for 24 h. Two different media were used for the isolation **medium 1** was composed of the following ingredients (g/l): KH_2PO_4 (2), $MgSO_4.7H_20$ (0.5), $MnSO_4.7H_2O$ (0.05), $FeSO_4.7H_20$ (0.01), $CaCl_2.2H_2O$ (0.01), trypticase (0.7), sucrose (20), agar (15.0), and thiamine-hydrochloride (0.002) sterilized separately by filtration [10] and **medium 2** 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), and yeast extract (0.1) [11].

The ingredients were dissolved in750 ml distilled water (soil sediments) or 750 ml seawater (marine sediment) and the pH was adjusted to 7.0. The final volume was completed up to one liter with distilled water. Media were sterilized by autoclaving at 121^{o}C for 15 min. The appeared colonies per plate of each sample were subjected to purification.

3. Production of polysaccharide

Isolates were screened for production of polysaccharide in a liquid medium composed of the following ingredients (g/l): peptone (4.0), yeast extract (2.0), and sucrose (20.0) [12]. The ingredients were dissolved in 750 ml seawater. After adjusting the pH, the final volume was completed up to one liter with distilled water. The medium was distributed in conical flask 250 ml. The flasks were autoclaved and inoculated using actively growing culture and incubated at 37ºC for 3 days. The culture medium was centrifuged at 5000 rpm for 10 min (Sigma-Laborzentrifugen, 2K 15) to remove bacterial cells. Trichloroacetic acid (5%) was added and left overnight at 4ºC and centrifuged at 5000 rpm again. The pH of the clear solution was adjusted to 7.0 with NaOH solution and dialyzed three times. The supernatant was completed to four volumes with acetone and left overnight at 4ºC. The precipitated polysaccharides were separated by centrifugation at 5000 rpm, washed twice with acetone and dehydrated by ether [13].

4. Antioxidant activity

A freshly prepared DPPH(1, 1-diphenyl-2-picrylhydrazyl) solution (20 mg/l) was used for the assay. Polysaccharide samples were dissolved in acetone and the acetonic DPPH served as a control [14].

A mixture of 2 ml of DPPH solution and 20, 40, 60, 80, and 100 mg/ml of samples were used. The mixture was shaken vigorously and left to stand for 30min in the dark, and the absorbance was measured at 517nm against blank using UV-2401 PC visible spectrophotometer (Shimadzu, Kyoto, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph plotted of inhibition percentage against compound concentration. The experiment was carried out in triplicate and averaged.

The scavenging activity was calculated as follows:

Scavenging ability (%) = (A517 of control - A517 of sample / A 517 of control) ×100.

5. Identification of bacteria

a. Cell morphology

Bacterial cells were stained with Gram's stain according to the method described by Shaffer and Goldin [15]. After staining, the morphology of bacterial cells; including shape and staining features; was examined by optical light microscope (10×90, Olympus CH40).

b. Biochemical tests

The pure isolated strain was identified according to the methods of Sneath [16] as described in Bergey's Manual of Systematic Bacteriology to the genus level.

RESULTS AND DISCUSSION

1. Collection and isolation of bacteria

In the course of a screening program for new bioactive polysaccharides, the present study was carried out on 53 isolates of marine samples and 30 isolates of soil samples, which were isolated from different areas in Egypt. The samples were collected from five areas (El-Ein El-Sokhna, Sidi Bisher beach (Alexandria), Marsa-Alam, EL kanater, and EL Fayoum).

The present study revealed that the majority of isolates were found in the Mediterranean Sea samples (Sidi Bisher beach, Alexandria) while the minimum isolates were detected in Red Sea samples (Marsa-Alam) (Table 1).

Table 1. Number of isolated bacteria from different habitats in Egypt

Sample sources	Number of isolates	Percentage (%)
Marsa-Alam (M)	9	10.43
El- Fayoum (F)	18	21.68
El-Ein El-Sokhna (E)	13	15.66
El-Kanater (K)	12	14.57
Sidi Bisher, Alexandria (S)	31	37.35
Total	83	100

2. Antioxidant activity of isolated exopolysaccharides from bacteria

The crude polysaccharides were examined by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical. The DPPH radical is one of the most commonly used substrates for fast evaluation of antioxidant activity because of its stability (in radical form) and simplicity of the assay. The free radical scavenging activity (RSA) of the isolated active polysaccharides from 83 bacteria isolates was assessed by the decolouration of an acetonic solution of DPPH radical. Twenty nine of the assessed precipitated EPS cultivars were able to reduce the stable, purple-coloured radical; DPPH· into the yellow-coloured; DPPH·-H. The effect of antioxidants on DPPH radical scavenging could be related to their hydrogen donating ability [17].

