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Research Article

Isolation, identification and evaluation of the anti-diabetic activity of secondary metabolites extracted from bacteria associated with the Persian Gulf sponges (*Haliclona* sp. and *Niphates* sp.)

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Abstract

Sponge-associated bacteria have a special position in marine biotechnology due to their unique biological activities. The main objective of this study was to isolate and identify the bacteria associated with sponges around Qeshm Island, Iran, which inhibit the activity of alpha-glucosidase and alpha-amylase enzymes by the produced metabolites. Samples were collected from sponge species living in the study area, including the genera *Haliclona* and *Niphates*. Isolation was performed using culture-dependent techniques. A total of 155 bacterial isolates were collected. The diversity pattern of bacteria in the sponge samples showed that the *Vibrio* and *Bacillus* constituted the predominant bacterial population. The assessment of alpha-glucosidase inhibitory activity of metabolites extracted from the isolated bacteria showed that 6 isolates could inhibit the enzyme activity with IC_{50} values ranging from 153.5 to 495.4 µg/ml, while 9 bacterial isolates inhibited the activity of alpha-amylase enzyme in IC_{50} values at the range of 112.9 to 670.9 µg/ml. The cytotoxic activity of the metabolites extracted on human umbilical cord endothelial cells showed the toxicity of the three extracts at effective concentrations, while seven isolates showed no toxicity. Genetic identification indicated 97% to 100% similarity of the potent isolates with the NCBI gene bank including *Bacillus pumilus, Bacillus safensis, Vibrio alginolyticus, Pseudomonas stutzeri, Vibrio parahaemolyticus, Pseudomonas lurida, Bacillus tequilensi*, and *Streptomyces enissocaesilis.* The results of this study provided a new understanding of the diversity pattern of cultivable sponge-associated bacteria and their inhibitory activity on alpha glucosidase and alpha amylase enzymes.

Keywords: Alpha-amylase, Alpha-glucosidase, Anti-diabetic, Persian Gulf, Secondary metabolites, Sponge-associated bacteria

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Introduction

In total, 34 of the 36 living animals live in the oceans, while only 18 groups live on land (Fenical, 2020). Due to the extraordinary difference between marine and terrestrial environments, it is estimated that marine bacteria have different characteristics from their soil origins. Therefore, they can produce a variety of bioactive compounds (Amelia *et al.*, 2022). Despite the wide biodiversity in marine environments, research on natural marine products is still in its infancy and remains unexplored compared to terrestrial habitats (Brinkmann *et al.*, 2017). Most studies have focused on the neurotoxic, antiviral, anti-cancer, anti-diabetic, antimicrobial, and cytotoxic properties of marine metabolites. There are some documents investigated regarding to natural feed additives as potential substitute for antibiotics in aquaculture nutrition (Pourmozaffar *et al.*, 2019). In general, natural marine resources have provided a great opportunity for the development of the pharmaceutical industry due to their diverse and unique bioactive compounds (Barzkar *et al.*, 2021).

Secondary metabolites produced by marine bacteria represent various biological activities (Manivasagan *et al.*, 2015; García-Jiménez *et al.*, 2018). These metabolites provide various activities, including antibacterial, antifungal, anti-cancer, anti-tumor, anti-diabetic, cytotoxic, cytostatic, anti-inflammatory, anti-parasitic, anti-malarial, anti-viral, antioxidant and anti-angiogenic, etc. (Gozari *et al.*, 2016; Gozari *et al.*, 2018; Gozari *et al.*, 2019a). Marine sponges belong to the foraminifera branch and feed by filtering large volumes of water through an advanced system. Thereby absorbing many microorganisms, algae, and organic particles and digesting them through phagocytosis (Nazemi *et al.*, 2017; Tamadoni Jahromi *et al.*, 2021). Sponges are able to efficiently retain different marine microorganisms in a coexistence relationship. Therefore, the presence of a large number of microorganisms in the mesophyll of sponges has been proven in various studies (Hofer, 2021). Bacteria can contain up to 60% of sponge biomass, equivalent to 108 to 109 bacteria per gram of sponge tissue (Posadas *et al.*, 2022). The high biosynthetic ability of sponges is due to symbiotic microorganisms (Zhang *et al.*, 2022). One of the major roles of sponge-associated bacteria is to participate in chemical defense mechanisms against invasive organisms and pathogens and to prevent bio adhesion by producing bioactive compounds (Gozari *et al*, 2020). By binding to the active site of the enzyme, these molecules inhibit the entry of the substrate and prevent the catalysis of the reaction by the enzyme. Relatively few enzyme inhibitors have been reported from marine bacteria (Lloyd, 2020). They are isolated from *Streptomyces* sp. SA-2289. Pyrrostatin A and B are inhibitors of the enzyme enacetyl beta-glucosamidase produced by *Streptomyces* sp. SA-3501 (Trang *et al.*, 2021). Diabetes is a metabolic disorder in the body, in which the body

