

Investigation of the antioxidant activity of some marine bacteria associated with some seaweeds from the Red Sea

N. Abdel-Wahab¹, Eman F. Ahmed², Hanan A.A. Taie³, Hossam M. Hassan⁴, M. S. Abdel Hameed¹, O. Hammouda^{1*}

¹Department of Botany, Faculty of Science, Beni-Suef University Egypt.

²Department of Chemistry of Natural and Microbial Products, National Research Centre, Cairo,

³Department of Plant Biochemistry-National Research Centre, 12311 Dokki, Cairo, Egypt.,

⁴Department of Pharmacognosy, College of Pharmacy, Beni-Suef University Egypt.

olahammouda@yahoo.com

Abstract: The aim of the present investigation is the isolation of endosymbiotic bacteria from the collected seaweeds from the Red Sea, identification them by 16s rDNA and evaluation the antioxidant activity of their extracellular metabolites by using DPPH assay. Two bacterial strains identified as *Bacterium SRCnm* & *Bacillus sp. JS* were isolated from *Acanthophora dendroides* & *Sargassum sabrebandum* respectively. The crude extract of the extracellular metabolites of two bacterial strains exhibited an interesting effect on scavenging DPPH free radical. The crude extract of *Bacterium SRCnm* exhibited a higher scavenging effect on DPPH radical (88.61%) than that by *Bacillus sp. JS* extract which exhibited (86.51%) at the same concentration 2 mg/ml, I_{c50} for the two bacterial extract was calculated as (1.129 & 1.360 mg/ml), respectively.

[N. Abdel-Wahab, Eman F. Ahmed, Hanan A.A. Taie, Hossam M. Hassan, M. S. Abdel Hameed, O. Hammouda.

Investigation of the antioxidant activity of some marine bacteria associated with some seaweeds from the Red Sea. *N Y Sci J* 2013;6(11):27-32]. (ISSN: 1554-0200). <http://www.sciencepub.net/newyork>. 4

Key words: Antioxidant activity, marine bacteria, seaweeds, Red Sea

1. Introduction

Free radicals (highly reactive species) are formed continuously in the cells as normal by-products of oxygen metabolism during mitochondrial oxidative phosphorylation, so the mitochondrion is the main source of free radicals (Przedborski and Jackson-Lewis, 1998). These free radicals as hydroxyl, superoxide and peroxyl radicals result in extensive oxidative damage of biomolecules, as DNA, which can lead to age related degenerative conditions, cancer and a wide range of other human diseases (Reaven and Witzum, 1996; Aruoma, 1999). Antioxidants are inhibiting and scavenging free radicals, thus providing protection to humans against various infections and degenerative diseases (Sharma, Gupta, 2008). Because of the possible toxicities of the synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), there is an increasing attention towards natural antioxidants (Naimiki, 1990).

Marine organisms are exposed to particularly high levels of 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 an effective antioxidant mechanisms. Many of them contain powerful plant-like or completely novel— antioxidant compounds

(Dunlap *et al.*, 2003). So that marine organisms could be expected to be an interesting source of antioxidant compounds.

Takao *et al.* (1994) isolated 112 marine bacterial strains from 12 marine animals had an antioxidant activity. The antioxidant potential of marine *Bacillus subtilis* has been demonstrated by Radha Krishna *et al.* (2011). Kalirajan Arunachalam *et al.* (2013) pointed out to the potential of the marine bacterium *Virgibacillus sp* associated with the sponge *Callyspongia diffusa* metabolites, in scavenging the free radicals *in vitro*.

The Red Sea represents one of the most diverse and exclusive rich places in the world with remarkable biodiversity and geographical variability. It is well documented that all marine organisms harbor symbiotic microorganisms such as bacteria, fungi and cyanobacteria. It was suggested that the seaweed-associated bacteria have a greater ability to produce bioactive agents more than that of its counterpart free living (Armstrong *et al.*, 2001). So that the bacteria isolated from invertebrates or algae become more interest than that from their counterparts in the surrounding water. Nevertheless, limited research has been conducted on the antioxidant activity of symbiotic bacteria associated with the Red Sea marine organisms.

The aim of our study is to isolate endosymbiotic bacteria from seaweeds collected from the red sea, identify them by 16s rDNA and

investigate their antioxidant activity by using DPPH assay.

2. Material and Methods

Collection and identification of the seaweeds

Seaweeds were collected from the Red Sea at depth 2 meters. The samples were placed in plastic bags at the underwater itself and transported to the laboratory immediately to the laboratory to be identified and deep frozen in order to isolate endosymbiotic marine bacteria from them.

