

Separation of YbdN Bioactive Protein from *Bacillus subtilis* Isolated from the Red Sea algae *Sargassum* sp. with Bioactivity against Antibiotic Resistant Bacterial Pathogens

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Abstract. Hundreds of bacterial isolates from Red Sea Jeddah coast included seawater, sediments, algae (*Halimeda* sp., *Sargassum* sp), sponges (*Siphonochalina* sp., *Leucetta chgosensis*), sea cucumber, (*Holothuria atra*), star fish (*Acanthaster planci*), mangrove (*Aveenia marina*) roots, sea anemone, (*Heteractis magnifica*), and jelly fish. One bacterial culture of them isolated from algae *Sargassum* sp identified as *B. subtilis* produced YbdN protein which known as antimicrobial compound against Methicillin-Resistant *Staphylococcus aureus*, Vancomycin-Resistant Enterococci and *Listeria monocytogenes*. *B. subtilis* production of YbdN protein was best in Colombia broth with pieces of synthetic polyurethane sponge at 28°C incubation temperature for 7 days, and shaking at 210 rpm.

Keywords: Red Sea; Algae; Pathogens; Bacteria; Bioactive Protein; *Bacillus subtilis*

Introduction

Natural and effective antibacterial compounds against antibiotic-resistant as well as new bacterial pathogens are continually sought after for application in human and veterinary medicine (Finch *et al.*, 2002). These natural products may exert their effect on bacterial cell membranes and/or specific protein targets (Stone and Williams, 1992; Butler, 2004; & Koehn and Carter, 2005). They normally have complicated structure that makes their artificial synthesis difficult and often expensive. This gives added advantage to the naturally occurring compounds if they could easily be extracted and purified in good quantities and with reasonable cost.

Marine organisms have been used as source of many useful natural compounds. The genus *Bacillus* is widely distributed in marine environment and produces a variety of useful products (Cherif *et al.*, 2001; and Aria *et al.* 2003). Members of this genus as well as many others need to be studied under varying culturing scenarios to determine conditions conducive to production of useful natural compounds. This is particularly important in the light of the ever spreading of antibiotic resistance by many strains of bacterial pathogens. These include methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) which often cause post surgery infection (Richards *et al.*, 2005). Consequently, this study was undertaken to recovery and characterization of compounds with effectiveness against antibiotic-resistant bacteria from Red Sea.

Materials and Methods

Bacterial Culture

The samples included the sea algae (*Halimeda* sp., *Sargassum* sp.), sponges (*Siphonochalina* sp., *Leucetta chgosensis*), sea cucumber, (*Holothuria atra*), star fish (*Acanthaster planci*), sea anemone, (*Heteractis magnifica*), jelly fish, mangrove (*Avecenia marina*) roots, sediment and seawater. With the exception of sediment and seawater, the samples were placed into 10 ml volumes of sterile (121°C/15 min) seawater in plastic bottles, transported on ice to the laboratory. Sediment and seawater were transported in pre-sterilized plastic bottles. Analyses were carried out at King Abdulaziz University, (Jeddah) Saudi Arabia and Heriot Watt University laboratory (Edinburgh) Scotland. Sub-samples, 1.0 g or 1.0 ml as appropriate, were transferred to 10 ml volumes of sterile seawater, and shaken vigorously for ~ 5 min to dislodge micro-organisms. Then, 10-fold dilution were prepared, and 10 µl of each dilution spread over plates of marine agar (2216E, Difco), tryptone soya agar (TSA) (Oxoid) supplemented with 1% NaCl (= TNA), diagnostic sensitivity test agar (DST) (Oxoid), and an equi-mixture of marine 2216 E agar and TSA. Media were incubated at room temperature for up to 14 days. Then, colonies representative of each colony type were streaked and re-streaked on fresh media to obtain pure cultures. Examination for inhibitory activity was performed on 376 bacterial