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loses the ability to produce insulin or becomes resistant to insulin production. Therefore, the insulin produced cannot function normally (Azam *et al.*, 2022). These biologically active compounds are members of different families including terpenoids, isoprenes, alkaloids, and flavonoids. Flavonoids are compounds that act as alpha-glucosidase inhibitors (Agrawal *et al.*, 2022). Despite advances in drug design, there is still an urgent need for new drugs with natural origins to combat various diseases such as diabetes. Therefore, this study aimed to evaluate the anti-diabetic activity of metabolites extracted from bacteria associated with some sponges in the Persian Gulf.

Material and methods

Sampling

Marine organisms were sampled in May 2020 from 10-15 m depth in Qeshm Island. About 25 samples of sponges were collected by diving and placed in sterile containers. The samples were kept to the ice until reaching the Persian Gulf and Oman Sea Ecology Research Institute laboratory in Bandar Abbas. The collected samples were isolated based on morphological features, including morphometric and morphometric features such as spicule identification by the Persian Gulf and Oman Sea Ecology Research Institute (Hooper, 2003). Sponge samples were identified from 2 species: *Niphates* sp. and *Haliclona* sp.

Preparation of the Samples

The samples were washed with sterile seawater just received at the laboratory to separate the attached particles. Subsequently, sponge samples were cut into 1 cubic cm pieces with sterile scissors under aseptic conditions and placed in a sterile mortar for homogenization. Then, serial dilutions up to 5-10 were prepared by sterile seawater dilution from homogenized samples (Gozari *et al.*, 2019b).

Cultivation and purification of bacteria

The sponges were cultured in culture media with formulations compatible with their natural conditions. Marine Zobell agar, Glycerol asparagine agar, and Marine sponge agar were used to isolate bacteria (Gozari *et al*, 2020).

Production and extraction of secondary metabolites from bacteria

The bacteria were cultured at 10^5 CFU/ml densities in a modified Nutrient Broth fermentation medium. After incubation at 30°C, the fermented liquid was removed and filtered using a vacuum pump (Gozari *et al.*, 2020).

Assaying the inhibition of alpha-glucosidase activity.

Different concentrations of the extracted metabolite were dissolved in sodium phosphate buffer (pH 6.8). 10 µL of each concentration was mixed with 20 µL of sodium phosphate buffer and 20 µl of para-nitrophenyl alpha-di-glucoside (2 mM) in a 96-well microplate and incubated at 37°C for 5 min. 10 µl of diluted alpha-glucosidase

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enzyme at a 0.2 U/ml rate was added to each well using 0.01 M sodium phosphate buffer. After incubation at 37°C for 15 min, the adsorption of the samples at 405 nm was recorded by a microplate reader. In the blank form, sodium phosphate buffer was used instead of the enzyme. Acarbose was assayed as a positive control (Chen *et al.*, 2016).

Assaying the inhibition of alpha-amylase activity

Alpha-amylase inhibition by metabolites was measured based on DNSA (Dinitrosalicylic acid) colorimetric method (Hansawasdi *et al.*, 2000).