Table 2. Radical scavenging activity (RSA) of exopolysaccharides produced by bacteria isolated from EL-kanater

	Radical scavenging activity (%)			
No. of isolates	**30 min**	**60 min**	**90 min**	**120 min**
K1	15.7800	17.9000	18.2000	18.6300
K2	-	-	-	-
K3	24.2200	27.5000	30.9100	32.1200
K4	42.5700	48.2100	50.8200	51.0400
K5	-	-	-	-
K6	-	-	-	-
K7	15.6000	17.001	17.500	17.6000
K8	-	-	-	-
K9	-	-	-	-
K10	27.5000-	28.7100	30.8900	31.5100
K11	-	-	-	-
K12	-	-	-	-

K= EL-kanater.*

Data in Tables (2-6) show the antioxidant activities of different bacterial exopolysaccharides at different times. It is clear that the antioxidant activity was higher at 120 min than at fewer times (30, 60, and 90 min). The highest antioxidant activities (98.1%) was recorded for exopolysaccharides from M7 isolate followed by these of M8 (97.34 %), S1 (95.83%), M3 (85.01 %), S22 (84.31%), M9 (83.26 %), M6 (81.50 %), M5 (80.42 %) and M4 (76.90) isolates.

It is obvious that the majority of the highest antioxidant isolates from marine habitats (Marsa Alam - Red Sea). Dunlap et al [18] mentioned that marine organisms are exposed to particularly 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 be have an effective antioxidant mechanisms. Many of them contain powerful plantlike or completely novel- antioxidant compounds. So that marine organisms could be expected to be an interesting source of antioxidant compounds.

These results are in a good agreement with those of Hu et al and Pilar et al [19,20] who reported that the polysaccharides play an important role as free radical scavengers and antioxidants for the prevention of oxidative damage in living organisms. Seng [21] reported that the polysaccharide extracts from *Ganoderma tsugae* possessed

good antioxidant properties except for their scavenging ability towards hydroxyl radicals and may be good candidates as a new dietary supplement and functional food, also it was reported that the selenium containing extracellular (EPS) and intracellular (IPS) polysaccharide showed excellent antioxidant activity that correlated well with increasing concentrations [22]. Hu [23] reported that two sulfated polysaccharide isolated from *Undaria pinnitafida* showed antioxidant acticity. The antioxidant activities of the polysaccharide were evaluated by *in vitro* antioxidant assays quantitating superoxide anion, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl radical-scavenging activity and metal chelating ability.

Table 3. Radical scavenging activity (RSA) of exopolysaccharides produced by bacteria isolated from EL-Fayoum

	Radical scavenging activity (%)			
No. of isolates	**30 min**	**60 min**	**90 min**	**120 min**
F1	-	-	-	-
F2	-	-	-	-
F3	-	-	-	-
F4	-	-	-	-
F5	-	-	-	-
F6	-	-	-	-
F7	-	-	-	-
F8	-	-	-	-
F9	-	-	-	-
F10	-	-	-	-
F11	-	-	-	-
F12	-	-	-	-
F13	39.92	42.0100	48.2100	49.31000
F14	-	-	-	-
F15	-	-	-	-
F16	-	-	-	-
F17	24.5000	27.4200	28.9100	29.003
F18	-	-	-	-

F= EL-Fayoum.

Table 4. Radical scavenging activity (RSA) of exopolysaccharides produced by bacteria isolated from Sidi Bisher beach (Alexandria)

	Radical scavenging activity (%)			
No. of isolates	**30 min**	**60 min**	**90 min**	**120 min**
S1	68.5	84.41	90.5	95.83
S2	30.11	38.21	42.1	45.18
S3	42.51	48.93	50.9	54.44
S4	-	-	-	-
S5	-	-	-	-
6S	-	-	-	-
S7	10.51	20.23	25.2	26.43
S8	-	-	-	-
S9	-	-	-	-
S10	-	-	-	-
S11	-	-	-	-
S12	-	-	-	-
S13	-	-	-	-
S14	-	-	-	-
S15	-	-	-	-
S16	-	-	-	-
S17	-	-	-	-
S18	-	-	-	-
S19	-	-	-	-
S20	-	-	-	-
S21	-	-	-	-
S22	50.84	64.82	78.2	84.31
S23	8.15	11.94	15.21	21.44
S24	-	-	-	-
S25	-	-	-	-
S26	-	-	-	-
S27	-	-	-	-
S28	-	-	-	-
S29	1.45	8.21	13.21	15.75
S30	-	-	-	-
S31	-	-	-	-

S= Sidi Bisher beach, Alexandria

Also, Chen et al [24] obtained a homogeneous exopolysaccharide possessed good *in vitro* antioxidant activity as evaluated by scavenging assays involving 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radicals.

Bacterium SRCnm isolated from Red Sea exhibited a higher scavenging effect on DPPH radical followed by *Bacillus sp. JS* [25].