Isolation of the endosymbiotic bacteria

The samples were washed with filtered and autoclaved seawater until they were visibly free from debris. Followed their surface were sterilized by a rapid wash of 70% ethanol and the inner parts were cut into small pieces 2 mm in thick. The resultant tissues were put in marine nutrient agar media and incubated for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates.

Identification of bacterial isolates by 16S rDNA sequencing

DNA was isolated from the bacterial isolates according to **Sambrook et al. (1989)**. The 16srDNA was amplified by polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16srDNA region. The forward primer was 5'AGAGTTTGATCMTGGCTCAG3' and the reverse primer was 5'TACGGYTACCTTGTTACGACTT3'. The PCR mixture consists of 30picomoles of each primer, 10ng of chromosomal DNA, 200 µM dNTPs and 2.5 Units of Taq polymerase in 50 µl of polymerase buffer. The PCR was carried out for 30 cycles in 94°C for 1 min, 55°C for 1 min and 72 °C for 2 minutes. After completion, a fraction of the PCR mixture was examined using agarose gel electrophoresis (**Ausuble et al., 1999**) and (Qiagen). DNA sequences were obtained using a 3130 X DNA Sequencer (Genetic Analyzer, Applied Biosystems, Hitachi, Japan), BigDye Terminator Cycle Sequencing (see details below). The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular phylogeny were performed using BioEdit software (**Hall, 1999**). The phylogenetic tree was displayed using the TREEVIEW program (**Page, 1996**).

DNA Sequencing

Automated DNA sequencing based on enzymatic chain terminator technique, developed by **Sanger et al., 1977**, was done using 3130 X DNA Sequencer(Genetic Analyzer, Applied Biosystems, Hitachi, Japan). The sequencing reaction was

performed with four different fluorescent labels identifying the ddNTPs, instead of the radioactive labels. These flurophores were excited with two argon lasers at 488 and 514 nm, respectively when the respective bands passed the lasers during the electrophoresis. The specific emissions were detected and the data were collected for analysis (**Prober et al., 1987 and Freeman et al., 1990**). The thermal cycling mixture was as follows: 8 µl of BigDye terminator mix, 6 µl of the sequencing primer (10 pmol) and 6 µl of the sample (PCR product or plasmid), then the reaction was run in the thermal cycler. The cyclic reaction composed of 1 min at 95°C, then 49 cycles of 30 sec at 95 °C, 10 sec at 52°C and 4min at 60°C. The products were purified using special column according to the instruction of the manufacturer. The elute were taken and add high dye formamide with (1:1)/volume ratio, run at 95 °C for 5 min for denaturation, shock on ice, then the sample become ready for sequencing in 3130 X DNA sequencer and analysis.

Fermentation

Bacterial isolates were inoculated in each flask with 100 ml marine nutrient broth containing 500 ml Erlenmeyer flask in a shaker (30 °C/250 rpm) for 72 hours and the cells were separated by centrifugation and the supernatant were extracted by using same volume of ethyl acetate three times, after separation the organic phase was concentrated in rotary evaporator at 40°C. The resulting crude extract was used for screening the antioxidant activity assay.

In vitro antioxidant assay

1, 1- diphenyl-2- picryl-hydrazil (DPPH) radical scavenging method:

The principle of this method depends on the advantage of (DPPH) to be strongly absorbed at the visible spectrum wavelength of 517nm, which characterized by deep violet color due to presence of free electrons, When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, it gives rise to the reduced form 1, 1-diphenyl-2-picryl hydrazine (reduced DPPH) with the loss of this violet color. The degree of discoloration is directly proportional to the free radical scavenging activity of the evaluated compound. The DPPH method is simple and rapid.

Procedure:

The free Radical scavenging activity using 1,1- diphenyl-2- picryl-hydrazil (DDPH) reagent was determined according to **Brand Williams et al. (1995)**. The bacterial crude extract dissolved in methanol to prepare different concentrations (0.5, 1.0, 1.5, 2.0 & 2.5 mg / ml). 1.5 ml of the extract sample was added to 1.5 ml of freshly prepared methanolic DPPH solution (20ug ml-1) with stirring.

The decolorizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control.

Antioxidant activity (DPPH scavenging effect %) = $\frac{(A_0 - A_1)}{A_0} \times 100$

Where, A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the bacterial crude extract.

3. Results and Discussion

Identification of the seaweeds

The seaweeds identified as *Acanthophora dendroides* (red algae) and *Sargassum sabrepandum* (brown algae) by Biological researchers, National Institute of Oceanography and Fisheries, Egypt.