strains against MRSA 9551 and MRSA J2407 and against VRE 788 and VRE 1349 (courtesy of S.G.B. Amyes, University of Edinburgh), *Listeria monocytogenes* National Collection of Type Cultures (NCTC), Colindale, London 10357 and 7973), *Escherichia coli* (laboratory culture), *Pseudomonas aeruginosa* (laboratory culture) and *Salmonella enteritidis* (laboratory culture) by using cross streaking method (Robertson *et al.*, 2000). Each marine culture were streaked at right angles across lines of inocula of the pathogens, and incubated for 24h at 37°C. Any production of antimicrobial compounds was indicated for each isolate by showing interruption spots of inhibition in otherwise continuous growth lines of the pathogens. The bioactive isolated marine cultures were identified by examination of key phenotypic traits (Sneath, 1986) and by 16S rRNA sequencing. Thus, the cultures were grown for 24 h at 28°C in tryptone soya broth supplemented with 1% NaCl (TNB), and the cells harvested by centrifugation for 10 min at 4°C and 5000 × g. The DNeasy tissue kit (Qiagen) and protocol was used to extract and purify the total DNA from animal tissues. Sequencing was carried out using universal primers amplifying a 1500 bp region of the 16S rRNA gene (9F: 5'-GAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGYTACCTTGTTAACGACTT-3') by MWG-Biotech. For identification of isolates, the 16S rRNA gene sequences were compared to data base using the basic alignment search tool (BLAST) (Altschul *et al.*, 1990).

Determination of Inhibitory Activity

Only one out of 376 isolates was found to produce growth inhibitory compound. Routine and gene sequencing tests showed the isolate to be *Bacillus subtilis* (see Results and Discussion below). The best growth was obtained in Columbia broth (Oxoid) with pieces of sponge, when incubated at 28°C for up to 7d with shaking at 210 rpm. The bioactive compound was extracted in supernatant following centrifugation at 10,000 × g for 15 min at 4°C, and filtration through 0.22 µm Millipore Millex porosity filters. Inhibitory activity was assessed against the bacterial cultures listed previously. This was carried out using well diffusion (Jack *et al.*, 1995) and by disc diffusion using 6 mm diameter Whatman No. 1 filter paper discs, which were impregnated with 110 µl volumes of the filtrate.

Isolation, Purification and Characterization of the Inhibitory Compounds

The growth conditions mentioned above were used to give the largest volume of bioactive supernatant. The filtered supernatant was concentrated by using a special molecular weights tube filter (Amicon Ultra -15; 10,000 MWCO) which collect all protein or molecules with molecular weights higher than 10 kDa. The supernatant concentrated after adding phosphate buffered saline (PBS; Oxoid; pH 7.4). Thereafter, concentrated proteins were applied to a Superose 12 HR 10/30 columns (Pharmacia, LKB) for gel filtration at a flow rate of 30 ml/h in PBS. The automatic fractions collector gave many 1-ml fractions; one of them was found to contain the inhibitory activity, for which it was tested as mentioned before.

Four milliliters of active gel filtrate each from a gel run were applied to a FPLC-MonoQ anion exchange column and eluted with a 0 to 1 M NaCl gradient in phosphate buffer (pH 7.0) at a flow rate of 1 ml min⁻¹. All the fractions were tested to assess the activity against different strains of MRSA, VRE and *Listeria monocytogenes*.

To determine the molecular weight of the active component, denaturing polyacrylamide gel electrophoresis (SDS_PAGE) was used (Laemmli, 1970). Continuous gel was used with 10% concentration, and 15 µl of chilled acetone-precipitated active component were loaded together with 5 µl volumes of SDS/PAGE molecular weight markers (Bio-Rad, Low range) (diluted 1:20 in SDS reducing sample buffer). Thereafter, electrophoresis was run (Bio-Rad, 1000/1500) at 100 mA for 30 min and 150 mA for 60 min. Gel was stained with Coomassie brilliant blue (CBB; 10.4% R-250: Sigma).

The protocols for MALDI-TOF-MS were carried out as per the technical manual provided by the manufacturer Amersham Pharmacia Biotech with minor modifications to identify the protein.

The effect of different physiochemical factors on the inhibitory activity of the supernatant was tested employing gel diffusion technique. Parameters tested included: Temperature (0-121°C for 30 min), pH (1-14 at 4°C for 24 h), 0.5 mg ml⁻¹ α- and β-amylase (Sigma), protease K (Sigma), pronase (Sigma), trypsin (Sigma), and Tween 20, 40, 60 and 80.

Results and Discussion

One bacterium out of 376 isolates was found to produce the bioactive compound. Based on physical, biochemical and gene sequencing, the isolate was identified as *Bacillus subtilis* (100% similarity by 16S rRNA gene sequencing). Inhibitory activity was demonstrated on MRSA 9551, MRSA J2407, VRE 788, VRE 1349 and *L. monocytogenes* (NCTC 7973 and NCTC 10357). The assignment of the bioactive compound to *B. subtilis* is easily accomplished by cross streaking. This technique could initially be readily used to reveal if such a compound is produced and to distinguish the bioactive compound producing isolate. For maximal growth and production of this extracellular product, Columbia broth provided with pieces of synthetic polyurethane sponge, and shaking at 210 rpm is recommended. The production was reduced when culturing is carried out in Columbia broth without shaking or in other type growth media.

