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Research Article

Enzymatic and antioxidant properties of bacterial symbionts of the sea cucumber *Holothuria forskali*

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Abstract: In several countries around the world, the sea cucumber fishery is currently in danger in view of the increasing demand for their nutritional benefits. Study associated bacteria provide a better understanding of sea cucumber biology, potential threats to its survival and root-active biomolecules synthesis.

In the present work, we investigated the biotechnological potential of symbiotic bacteria associated with the intestine of the sea cucumber, *Holothuria forskali*. An important diversity was revealed with the isolation of 143 bacterial isolates on Zobell agar medium. The study of the Internal Transcribed Spacer (ITS) of the strains collection shows 19 different haplotypes. Molecular identification by 16S gene sequencing permitted the affiliation of the symbiotic bacteria of the holothurian intestine to the *Vibrio*, *Halomonas*, *Pseudomonas*, *Staphylococcus* and *Bacillus* genera. Particularly *Vibrio* and *Bacillus* strains exhibited the greatest amylase, protease, cellulase, esterase, and agarase activity, which reflects their involvement in the degradation of several polymers inside the cucumber tract.

The supernatant of these bacteria also shows an important ROS (reactive oxygen species) scavenging ability, exceeding 80% in some *Bacillus* strains. To confirm the safety and effectiveness of these strains in vivo, further studies are needed.

Keywords: bacteria; symbionts; biodiversity; antioxidant; intestines.

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1. Introduction

Holothurians (Echinodermata), known as sea cucumbers, are a popular luxury food in Asia and represent an economically important market as a high-value seafood product (Pangestuti and Arifin 2018). Their nutritional benefits have also been widely explored in traditional medicine (Vergara and Rodriguez 2016) and pharmacology and cosmetics due to their bioactive compounds with antitumor,antioxidant, and anti-inflammatory properties (Telahigue et al. 2020).

Moreover, sea cucumbers have an important ecological role in marine ecosystems due to their feeding behaviour as deposit feeders and detritivore animals (Purcell et al. 2016). They are candidates for aquaculture bioremediation and for integrated multi-trophic aquaculture (Tolon et al. 2017).

As a result of increasing commercialization of sea cucumbers and uncontrolled exploitation of wild stocks in the past decades, some species have been classified as threatened (Purcell et al. 2016).

The black sea cucumber, *Holothuria forskali* is one of the most widespread sea cucumbers in the North East Atlantic Area and the Mediterranean Sea, particularly along the Tunisian coasts (Telahigue et al. 2019). This sea cucumber is newly targeted to supply the Asian market. As a result, developing aquaculture is a sustainable way to meet market demand while also creating new economic activities in coastal areas. Nevertheless, a number of issues, particularly bacterial infections, provide substantial challenges to sea cucumber farming (Lavitra et al. 2009). In the other way, various studies have demonstrated that some beneficial strains can perform vital functions for the host (Wang and Shao 2021).

Several authors have established that intestinal microbiota is an essential component of the host because it regulates food intake, modulates immune response, and increases intestinal epithelial proliferation. Mutualistic microorganisms often do not fulfill their role directly, but through enzymatic excretion.

The use of some bacteria, found in the culture media and the bodies of organisms such as shrimp and fish, as probiotics, has shown a positive effect on the growth and health of the fish through the improvement of their body microbial balance (Koga et al. 2022).

Over the past decade, research on microbiota in different tissues of sea cucumbers started to develop. Pagán-Jiménez et al. (2019) reported that the gut microbiota of *Holothuria glaberrima* is dominated by bacteria from the phylum Proteobacteria. Wibowo et al. (2019) have isolated 127 bacterial strains from the internal and external parts of *Holothuria leucospilota* and *Stichopus vastus*. Protobacteria was the dominant phylum among the 12 detected phyla. The authors have reported also that the isolated bacterial strains are able to produce compounds with high biotechnological potential. Recently, Sembiring et al. (2021) isolated three potential probiotics (*Gamma proteobacterium*, *Bacillus subtilis,* and *Bacillus sp*.) that can be used to enhance raised sea cucumbers productivity.