Molecular identification of the bacterial isolates

Molecular characterization of the two bacterial strains was carried out by PCR amplification of 16S rDNA gene, a fraction of the PCR mixture was examined using agarose gel electrophoresis. According to sequencing similarities and multiple alignment, the bacterial strain isolated from *Acanthophora dendroides* were found to be in a close relation to *Bacterium SRCnm* (ac: GQ979939.) and these isolated from *Sargassum sabrepandum* was similar to *Bacillus sp. JS*, (ac: CP003492), with 100% identity. The phylogenetic tree of *Bacterium SRCnm* was displayed using the TREEVIEW program (Fig 1).

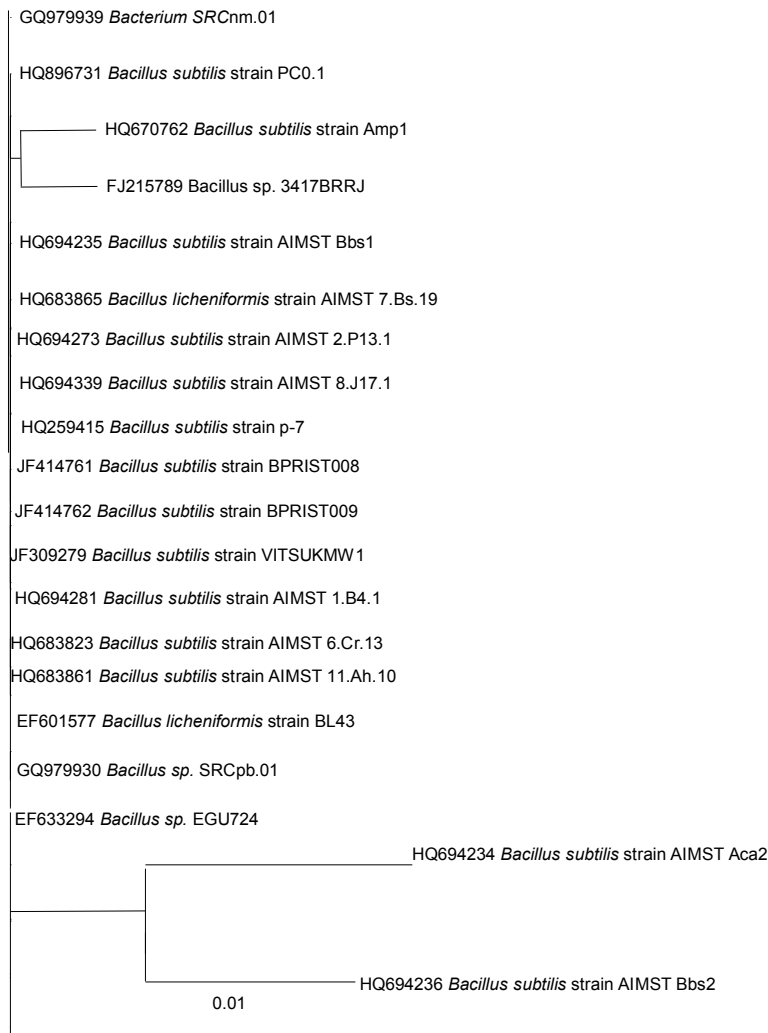


Fig. 1: The phylogenetic tree based on PCR product sequencing of DNA isolated from the endophytic *Bacterium SRCnm*.

In accordance with our results, endobiotic *Bacillus sp.* was previously isolated from *Sargassum sp.* (Claudia *et al.*, 2010 & Jamal and Mudarris 2010). Furthermore, Sutha *et al.* (2011) isolated Endosymbiotic *Bacillus subtilis* & *staphylococcus aureus* from the red seaweed *Acanthophora spicifera*.

It was demonstrated that *Bacillus sp.* showed a wide distribution among marine organisms 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, and this is thought to ensure their survival within the marine invertebrates (Ettoumi *et al.*, 2009).

It was found that the surface or cavum of marine organisms such as seaweeds and invertebrates are more nutritious than inanimate material and seawater, and large number of bacteria could live on it (sponga *et al.*, 1999). The relationship between the bacteria and the marine organisms could be explained on the basis that bacteria could acquire their necessary nutrition such as, vitamins, polysaccharides and fatty acids from their hosts (invertebrates or seaweeds). Meanwhile, they could secrete products as, amino acids, antibiotic and toxins for the development and metabolism of the host or to improve the chemical defense capability of their host (Armstrong *et al.*, 2001).