Evaluation of the cytotoxic activity of inhibitory metabolites

The cytotoxic activity of metabolites was investigated at different concentrations and had enzymatic inhibitory activity against normal human umbilical cord endothelial cell lines (HUVEC). MTT tetrazolium reagent was used for this purpose. IC_{50} of each extract was determined (Peng and Zhao, 2009).

Identification of isolates producing secondary inhibitory metabolites

The microscopic morphology of the selected productive isolates was investigated using the gram stain method. Macroscopic morphology, including strain growth rate, colony color, spore formation, and water-soluble pigment production isolates with inhibitory activity, were identified based on the polyphasic identification strategy of bacteria, which includes morphological, physiological, biochemical, and genetic characteristics (Goto, 2005).

Investigation of the production of some enzymes

The production of hydrogen sulfide, nitrate reductase, and amylase enzymes was performed using a modified broth neutrinos medium. The production of oxidase enzyme was also measured using 1% tetramethyl - phenylenediamine reagent and 3% oxygenated catalase (Pollack *et al.*, 2018).

Genetic identification DNA extraction of productive isolates

Genomic DNA extraction of productive isolates was performed using the CTAB extraction method. For this purpose, different stages of cell lysis, de-proteinization, and purification of genomic DNA were performed after preparing the appropriate biomass. The amount of DNA extracted at 230, 260, and 280 nm was examined by spectrophotometry, and the concentration of genomic DNA was calculated. The quality of DNA extracted by electrophoresis on 1% agarose gel was evaluated (Kieser *et al.*, 2000).

16S rRNA gene amplification of selected isolates

Genetic identification of productive isolates was based on the similarity of the 16S rRNA protected gene of

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productive isolates. Primers 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ ACGGCTACCTTGTTACGA 3′) were used to amplify the gene (Heuer *et al.*, 1997). The optimal temperature chain of the PCR reaction is shown in Table 1. After performing the reaction, the quality of the PCR product was evaluated using agarose gel electrophoresis (Fig. 4). 16S rRNA gene sequencing was performed using the Sanger method and a Dye Deoxy Terminator Cycle Sequencing kit technique (Tamadoni Jahromi *et al.*, 2019).

Phylogenetic analysis of productive isolates

Genetic and evolutionary distance of the selected isolates was calculated by comparing them with the closest strains in the NCBI gene bank using phylogenetic analyses. The phylogenetic tree was mapped by MEGA X software based on the neighbor-joining model (Kumar *et al.*, 2018).

Statistical analysis

The IC_{50} of the samples was determined by plotting the dose-response curve using GraphPad Prism 6 software. Results were expressed as mean, standard error (SE). Mean means were compared using one-way ANOVA and the Bartlett test. Data differences at the level of $p<0.05$ were considered statistically significant. The amount of inhibitory activity was calculated by the following equation (Le Berre *et al.*, 2022).

Results

Biodiversity of bacteria isolated from sponge samples

We obtained 106 bacterial isolates from the sponge *Haliclona* sp. and 49 isolates from sponge *Niphates* sp. Collected from Qeshm Island. The results of bacterial identification showed that the diversity pattern was different in two sponge species (Fig. 1).