In a previous study, we have listed chemical compounds from *H. forskali* that derive from the ability of sea cucumbers to produce bioactive metabolites in response to various environmental stresses and avoidance of predation (Telahigue et al. 2020). The analytical screening revealed some interesting molecules that can be explored for their potential uses as ingredients in functional foods or as raw materials for new drug forms. As reported by numerous studies, we have also reported that *H. forskali* extracts have high inhibitory activity against both *Escherichia coli* and *Bacillus subtilis*, at concentrations of 2 and 4 mg m^{-1}, respectively (Telahigue et al. 2020). Based on the idea that *H. forskali* microbiome is behind detected activities, we aimed in the current study to investigate associated bacteria by isolation, enzymatic assay, and measurement of their antioxidant activity. This research is a first attempt aiming to fill in the gaps in our understanding of this species' microbiota in the wild, setting the path for future research into the changes in bacterial assemblages involved, among others, with the intestine regeneration process.

2. Materials and Methods

2.1. Sampling

Sea cucumbers were sampled in the Gulf of Tunis at Sidi Rais, which has a rocky bottom overgrown with seagrass meadows. Sea cucumbers were collected during May 2019, by scuba diving at depths ranging from 3 to 4 m. Samples were brought back to the laboratory alive. The identification of the species was carried out by experts in Mediterranean flora and fauna. Once in the lab, the samples were kept in well-ventilated seawater aquariums until they are examined. Dissection is performed in an aseptic environment. Individual digestive tracts were removed under the same conditions, and a fragment of the posterior part of each individual's digestive tract was used for bacterial isolation, while the remaining part was immediately frozen at -80°C for molecular analysis.

2.2. Isolation and characterization of bacterial strains

To investigate the diversity of bacteria associated with the intestine of the sea cucumber *H. forskali*, dilutions from 10^{-1} to 10^{-7} were obtained based on 1 mL macerated animal intestine suspended in 7 ml sterile seawater. Diluted bacteria were isolated with the pour plate technique on Zobell agar medium and incubated for 48 h (Girsang et al. 2020). Characteristics of the bacterial colony were observed to identify form, elevation, margin, size, and color. Each colony was purified and incubated for 48 h to obtain single colonies (Singh et al. 2019). The pure colonies were examined using a compound microscope (Leica) with a magnification of 1000X and oil immersion after Gram staining.

2.3. DNA extraction, amplification of ITS 16S-23S and 16S rRNA gene

Genomic DNA from pure strains is extracted using a DNA extraction kit (Promega kit). DNA concentrations were measured using a Nanodrop Spectrophotometer (Bio-Rad). Amplification of the 16S–23S internal transcribed spacer region (ITS) was performed as previously described by Daffonchio et al. (2003) using, respectively, the universal primers S-D-Bact-1494-a20 (5’-GTCGTAACAAGGTAGCCGTA3-’) and L-D-Bact0035-a-15 (5’-CAAGGCATCCACCGT-3’). The amplification reaction mixture consists of 1x PCR reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 0.3 mM of each primer, 1 U Taq DNA polymerase, and 1 µl of total DNA. The PCR program consists of a 3-min initial step at 94°C, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and elongation at 72°C for 2 min, followed by an 8-min final elongation step at 72°C. The ITS-PCR amplification patterns were transferred to 1% agarose gels in 0.5x Tris-borate-EDTA buffer and stained in 0.5 mgL^{-1} ethidium bromide solution for 30 min. ITS-PCR profiles were compared, and the strains showing comparative profiles were considered identical haplotypes. The 16S rRNA gene was amplified using universal primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3‘) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) as forward and reverse primers, respectively (Isnansetyo and Kamei 2003). The reagent concentrations are as follows: 1X PCR buffer, 0.5 µM of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1U of Taq DNA polymerase, and 200 ng of purified DNA in a reaction volume of 25 µl. The amplification program includes an initial denaturation step of 10 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, hybridization for 1 min at 55°C, elongation for 1 min at 72°C, and a final elongation step of 10 min at 72°C (Turki et al. 2013). Purified and sequenced amplification products were used to identify bacterial strains.