Table 5. Radical scavenging activity (RSA) of of exopolysaccharides produced by bacterial isolates from Marsa-Alam (mangrove tree)

	Radical scavenging activity (%)			
No. of isolates	**30 min**	**60 min**	**90 min**	**120 min**
M 1	15.9700	18.9900	20.1500	20.8900
M2	-	-	-	-
M3	77.6300	82.0000	84.2000	85.0100
M4	71.0410	75.1300	76.1100	76.9000
M5	67.5600	77.8200	78.1000	80.4200
M6	69.5600	72.1000	74.8000	81.5000
M7	92.3400	96.7600	97.2000	98.1000
M8	90.5000	92.0700	95.9000	97.3400
M9	78.4200	80.9600	81.1000	83.2600

M*= *Marsa-Alam (mangrove tree).

Table 6. Radical scavenging activity (RSA) of exopolysaccharides produced by bacterial isolates from El-Ein El-Sokhna

		Radical scavenging activity (%)			
	No. of isolates	**30 min**	**60 min**	**90 min**	**120 min**
	E1	58.921	60.11	65.542	67.155
	E2	-	-	-	66.977
	E3	49.511	58.221	63.210	68.899
	E4	57.921	60.121	66.211	68.899
	E5	30.821	39.910	41.421	44.036
	E6	-	-	-	-
	E7	58.404	60.321	67.210	69.633
	E8	48.138	52.243	57.010	60.100
	E9	46.129	50.351	55.501	58.440
	E10	-	-	-	-
	E11	-	-	-	-
	E12	-	-	-	-
E	**E13**	-	-	-	-

El-Ein El-Sokhna

The nine isolates have the higher antioxidant activities are shown in Table (7).

Table 7. The most active isolates as radical scavenging free radical of different bacterial exopolysaccharides

	Radical scavenging activity (%)			
No. of isolates	**30 min**	**60 min**	**90 min**	**120 min**
M7	92.3400	96.7600	97.2000	98.1000
M8	90.5000	92.0700	95.9000	97.3400
S1	68.5000	84.4100	90.5000	95.8300
M3	77.6300	82.0000	84.2000	85.0100
S22	50.8400	64.82	78.2000	84.3100
M9	78.4200	80.9600	81.1000	83.2600
M6	69.5600	72.1000	74.8000	81.5000
M5	67.5600	77.8200	78.1000	80.4200
M4	71.0410	75.1300	76.1100	76.9000

3. Identification of the selected bacteria isolates.

Data in Table (8) show the morphological and the physiological parameters and identified bacteria isolates of the nine bacterial isolates. The data represent the nine identified bacterial isolates according to the obtained parameters which compared with those mentioned by Sneath [16].

It has been shown that the seven marine isolates were identified as *Bacillus licheniformis*, *Bacillus insolitus*, *Bacillus polymyxa*, *Bacillus marinus*, *Bacillus anthracis*, *Staphylococcus sp*, *Bacillus brevis* and the soil isolates identified as *Bacillus alvei* and *Bacillus polymyxa*. It was demonstrated that *Bacillus sp.* showed a wide distribution among marine habitats which could be explained on the basis of that many Gram-positive bacteria are known to generate spores under adverse conditions, such as those encountered in marine ecosystems [25].

The data as in accordance with Yaowei et al and Ying et al who produced antioxidant exopolysaccharidess (EPSs) by *Bacillus licheniformis* UD061 in solid state fermentation and *Bacillus* sp. respectively [27,28] .

It was stated that the rhizospheric bacteria *Bacillus polymyxa* produced polysaccharides showed ability to activate multiple plant defense mechanisms against wheat powdery mildew caused by *Blumeriagraminis* [29].

Table 8. Identification of the most active bacteria showed high antioxidant activity

Isolate	**isolate*M7**	**isolate*M8**	**isolate*S1**	**isolate*M3**	**isolate *S22**	**isolate*M9**	**isolate*M6**	**isolate*M5**	**isolate*M4**
Characteristics	***Bacillus circulans***	***Bacillus licheniformis***	***Bacillus alvei***	***Bacillus insolitus***	***Bacillus polymyxa***	***Bacillus marinus***	***Bacillus anthracis***	***Staphylococcus***	***Bacillus brevis***
Physiological									
Gram stain reaction	+	+	+	+	+	+	+	+	+
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	+	+	-	-	-	+	+	-	-
Reduction of nitrate to nitrite	+	+	+	+	+	+	+	+	-
Production of indole	-	-	+	-	-	-	-	-	-
7.5% NaCl	-	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	-	+	-	+	-	+

**M = Marsa-Alam (mangrove tree) *S= Sidi Bisher beach, Alexandria*

CONCLUSION

Marine bacteria are an interesting source of bioactive secondary metabolites with interesting activity as antioxidant activity. The present investigation pointed out the potential of the metabolites produced by bacteria in scavenging the free radicals DPPH. The nine marine bacterial isolates exhibited antioxidant activities which revealed a potential source of novel antioxidant compounds. Therefore, further investigation on its antioxidant properties in vivo, in addition to separation of individual compounds are recommended to identify the exact compound responsible for its potential free radical scavenging activity.

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