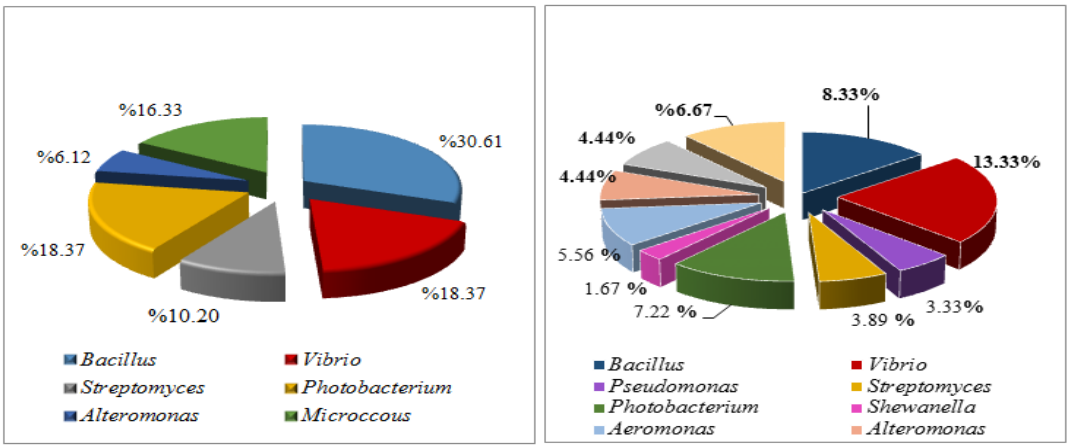


Figure 1: Biodiversity of bacteria isolated from *Haliclona* sp. (right) and *Niphates* sp. (left) collected from Qeshm Island

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Measurement of inhibition of alpha-glucosidase activity

Evaluation of the inhibitory activity of metabolites extracted from 155 isolates obtained from sponge samples showed that 9 isolates producing alpha-glucosidase inhibitory compounds with IC_{50} values were less than 1250/g/ml. The IC_{50} inhibitory properties of the metabolites extracted from the generator isolates are listed in Table 1-3. Among the extracted metabolites, the highest inhibitory activity belonged to QH 26 isolate with IC_{50} value of 177.6 µg/ml. While the lowest level of IC_{50} belonged to the metabolites extracted from QN 63 isolate (495.4) (Table 1).

Table 1: Assessment of alpha-glucosidase inhibitory activity of the extracted metabolites.

Isolate	IC_{50} ±SE (µg/ml)
QH 26	177.6±11.38
QH 30	453.8± 19.73
QH 36	153.5± 15.02
QH 37	441.0± 12./20
QN 45	341.8± 14.54
QN 63	495.4±20.13

Measurement of inhibition of alpha-amylase activity

Evaluation of the inhibitory activity of metabolites extracted from 155 isolates obtained from sponge samples showed that 6 isolates producing amylase inhibitory compounds with IC_{50} values were less than 1250/g/ml. The IC_{50} inhibitory properties of the metabolites extracted from the generator isolates are listed in Table 3. Among the extracted metabolites, the highest inhibitory activity belonged to QH 26 isolate with an IC_{50} value of 112.9 µg/ml. While the lowest IC_{50} level belonged to the metabolites extracted from QN 45 isolate at the rate of 411.1 µg/ml (Table 2).

Measurement of inhibition of alpha-amylase activity

Evaluation of the inhibitory activity of metabolites extracted from 155 isolates obtained from sponge samples showed that 6 isolates producing amylase inhibitory compounds with IC_{50} values were less than 1250 g/ml. The IC_{50} inhibitory properties of the metabolites extracted from the generator isolates are listed in Table 3. Among the extracted metabolites, the highest inhibitory activity belonged to QH 26 isolate with an IC_{50} value of 112.9 µg/ml. While the lowest IC_{50} level belonged to the metabolites extracted from QN 45, isolate at the rate of 411.1 µg/ml (Table 2).

Table 2: Assessment of inhibitory activity of amylase enzyme by extracted metabolites.

Isolate	IC_{50} ±SE (µg/ml)
QH 26	9.03±112.9
QH 30	25.01±210.8
QH 36	15.56±314.8
QH 37	19.32±347.8
QN 45	21.53 ±411.1
QN 63	201.9±17.17
Acarbose	78.32± 15.14

Table 3: Determination of IC50 cytotoxic activity of extracted metabolites against HUVEC cell line.