2.4. Phylogenic analyses

The sequences obtained were compared with the 16S rRNA sequences available in the Microbial Nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov) of the National Center for Biotechnology Information database (NCBI) (http://www.ncbi.nlm.nih.gov) (Cole et al. 2014). Multiple sequence alignment was performed using ClustalW (Thompson et al. 2002). The maximum likelihood method was used to calculate evolutionary distances. The phylogenetic dendrograms were constructed using MEGA 11 (Molecular Evolutionary Genetics Analysis) (Tamura et al. 2021) to perform analyses of sequence data and phylogenetic relationships.

2.5. Screening for digestive enzymes

The isolated bacterial strains were screened for their ability to secrete digestive enzymes on agar plates with the substrate corresponding to each evaluated enzymatic activity. Amylase activity was assayed on plates containing starch agar (10 g tryptone, 10 g starch, 5 g meat extract, 15 g Agar-Agar, and 1000 ml distilled water, pH 7.4 ± 0.2). The plates were incubated for 24 h at 42°C, then refrigerated for 24 h at 8°C before being stained for 15 min with a 2% iodine/potassium iodide solution to confirm the substrate degradation halo (Xavier et al. 2017). A distinct halo development surrounding the colony shows amylase production. Lipase activity was detected by the development of a turbid halo around the inoculum on Tryptic soy agar plates treated with 1% Tween 80, as stated by Rollof et al. (1987). Protease activity was assessed on Muller-Hinton agar containing 3% skimmed milk. Plates were streaked with test strains, followed by incubation at 37°C for 24 h. The presence of a halo around the colonies indicated caseinase activity (Burke et al. 1991). For the isolate cellulase activity, CMC Plate Assay was used as described in Ray et al. (2007). The plates were incubated at 37°C for 5 days to facilitate the secretion of cellulase. After incubation, the agar medium was overflown for 15 min with an aqueous solution of Congo red (1% w/v). After discarding the Congo red solution, the plates were further treated by flooding with 1 M NaCl for 15 min. The clear zone of hydrolysis showed cellulose degradation (Shaikh et al. 2013). For agarase activity, the plates were stained with Lugol's Iodine solution (1% I_2, 2% KI), then washed with distilled water to remove excess Lugol's Iodine solution. Because the Lugol's Iodine solution only stains polysaccharides, agar degradability was detected surrounding the bacterial colonies (Kim et al. 2017).

2.6. Antioxidant activity assay

The isolated bacterial strain supernatants were screened for their 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging using the method reported by Brand-Williams (1995). This method involved the capture of DPPH radicals by antioxidants, producing a decrease in absorbance at 515 nm. The DPPH was dissolved in methyl alcohol at a concentration of 60 mM. Once homogenized, the solution was transferred into a dark glass bottle and used only the day of the test. A homogenization step followed by agitation was carried out by transferring 0.1 ml of each sample (culture supernatant) to a tube containing 3.9 ml of DPPH radical solution. As a control, 3.9 ml of DPPH solution was combined with 50% methyl alcohol, 50% acetone, and 70% water. Methyl alcohol was used as a blank. We calculated the half maximal effective concentration EC50 (g ml^{-1}) using the standard curve, which is the minimum concentration needed to reduce 50% of the initial DPPH reaction after the extract reached stability. The percentage of radical scavenging activity of DPPH was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{Ac - As}{Ac} x100$$

Where, Ac and As were the absorbance of the control and sample, respectively. For all strain supernatants, the antioxidant assay was conducted in triplicates.

