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

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**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 extractof *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, Ic50 for the two bacterial extract was calculated as (1.129 & 1.360 mg/ml), respectively.

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**Key wards**: 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-Lewwis, 1998)**. These free radicals as hydroxyl, superoxide and peroxyl radicals result in extensive oxidative damage of bio-molecules, 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 hyroxytoluene (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 be 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 bacteriun *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 ºC 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 with100 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 %) = ((A0- A1)/ A0) x 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 *Sarggassum 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 *Sarggassum 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).

|  |
| --- |
| 0.01  GQ979939 *Bacterium SRC*nm.01  HQ896731 *Bacillus subtilis* strain PC0.1  HQ670762 *Bacillus subtilis* strain Amp1  FJ215789 Bacillus sp. 3417BRRJ  HQ694235 *Bacillus subtilis* strain AIMST Bbs1  HQ683865 *Bacillus licheniformis* strain AIMST 7.Bs.19  HQ694273 *Bacillus subtilis* strain AIMST 2.P13.1  HQ694339 *Bacillus subtilis* strain AIMST 8.J17.1  HQ259415 *Bacillus subtilis* strain p-7  JF414761 *Bacillus subtilis* strain BPRIST008  JF414762 *Bacillus subtilis* strain BPRIST009  JF309279 *Bacillus subtilis* strain VITSUKMW1  HQ694281 *Bacillus subtilis* strain AIMST 1.B4.1  HQ683823 *Bacillus subtilis* strain AIMST 6.Cr.13  HQ683861 *Bacillus subtilis* strain AIMST 11.Ah.10  EF601577 *Bacillus licheniformis* strain BL43  GQ979930 *Bacillus sp.* SRCpb.01  EF633294 *Bacillus sp.* EGU724  HQ694234 *Bacillus subtilis* strain AIMST Aca2  HQ694236 *Bacillus subtilis* strain AIMST Bbs2 |

**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****.* (Cla´udia *et al.,* 2010 & Jamal and Mudarris 2010)**. Furthermore, **Sutha *et al.* (2011)** isolatedEndosymbiotic *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 bacteriaextracts using DPPH assay~~:~~**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  |  |  | | --- | --- | --- | | Concentrations (mg/ml) | Antioxidant Activity% | | | *Bacterium SRCnm* | *Bacillus sp. JS* | | 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 & Sarggassum 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~~.~~

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