Isolate	IC_{50}± SE (µg/ml)
QH 36	196.7± 25.93
QH 45	510.6± 11.27

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Evaluation of the cytotoxic activity of extracted metabolites

The results of the cytotoxic activity assay of enzymes inhibiting enzyme activity showed that isolates of QH 36 and QH 45 against the cervical endothelial cell line of HUVEC showed cytotoxic activity with lethal values. The results showed cytotoxic activity dependent on the concentration of these isolates (Table 3). Determination of IC_{50} of the extracted metabolites showed that QH 36 isolate represented high cytotoxic activity against the HUVEC cell line with an IC_{50} value equal to 196.7 g/ml (Table 2). Examination of microscopic images of treated cells and treatment without treatment confirmed a significant reduction in the number of living cells and their morphological changes (Fig. 2).

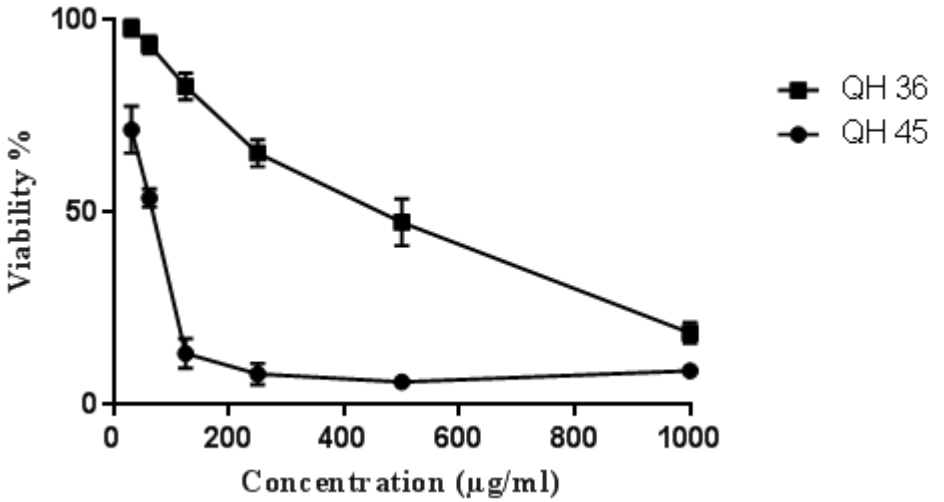


Figure 2: Dose-response diagram of cytotoxic activity of inhibitory metabolites against Human Umbilical Vein Endothelial Cells (HUVEC cells).

Genetic identification of inhibitory isolates

The results of genomic DNA electrophoresis extracted from the isolates confirmed the success of the extraction process. A comparison of the length of genomic DNA extracted from selected isolates with a gene ruler (Ladder) showed that the size of the extracted sequences was more than 10 kbp. As shown in Figure 3, the integrity and structure of the genomic DNA are protected during extraction. Also, the results of PCR gene electrophoresis of 16S rRNA of selected isolates indicated the production of sequences with a length of about 1500 bp. These results showed that the 16S rRNA gene amplification process was performed specifically by the polymerase chain reaction (Fig. 4).

16S rRNA sequence matching assay of productive isolates

The 16S rRNA sequence Blastn analysis results showed about 97 to

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100% homology between 6 selected isolates with the type strains registered in the gene bank. The results showed that QH 26 strain had the highest homology with *Bacillus pumilus* strain ATCC 7061 strain at 100%. QH 30 isolate showed the highest homology at 99.88% with *Bacillus safensis* strain FO-36b. The 16S rRNA sequence of the QH 36 isolate showed 99.12% similarity with Vibrio alginolyticus strain ATCC 17749. Also, 99.88% similarity of QH 37 isolates with *Pseudomonas stutzeri* strain CCUG 11256 was confirmed. The results of this study also showed that QN 63 isolate had the highest similarity with *Vibrio alginolyticus* strain NBRC 15630 with 99.87%. While the 16S rRNA gene belonging to QN 45 strain showed the highest similarity with *Vibrio parahaemolyticus* strain ATCC 17802 strain at 100%.