3. Results and discussion

3.1. Bacterial isolation and characterization

A total of 143 bacterial samples isolated from *H. forskali* specimens were subjected to genetic identification procedures including DNA extraction, PCR amplification of the 16S rRNA and the ITS 16S-23S genes, and DNA sequencing. The study was completed by the sequencing of the 16S rRNA gene determine the affiliation of the isolated strains. Figure 1 shows the agarose gel electrophoresis of ITS-PCR products of different isolates. A total of 19 different ITS- haplotypes designated Sc1, Sc2, Sc3, Sc4, Sc5, Sc6, Sc7.1, Sc7.2, Sc8, Sc9.1, Sc9.2, Sc9.3, Sc10, Sc11.1, SC11.2, Sc12, Sc13,

Sc14, and Sc20 were distinguished. ITS-PCR patterns show different bands ranging from 250 to 1000 bp. The representative isolates of each ITS-haplotype were identified at species level by 16S rDNA gene sequence analysis. The phylogenetic tree based on the 16S rRNA gene sequences of bacterial isolates is shown in figure 2. Isolates are clustered into two distinct phylogenetic groups. The first group includes members of the Gammaproteobacteria subdivision distributed into four genera (*Vibrio*, *Klebsiella*, *Halomonas* and *Pseudomonas*). The second group includes Bacillales from the Firmicutes division distributed into two genera: *Bacillus* and *Staphylococcus*. Homology analysis of 16S rDNA sequences revealed a large similarity between the Sc20 isolate and *Vibrio tubiashii* (MH386389).

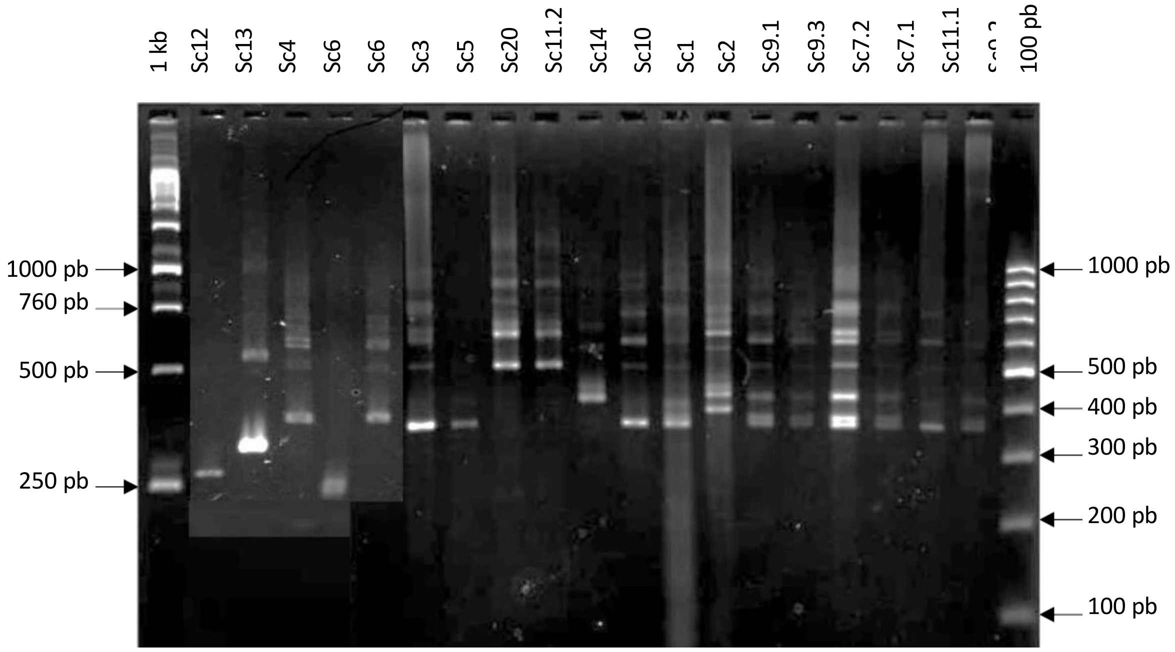

Figure 1. Electrophoretic patterns of ITS-PCR 16S-23S bacterial strains isolated from *Holothuria forskali* intestine.