3.6. Antioxidant activity:

The scavenging of the DPPH radical design is a widely used method to evaluate the free radical

scavenging ability of various samples. The effect of antioxidants on DPPH radical scavenging could be related to their hydrogen donating ability (Spandana *et al.*, 2012). The results in Table (1) demonstrated a decrease in the concentration of DPPH radical due to the scavenging ability of the two bacterial crude extracts. The crude extract of *Bacterium SRCnm* & *Bacillus sp JS* showed concentration dependent DPPH scavenging activity, where the scavenging activity was increased linearly with the gradual increase in concentration of bacterial extract and nearly constant values were obtained at concentration of (2.0 and 2.5) mg/ml. The highest scavenging effect was produced by *Bacterium SRCnm* extract (88.61%) followed by *Bacillus sp. JS* extract (86.51%) at the same concentration 2 mg/ml and the Ic50 of the two isolates were (1.129 & 1.360 mg/ml), respectively.

Takao *et al.* (1994) isolated 112 bacterial strains from different marine organisms which exhibited antioxidant activity, they correlated this antioxidant activity to the presence of uric acid, indole, 3,4- dimethoxyphenol and 3-hydroxyindolin-2-one in their fermentation broth which isolated guiding by decoloration of DPPH sprayed on silica gel TLC. They explained the bacterial production of such antioxidant compounds, as a kind of adaptation to the aerobic conditions. The antioxidant activity of indole and its derivatives confirmed previously by Tabor & Coats (1991). Its activity could be explained on the basis of its radical scavenging ability.

Table (1): Antioxidant activity of isolated marine bacteria extracts using DPPH assay:

Concentrations (mg/ml)	Antioxidant Activity%	
	<i>Bacterium SRCnm</i>	<i>Bacillus sp. JS</i>
0.5	48.89	46.13
1.00	59.73	57.95
1.50	75.34	73.82
2.00	88.61	86.51
Ic50 mg/ml	1.129	1.360

Shoudong Guo *et al.* (2010), isolated two extracellular polysaccharides with antioxidant activity from marine bacteria *Edwardsiella tarda*. The scavenging ability of the polysaccharides on DPPH radical could be attributed to its hydrogen donating ability (Zhao *et al.*, 2006).

Furthermore, our result is in accordance with that obtained by Radha Krishna *et al.*, 2011,

who reported that crude extract of *Bacillus subtilis* (MTCC No.10619) showed a highly antioxidant activity. At the same manner Kalirajan & Ranjitsingh (2013) referred to the potential of crude metabolites of *Virgibacillus sp.* associated with the marine sponge *C. diffusa*. in scavenging the free radicals in vitro.

Conclusion:

Marine endosymbiotic 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 marine bacteria *Bacterium SRCnm* & *Bacillus sp. JS* associated with *Acanthophora dendroides* & *Sargassum sabrebandum*, respectively, in scavenging the free radicals DPPH. The two 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-