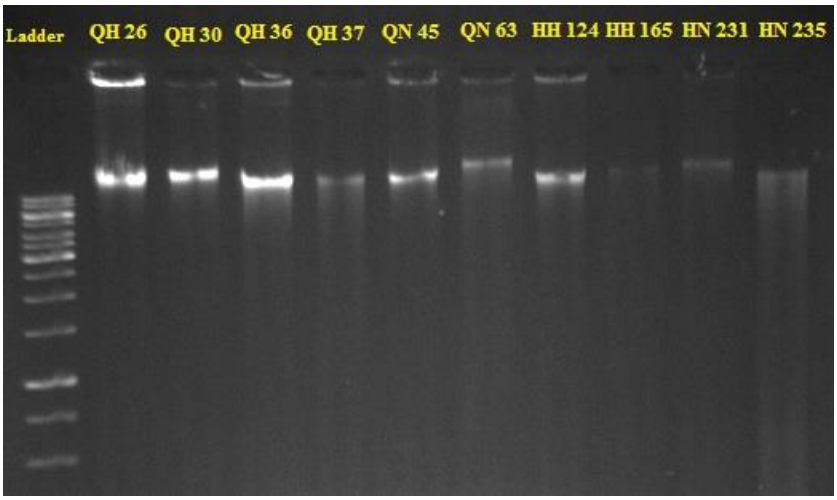


Figure 3: Genomic DNA electrophoresis extracted from selected isolates on agarose gel.

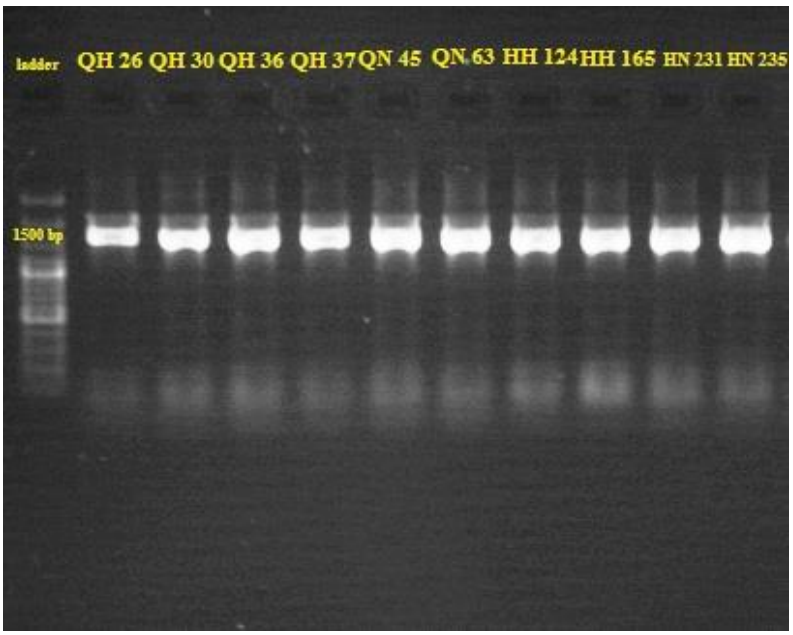


Figure 4: PCR product electrophoresis of 16S rRNA amplification of selected isolates.

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Phylogenetic analysis of productive isolates

The results of phylogenetic analysis between inhibitory metabolite-producing strains and the nearest index strains based on 16s rRNA gene sequence or using Neighbor-joining pattern showed that the strains in the phylogenetic tree drawn were located in 4 separate clusters. The QN 63, QN 45, and QN 36 strains were found in a common cluster, although the QN 45 strain formed a separate evolutionary pathway. In the second cluster, strains belonging to the genus Pseudomonas were placed, forming a separate evolutionary path from the other strains (Fig. 5).