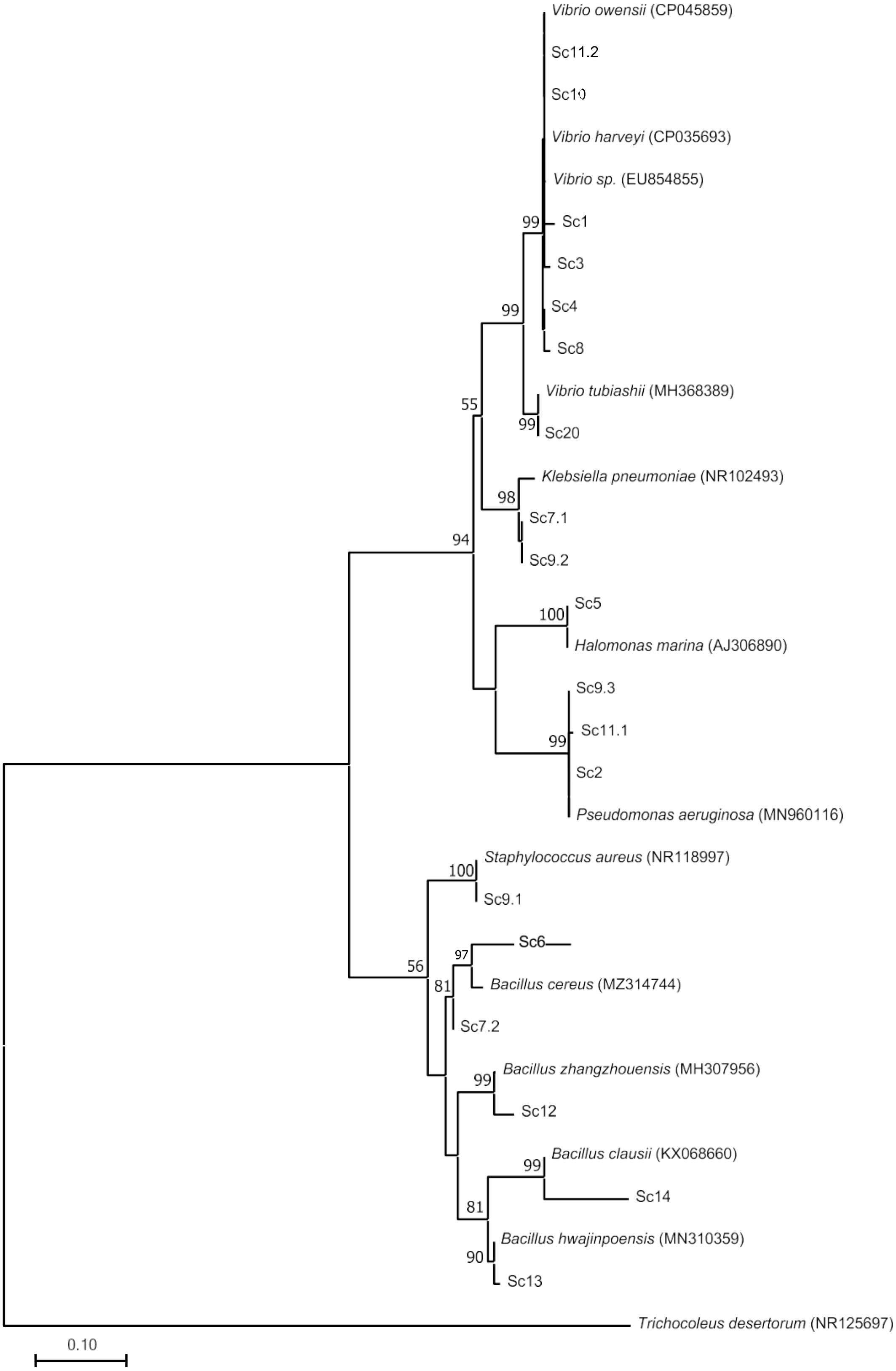

Figure 2. Phylogenetic position of the isolated bacterial strains with nine closest reference strains (NCBI). The topology shown in the tree was obtained using the neighbor-joining method. The scale bar represents 0.10 substitutions per base position. Bootstrap values (%) are represented by the numbers supplied at the nodes. *Trichocoleus desertorum* (NR 125697) is used as an outgroup.