The beneficial effect of shaking may be due to the even distribution of the isolate throughout the broth medium or due to added aeration. The sponge pieces definitely provide more surface area and promote biofilm formation (Brindle *et al.*, 1998). They also enhance the production of the bioactive protein (Jamal *et al.*, 2006).

The physiochemical factors provide evidence that the bioactive compound is a protein; as it was affected by either Tween 20, 40, 60 or 80, or α - or β -amylase, it was sensitive to protease K, pronase and trypsin and it was not affected by heating to 40°C or by exposing to pH of value range of 5-14. Also, its absorbance is maximal at 280 nm wave length (Fig. 1a), and only fractions with A 280 maxima showed inhibitory activity (Fig. 1b).

Molecular weight determination showed a single band in the bioactive fraction corresponding to 30.7 kDa (Fig. 1c), which was inhibitory to the cultures of MRSA and *L. monocytogenes* (Fig. 1d). Also, one of the filtrate of ion-exchange chromatography showed the same single band of 30.7 kDa on the electrophoresis after testing bioactivity at 400 mM NaCl (Fig. 2 a-c). Identification of the purified

protein By MALDI-TOF-MS-PRO analysis and comparison with the NCBI database showed that the bacteriocin is homologous with YbdN protein. This protein is known to be produced by *B. subtilis*, by *E. coli* O157:H7 EDL933, and by *B. licheniformis*. Therefore, the extracellular product extracted from *B. subtilis* is the YbdN protein and with antimicrobial function against MRSA, VRE and *L. monocytogenes*.

It is known that the marine bacteria bacilli have long been associated with the production of antibacterial compounds. These bacteria are widely distributed in the environment, and are used in the biotechnology industry to manufacture enzymes, antibiotics, biochemical's and consumer products (Erickson, 1976; Alexander, 1977; Eveleigh, 1981; and Austin, 2001). Another bacteriocin, (thuricin 7), with a molecular weight of 11.6 kDa is produced by *B. thuringiensis*, and is effective against *B. cereus*, *Streptococcus pyogenes* and *L. monocytogenes* (Cherif *et al.*, 2001). Antimicrobial compounds which are effective against MRSA and VRE were also produced by *B. licheniformis* strain EI-34-6, which was isolated from the surface of marine alga (Yan *et al.*, 2003).

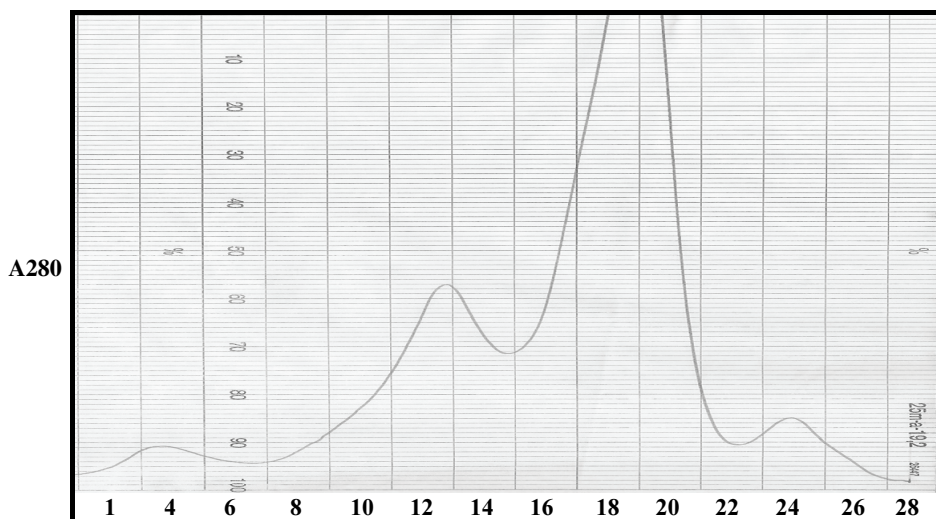


Fig. 1(a). A280 profile of the supernatant from gel filtration.