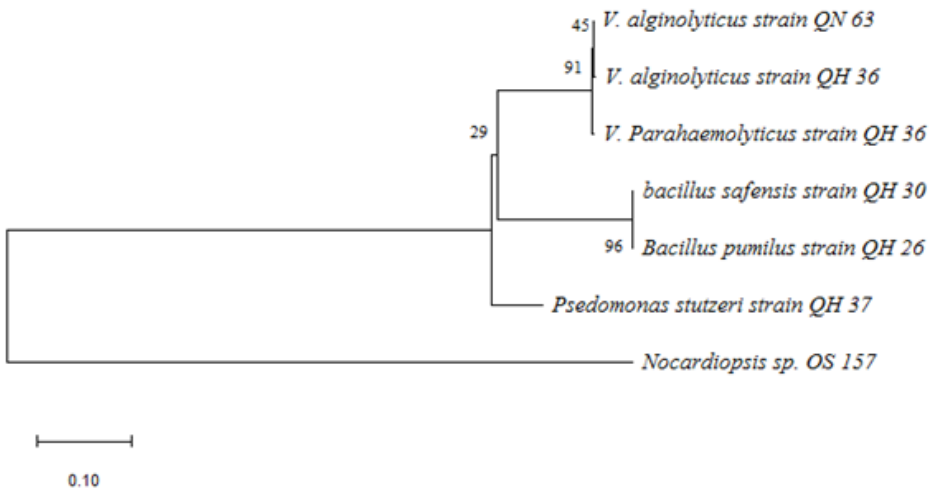


Figure 5: Phylogenetic tree of the potent strains based on Neighbor joining method. The numbers shown next to the nodes indicate the Bootstrap value. The scale bar indicates the replacement of 5 nucleotides per 100 nucleotides.

Discussion

In recent years, extensive programs have been designed and implemented to explore natural compounds, especially enzyme-inhibiting compounds from marine resources worldwide (Barzkar *et al.*, 2022). The present project was planned and implemented to isolate the bacteria that produce inhibitory compounds of enzymes related to diabetes. In this project, an attempt was made to isolate sponge samples from Qeshm Island. Bacteria associated with sea sponges have been prominent in microbial biotechnology and were screened for this purpose (Manivasagan *et al.*, 2015).

Examination of the biodiversity pattern of bacteria from two genera (*Haliclona* and *Niphates*) in both sampled areas showed that the samples belonging to the genus *Haliclona* hosted a more diverse range of bacteria. In *Haliclona* sponge samples, about 10 different genera of different bacteria were present, while in *Niphates* sponge samples, this diversity was limited to 6 bacterial genera. It is remarkable that the reported pattern is limited to culturable bacteria only with the

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isolation methods used in this study and cannot indicate bacterial diversity in the sponge microbiome, especially non-culturable species. However, to compare the diversity of culturable bacteria in the studied samples, the isolation techniques are significant.

In this study, *Vibrio* and *Bacillus* genera predominance in the studied samples was significant. In other studies aimed at isolating actinobacteria, the predominance of Streptomyces in sponge samples has been reported. For example, a study reported that 73.6% of the isolates obtained from the Hymeniacidon perceive sponge belonged to *Streptomyces* (Zhang *et al.*, 2022). Results of another study on the biodiversity of actinobacteria associated with the marine sponge Iotrochota sp. showed that members of the genus Streptomyces formed the predominant population of actinobacteria associated with this sponge (Jiang *et al.*, 2008). The results of a biodiversity study of actinobacteria associated with *Dendrilla nigra* sponge showed that Streptomycetes accounted for 23.91% of actinobacteria isolates (Selvin *et al.*, 2009). Achieving enzymatic activity-inhibiting bacteria is difficult, complex, and important in at least two respects. In the first aspect, the bacterial profile of sponges in different sampling locations varies according to various parameters, and in the second aspect, most bacteria are in vitro uncultivable (Schirmer *et al.*, 2005). Therefore, studying the discovery of natural products produced by bacteria that coexist with marine invertebrates is a promising risk. The biggest challenge for the systematic development of marine organisms' bioactive secondary metabolites industry is access to large-scale source organisms. Given the ease and possibility of sustainable drug production by microorganisms on an industrial scale, the achievement of symbiotic microorganisms as the main source of production of target metabolites presents a clear perspective in this area. The results of inhibitory activity of the extracted metabolites showed that 9 and 10, equivalent to 3.46 and 3.84% of bacterial isolates, respectively, inhibiting the activity of alpha-glucosidase and alpha-amylase inhibitors, were effective. Compounds that inhibit the activity of these enzymes can play an important role in reducing blood glucose uptake by inhibiting the activity of alpha-amylase or alpha-glucosidase enzymes as carbohydrate hydrolyzing enzymes. The IC50 activity of these metabolites ranged from 112.9 to 670.9 µg/ml for alpha-amylase. However, this rate has been reported for algal extracts sampled from the Persian Gulf, about 420 µg/ml to 7.5 mg/ml (Pirian *et al.*, 2017). Another study reported the IC_{50} inhibitory activity of metabolites extracted from *Streptomyces longisporoflavus* at 162.3 µg/ml (Akshatha *et al.*, 2014). In the present study, the toxicity test was used on human endothelial human umbilical cord cell lines to evaluate the toxicity of the compounds. The cytotoxic activity of the secondary metabolites extracted