A large similarity is also observed between Sc11.2, Sc10, Sc1, Sc4 and Sc8 isolates and *Vibrio owensii* (CP045859). The isolate Sc3 is affiliated with *Vibrio harveyi* (CP035693) with 98% of sequence similarity. Two isolates (Sc7.1 and Sc9.2) showed a similarity of 98% with *Klebsiella pneumoniae* (NR036794). A total similarity was revealed between Sc5 and *Halomonas marina* (AJ306890). Sc2, Sc9.3 and Sc11.1 can be attributed to *Pseudomonas aeruginosa* (MN960116) with a similarity of 99%. The Sc9.1 isolate corresponds to *Staphylococcus aureus* (NR118997). Both Sc6 and Sc7.2 were like *Bacillus cereus* (MZ314744). However, Sc12, Sc13 and Sc14 were affiliated with *Bacillus zhangzhouensis* (MH307956), *Bacillus hwajinpoensis* (MN310359) and *Bacillus claussi* (KX068660) respectively.

3.2. Screening for digestive enzymes

The bacterial collection has been screened for cellulase, amylase, esterase, protease, and agarase activities on a variety of substrate-agars. The results are summarized in Table 1.

Table 1. Screening for enzymatic and antioxidant activities of *Holothuria forskali* associated bacteria.

Isolate	Amylase	Lipase	Protease	Cellulase	Agarase	Antioxidant activity (%)
Sc1	++	+	++	-	+++	3.4
Sc2	+++	++	+++	-	++	7.6
Sc3	++	++	+++	-	+++	4.3
Sc4	+	++	++	-	++	3.2
Sc5	+	++	++	-	+++	5.8
Sc6	+	+	++	-	++	79.6
Sc7.1	++	++	++	-	+++	2.9
Sc7.2	++	+	+	-	+++	81.2
Sc8	+	++	+++	-	++	11.3
Sc9.1	++	++	++	-	+++	5.1
Sc9.2	++	++	++	-	+++	2.8
Sc9.3	+++	+	++	-	++	8.4
Sc10	+	++	++	-	+++	4.7
Sc11.1	++	+	+	-	+++	2.5
Sc11.2	+++	+++	+++	-	+++	1.8
Sc12	+	+	+	+	-	80.5
Sc13	-	-	++	-	++	81.8
Sc14	-	-	-	-	-	79.9
Sc20	++	+++	++	-	+++	9.3

+++ important activity (halo diameter ≥ 25 mm)
++ intermediate activity (11 < halo diameter < 24 mm)
+ low activity (halo diameter < 11 mm)

Proteolytic activity was observed with nearly all tested strains. In this collection, only the strain Sc14, which is affiliated with *B. claussi*, was not able to hydrolyze casein. Belonging to the *Bacillus* genus, Sc6, Sc12 and Sc13 showed moderate activity. The most important caseinase activity was observed with *Vibrio* strains Sc2, Sc8, Sc3 and Sc11.2, where the diameter of the degradation areas exceeded 30 mm in diameter. The *Bacillus* strains, Sc13 and Sc14, did not degrade Tween 80, while Sc12 (*B. claussi*) showed low lipase activity.