References

1. **Armstrong, Liming Yan, Kenneth G. Boyd, Phillip C. Wright & J. Grant Burgess (2001).** The symbiotic role of marine microbes on living surfaces. *Hydrobiologia* 461: 37–40
2. **Aruoma, I. O. (1999).** Antioxidant action of plant foods. Use of oxidative DNA damage, as a tool for studying antioxidant efficacy. *Free Radical Research* 30: 419- 427.
3. **Ausubel FM, Brent R, Kingston RE, More DD, Seidam JG, Smith JA and Struhl K.(eds). (1999).** Short protocols in Molecular Biology. John Willey and Sons, Inc. NY.biol. 29: 130D135.
4. **Brand-Williams W, Cuvelier ME, Berset C: Use of free radical method to evaluate antioxidant activity (1995).** *Lebensmittel-Wissenschaftund-Technol* 1995; 28:25-30.
5. **Claudia B. A. Menezes, Rafaella C. Bonugli-Santos, Paula B. Miqueletto, Michel R. Z. Passarini, Carlos H. D. Silva, Mariana R. Justo, Rebeca R. Leal, Fabiana Fantinatti-Garboggini, Vale´ria M. Oliveira, Roberto G.S. Berlinck, Lara D. Sette. (2010).** Microbial diversity associated with algae, ascidians and sponges from the north coast of Saõ Paulo state, Brazil. *Microbiological Research* 165; 466—482.
6. **Dunlap, W.; Llewellyn, L.; Doyle, J.; Yamamoto, Y. A (2003)** Microtiter Plate Assay for Screening Antioxidant Activity in Extracts of Marine Organisms. *Marine Biotechnology* 2003, 5, 294-301.
7. **Ettoumi B, Raddadi N, Borin S, Daffonchio D, Boudabous A, Cherif A (2009).** Diversity and phylogeny of culturable spore-forming Bacilli isolated from marine sediments. *J Basic Microbiol*, 49 Suppl 1:S13-23
8. *Food Sci. Nutr.*, 29: 273-300.
9. **Freeman M, Baehler C and Spotts S. (1990).** Automated Lazer fluorescence sequencing. *Biotechnol.*; 8: 147-148.
10. **Hall T.A. BioEdit: (1999).** a user- friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid. Symp. Ser.* 41: 95-98.
11. **Jamal M. T. and Mudarris M. S. A. (2010).** Separation of YbdN Bioactive Protein from *Bacillus subtilis* Isolated from the Red Sea algae *Sargassum sp.* with Bioactivity against Antibiotic Resistant Bacterial Pathogens. *JKAU: Mar. Sci.*, Vol. 21, No. 1, pp: 53-64.
12. **Joshi R, Sharma A, Jat BL (2009).** Analysis of antioxidant activity in extracts of *Calotropis procera* (Ait.) R. Br. *J. Appl. Bio. Sci.*, 17: 899-903.
13. **Kalirajan Arunachalam, R anjitsingh Amirtham and Jacob Appadorai (2013).** Antioxidant potential and biochemical evaluation of metabolites from the marine bacteria *Virgibacillus sp.* associated with the sponge *Callyspongia diffusa*, *Free Radicals and Antioxidants*, (3)1, 47-51.
14. **Naimiki M (1990).** Antioxidant / antimutagens in foods. *CRC Crit. Rev.*
15. **Page RDM. TREEVIEW: (1996).** An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences.*12: 357-358.
16. **Prober JM, Trainor GL, Dam RJ, Hobbs FW, Robertson CW, Zagursky RJ, Cocuzza AJ, Jensen MA, Baumeister K. (1987).**A system for rapid DNA sequencing with fluorescent chain-terminating dideoxynucleotides *Science.* 238:336-341.
17. **Przedborski S. and Jackson-Lewis V. (1998).** Experimental developments in movements disorders: update on proposed free radical mechanisms. *Current Opinion Neurol.* 11: 335-339.
18. **Radha Krishna E., Shamsher Kumar P& Veerendra Kumar B. (2011)** Study on Antioxidant activity and strain development of *Bacillus subtilis* (MTCC No.10619). *Journal of Agricultural Technology.* Vol. 7(6): 1693-1703
19. **Reaven, P. D. and Witzum, J. L. (1996).** Oxidised LDL in atherogenesis. Role of dietary modification. *Annual Review of Nutrition* 16: 51-71.
20. **Sambrook J, Fritsch E. Fand Maniatis T. (1989).** *Molecular cloning A. Laboratory*

- Manual. Cold Spring Harbor Laboratory, NY1989.
21. **Sanger F, Nicklen S, Coulson AR. (1977).** DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. 74:5463-5467.
 22. **Sharma S K, Gupta V. K. (2008).** Phcog mag. Research article, 13 (4):70-74.
 23. **Shoudong Guo, Mao Wenjun, Han Yin, et al. (2010).** Structural characteristics and antioxidant activities of the extracellular polysaccharides produced by marine bacterium *Edwardsiella tarda*. Bioresour Technol.;101: 4729-4732.
 24. **Spandana M., Ratna madhuri T., Aruna kumari D. and Susmitha K. (2012).** Investigation on antioxidant and antimicrobial activities of marine actinomycetes. IJPWR Vol 3 ISSUE 2 (Mar-June).
 25. **Sponga F., Cava lettil., Lazzarini A., Losi D., Marine F. (1999).** Biodiversity and potentials of marine derived microorganisms. J. Biotechnol., 70:65-69.
 26. **Tabor M. W., Sainsbury M., Shertzer H. G. and Coats E. (1991).** Antioxidation potential of indole compounds-structure activity studies. Adv. Exp. Med. Biol. 283, 833-836.
 27. **Takao T., Futoshi Kitatani, Naoharu Watanabe, Akihito Yagi and Kanzo Sakata (1994).** A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish, Biosci, Biotech, Biochem, (58)10, 1780-1783.
 28. **Zhao, T.T., Zhang, Q.B., Qi, H.M., Zhang, H., Niu, X.Z., Xu, Z.H., Li, Z.E., (2006).** Degradation of porphyran from *Porphyra haitanensis* and the antioxidant activities of the degraded porphyrans with different molecular weight. Int. J. Biol. Macromol. 38, 45-50.

11/2/2013