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was assessed by MTT cell proliferation assay. This method has played an important role in studying new marine anticancer drugs in the last 30 years (Nga *et al.*, 2020). The results of cytotoxic activity showed that the secondary metabolites extracted from isolates QH 36 and QN 45 against the HUVEC cell line showed cytotoxic activity equivalent to 196.7 and 510.6 µg/ml, respectively. Therefore, these three bacteria will be excluded from future studies due to the toxicity of metabolites. The production of cytotoxic compounds by bacteria that coexist with sponges has been reported in various studies. Marine organisms, such as sponges, produce cytotoxic compounds to exert their defense strategies against predators and pathogens in their habitats. According to the theory of the microbial origin of some of the secondary metabolites of marine organisms, certain species of symbiotic bacteria must be permanently and specifically associated with the host organism and function (Sabrina Pankey *et al.*, 2022). According to the results of measuring the inhibitory activity of the extracted metabolites against alpha-glucosidase and alpha-amylase enzymes, the multiphase identification of the isolates was performed up to the strain level. Genetic identification of capable isolates was performed following the study of morphological, physiological, and biochemical characteristics of the obtained isolates. The results of genetic identification based on a comparison of 16S rRNA gene sequence showed that the potential isolates from the production of inhibitory compounds of the target enzymes had a 97 to 100% similarity with the index strains registered in the NCBI database. According to the results of multiphase identification tests, the results showed that most of the selected isolates are different strains from the index strains and can be further studied as new sources of bioactive metabolites. Phylogenetic analysis of selected strains with the closest strains was performed based on the 16s rRNA gene. The sequence of this gene is highly conserved and provides accurate information for species and genus differentiation (Abellan-Schneyder *et al.*, 2021). The phylogenetic analysis results based on the Neighbor-joining distance method indicated the existence of 4 separate clusters. It seems that evolved strains distinct from their counterparts in adapting to the conditions of their microenvironments within the host have undergone mutations. Of course, these mutations do not only target the 16s rRNA evolutionary genes and may be involved in the other biosynthetic genes. Therefore, it is possible to alter the biosynthesis pathways of secondary metabolites. The present study led to a new understanding of the biodiversity of cultivable bacteria associated with sponge communities in the Persian Gulf around Qeshm Island. It also showed the potential inhibitory activity of metabolites produced by these bacteria against alpha-amylase and alpha-glucosidase enzymes. These results can further prove the ecologically active

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role of significant sponge-related bacterial populations. The present study presented an efficient strategy for isolating bacteria from marine sponges with different culture media. The result of this study was the acquisition of 7 strains of bacteria that produce inhibitory compounds against enzymes involved in diabetes. The results of this study confirmed the non-toxicity of the extracted metabolites against normal human cells. Therefore, if the pharmacological tests are approved, the mentioned strains can be used as a candidate to study the native sources of anti-diabetic drugs.

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