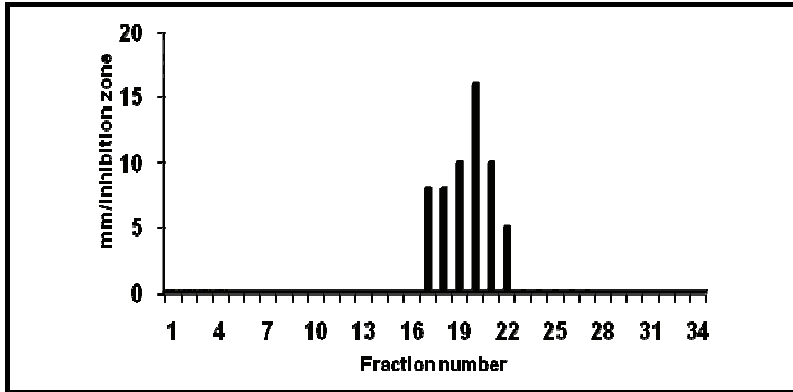


Fig. 1(b). Inhibitory profile of gel filtration fraction 1 to 34 showing inhibitory activity corresponding to the A280 profile in Fig. 1(a).

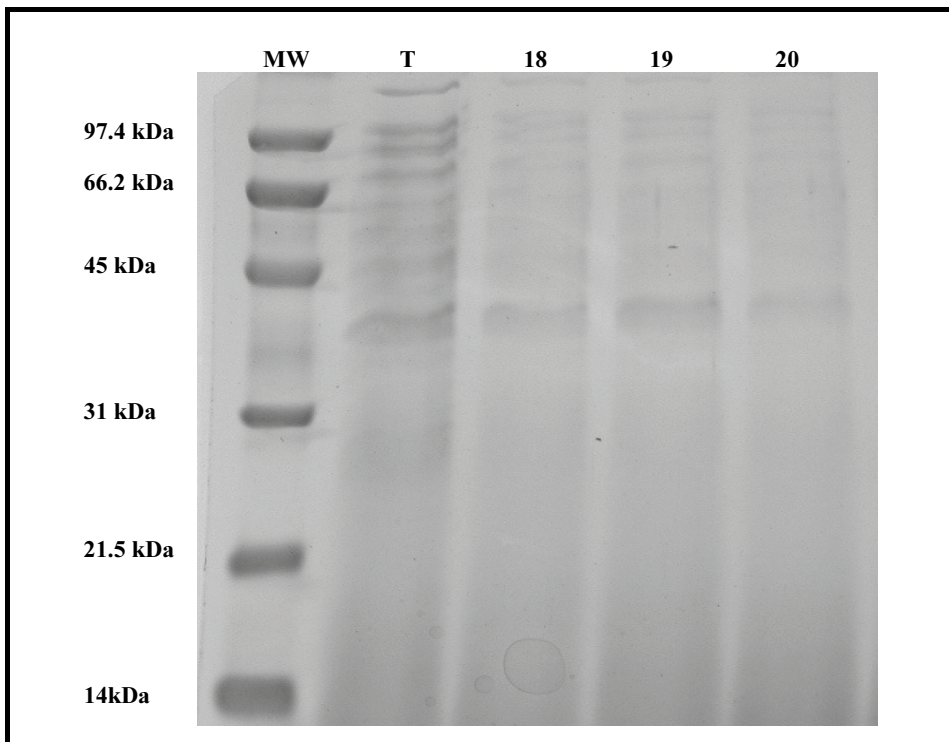


Fig. 1(c). Lanes 18-20, SDS-PAGE analysis of the active antibacterial fractions after gel filtration. Lane T contains the supernatant before fractionation. MW contains the molecular weight markers.

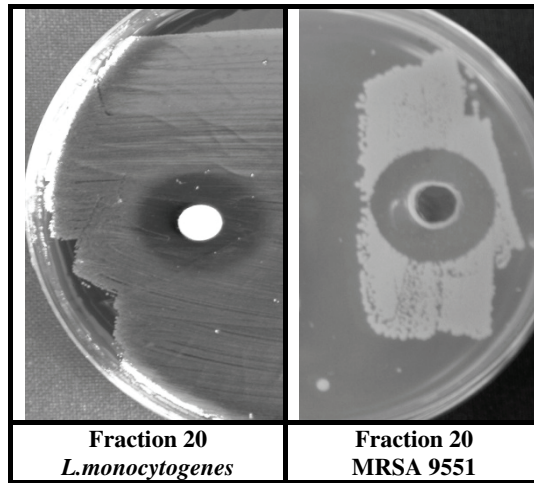


Fig. 1(d). Zones of inhibition resulting from fraction 20.