The most significant lipase activity in *Vibrio* strains, was observed in strains Sc11.2 (*V. owensii*) and Sc20 (*V. tubiashii*). All *Vibrio* strains, in addition to Sc12 (*B. zhangzhouensis*) have starch-degrading capacity. The most important amylase activity was observed in the Sc2 and Sc11.2 strains belonging to the *Vibrio* genus. *B. hwajinpoensis* (Sc13) and *B. claussi* (Sc14) are devoid of amylases. Significant agarolytic activity was detected in all strains except Sc14 and Sc12 which are agarase negative. Among the strains, the only one that can hydrolyze cellulose is Sc12 (*B. zhangzhouensis*).

3.3. Antioxidant activity

Results have revealed that the isolated bacterial strains Sc6, Sc7.2, Sc12, Sc13, and Sc14 possess greater antioxidant activities than those of the other strains. The molecular identification has shown that all these strains are affiliated to the *Bacillus* genus (Figure 2).

3.4. Discussion

In this work, 143 bacterial strains were isolated from the intestine of *H. forskali.* Among them, four showed greater than 97% sequence similarity with the genus *Bacillus* and were affiliated with the species *B. thuringiensis*, *B. zhangzhouensis*, *B. clausii* and *B. hwajinpoensis*. Species belonging to the genus *Bacillus* are gram-positive, spore-forming, rod-shaped motile bacteria that are present in various environmental conditions, but mainly in soil (Foysal and Lisa 2018). Some species of *Bacillus* have symbiotic relationships with plants and animals. Symbiotic bacteria of the genus *Bacillus* can play several roles in maintaining the integrity of the host organism. Some of them protect their hosts against microbial infections through the secretion of antibiotics, enzymes, and volatile compounds (Soares et al. 2016).

Our results revealed that the Sc6 strain isolated from *H. forskali* is affiliated with *B. thuringiensis* species. This species was also isolated from the sea cucumber *H. leucospilota* and the surrounding sediment by Lutfi et al. (2018). *B. thuringiensis* strains are of very significant biotechnological interest. During the vegetative and sporulation phases, *B. thuringiensis* generates insecticidal proteins such as crystal proteins, cytolytic toxin proteins, and vegetative insecticidal proteins (Adang et al. 2014). Sc6 strain has amylase, lipase, protease, and agarase hydrolytic activities, implying an auxiliary role in sea cucumber digestion. As for the Sc14 strain, it is affiliated with *B. clausii* (99%). Several studies have confirmed the antimicrobial activity of *B. clausii* strains against Gram-positive bacteria, particularly against *Staphylococcus aureus*, *Enterococcus faecium* and *Clostridium difficile* (Urdaci et al. 2004). *B. clausii* is distinguished from the other species of the genus by its ability to withstand high pH environments and its exceptional tolerance to antibiotics (Urdaci et al. 2004).

Moreover, *B. clausii* was the main constituent of a probiotic marketed for more than 60 years for the prevention and treatment of diarrhea "Enterogermina®" (Ianiro et al. 2018). The effectiveness of *B. clausii* in the prevention of recurrent respiratory infections has also been demonstrated (Marseglia et al. 2007). According to this data, it is quite possible that this germ has a mutualistic relationship with the sea cucumber *H. forskali*. In this relationship, the bacterium *B. clausii* (Sc14) finds suitable conditions for its development (alkaline pH and richness in organic polymers) and for the secretion of molecules with antimicrobial effect that limit the growth of pathogenic germs. Our results showed that strain Sc13, affiliated with *B. hwajinpoensi*, possesses protease and agarase activity. This spore-forming species was also isolated from seawater (Yoon et al. 2004) and sponges like *Haliclona simulans*, *Geodia cydonium* and *Ircinia muscarum* (Zuppa et al. 2014).

The agarase activity of the *B. hwajinpoensi* strain is demonstrated for the first time in this work. Since seaweed is rich in agar, the agarase activity of the strain is proof of its perfect adaptation to aquatic environments.