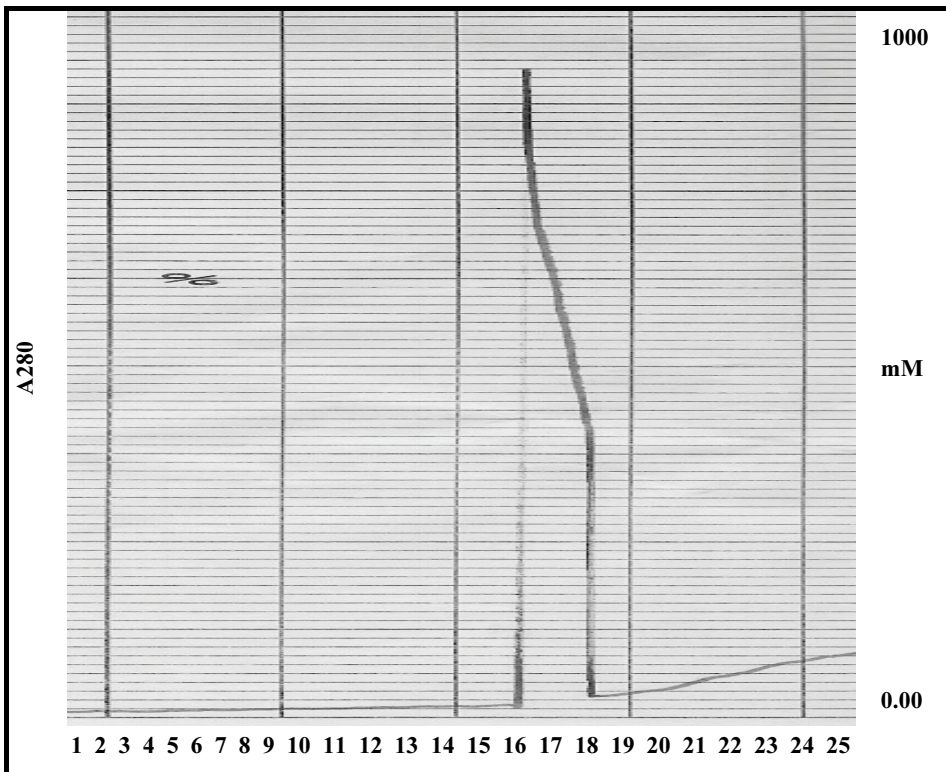


Fig. 2(a). A single protein peak that eluted at 64 % to 72% (wt/vol) NaCl was revealed by anion exchange chromatography of the active gel filtration fractions (Fig. 1).

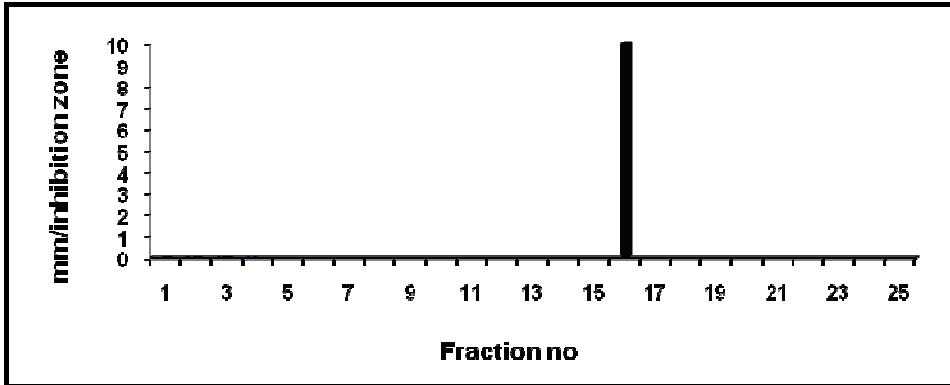


Fig. 2(b). Inhibitory profile of ion-exchange fractions corresponded with the protein peak of Fig. 1(a).

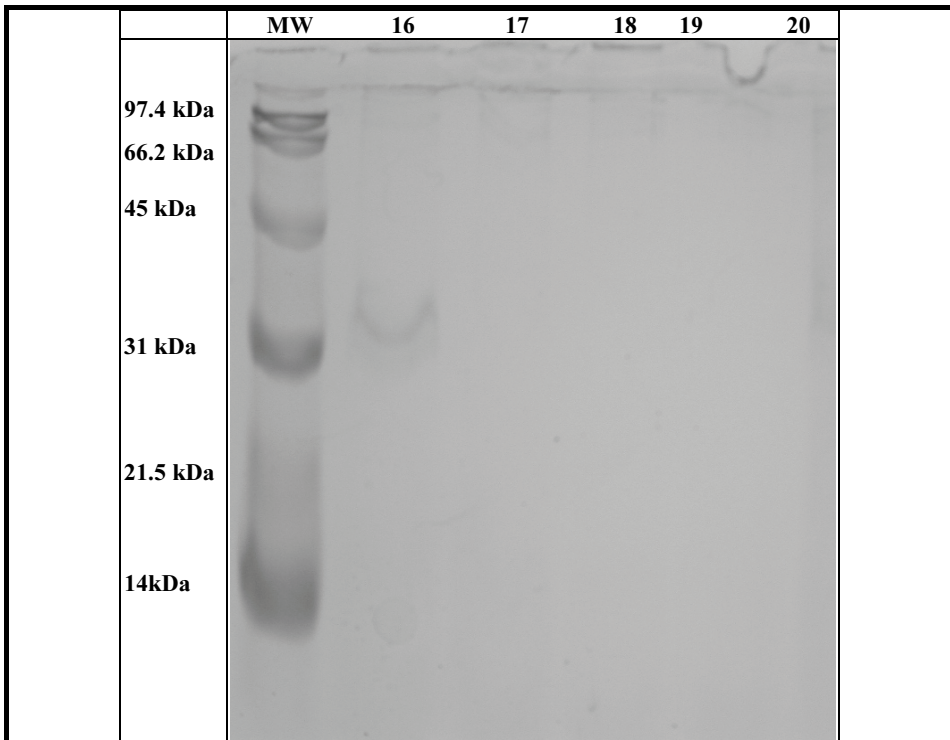


Fig. 2(c). SDS-PAGE analysis after anion-exchange of fractions 16 to 20, with inhibitory activity in fraction 16. Lane MW contains the molecular weight markers.

Conclusions

- An extracellular product with antimicrobial activity was extracted from broth culture of *Bacillus subtilis* in Columbia broth.
- The extracellular product was identified as YbdN protein and demonstrated inhibitory effect on the growth of methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, and *Listeria monocytogenes*.
- Best production of the protein was achieved when the broth culture was provided with synthetic polyurethane sponge pieces with culture shaking.

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فصل بروتين (YbdN) من بكتيريا *Bacillus subtilis* المعزولة
من طحالب *Sargassum* sp. من البحر الأحمر ذي فاعلية
مضادة لجراثيم ممرضة و مقاومة للمضادات الحيوية

مدوح طه جمال، و محمد سعيد مدرس

كلية علوم البحار - جامعة الملك عبد العزيز، ص. ب. ٢٠٧، جدة ٢١٥٨٩
المملكة العربية السعودية

المستخلص. من مئات المعزولات البكتيرية من ماء وروسوبيات
شاطئ البحر الأحمر لمدينة جدة بالمملكة العربية السعودية وكذلك
من مختلف الكائنات البحرية مثل الطحالب (*Halimeda* sp.,
Sargassum sp.), والأسفنج (*Leucetta*)، و *Siphonochalina* sp.,
chgosensis), وخيار البحر (*Holothuria atra*), ونجم البحر
(*Acanthaster planci*), وجذور نباتات الشورى (*Avecenia*
marina), وشقائق النعمان (*Heteractis magnifica*), وسمك الجلي،
وجدت معزولة بكتيرية واحدة من طحالب *Sargassum* sp. صنفت
باسم *B. subtilis* والتي استخلص منها بروتين (YbdN) المعروف
بفاعلية المضادة لسلاسل بكتيرية ممرضة للإنسان ومقاومة
للمضادات الحيوية مثل :

Methicillin-Resistant *Staphylococcus aureus* Vancomycin-
Resistant Enterococci and *Listeria monocytogenes*.

إن أفضل ظروف لإنتاج البروتين كانت عن طريق زراعة النوع
البكتيري المذكور بعاليه في وسط من نوع (Colombia broth) وفي
وجود قطع من اسفنج (synthetic polyurethane) في درجة حرارة
٢٨ درجة مئوية لمدة سبعة أيام في حاضنة ذات اهتزاز دائري
بسرعة محددة ٢١٠ دائرة / دقيقة.