Sc12 strain affiliated with *B. zhangzhouensis* has amylase, lipase, protease, and cellulase activities. Moreover, it is the only strain capable of degrading cellulose among the isolated strains in this work. Ruginescu et al. (2018) isolated a similar strain from Salt Lake in Romania. This strain has an active endoglucanase in a high salt concentration (3M NaCl) (Ruginescu et al. 2018). In addition to its role in pathogen defense, this strain is involved in the degradation of various polymers.

As well as the strains affiliated with *Bacillus,* there was great diversity among the strains associated with *Vibrio*. Many species of this genus are widespread in marine sediments, marine animals, and other diverse environments (Lutfi et al. 2018). Detritus is made up of organic debris that provides food for detritus feeders (Hagen et al. 2012). In this regard, many isolates showed various substrate digestion activities that could be involved in the sea cumber nutrition process. Our results are in accordance with those of several authors (Kim et al. 2017; Lutfi et al. 2018). It was demonstrated that *Vibrio* can degrade a wide range of macromolecules, including starch, alginate and agar (Li et al. 2020).

The strain Sc5 is affiliated with *Halomonas marina* (100%). The genus *Halomona* was also detected in the intestine of the sea cucumbers *Holothuria leucospilota* and *Apostichopus japonicusi* (Bogatyrenko and Buzoleva 2016). It was reported that *Halomonas* strains could perform aerobic denitrification and heterotrophic sulfide oxidation, and they play a substantial role in the nutrient cycle (Wang and Shao 2021).

The strains Sc2, Sc9.3 and Sc11.1 are affiliated with *Pseudomonas aeruginosa* (99%). Using the nematode *Caenorhabditis elegans* as a host model organism, Lee et al. (2009) proposed that *Holothuria atra* has anti-infective properties against *P. aeruginosa*. In their study, the authors demonstrated that the bioactive fraction of *H. altra* inhibits the production of pathogen virulence factors.

The strain Sc9.1 is affiliated with *Staphylococcus aureus* (100%). This indicates that sea cucumbers *H. forskali* were contaminated with potentially pathogenic bacteria. Our results are in accordance with those of Lew et al. (2020) who reported that raw or undercooked sea cucumber should be avoided by consumers.

The *Bacillus* affiliated isolates (Sc6, Sc7.2, Sc12, Sc13, and Sc14) showed an important antioxidant activity. Our results are consistent with several authors (Petruk et al. 2018; Kotowicz et al. 2019). *Bacillus* produce a number of metabolites that carry antioxidant properties including carotenoids that are produced in some species and present in the cell wall. It is known that bacteria contain protective proteins that can detoxify reactive oxygen species (ROS) and counter damage (Zhao and Drlica 2014). The synthetized metabolites are responsible for obstructing damaging radicals associated with biochemical processes in biological systems.

Sea cucumber extracts have been reported to have substantial antioxidant properties. (Hossain et al. 2022). Sea cucumbers may absorb phenolic compounds generated from algae and/or microalgae, and phenolic-rich particles derived from decaying marine macroalgae (Mamelona et al. 2007). These phenolics may be responsible for sea cucumbers' remarkable antioxidant activity. Furthermore, the associated bacteria may reinforce this antioxidant effect. To confirm the bacterial strain's involvement, more investigations are needed.

Overall, our results imply that the intestine *of H. forskali* is one of the suitable habitats for the identified bacteria. However, detected bacterial strains need to be characterized in terms of how they provide nutrition aid and protect against pathogens.

4. Conclusion

The isolation technique yielded 19 isolates of bacteria associated with the sea cucumber, *H. forskali*. The intestine of this sea cucumber was one of the suitable habitats for *Bacillus* and *Vibrio* strains which are the dominant identified genera among bacterial isolates. *B. thuringiensis*, *B. zhangzhouensis*, *B. clausii*, and *B. hwajinpoensis* in particular, have shown strong antioxidant activity. The genus *Vibrio* was represented by *V. owensii*, *V. harveyi* and *V. tubiashii*. In vivo studies should be conducted to confirm whether these strains are safe and effective as probiotics.

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