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# Identification, Screening, and Molecular Characterization of Bacterial Microbiota in the guts of *Epinephelus* sp.

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## Abstract

Bacterial microbiota is predominantly present in all living organisms. Most of the bacteria present in the gut of the fish are contaminating the food chain. In the present study, we aimed to isolate and characterize the bacteria in the gut of *Epinephelus* sp. in the red sea of Jeddah, Kingdom of Saudi Arabia. Bacteria were isolated from the guts of 10 fish samples and were grown on Luria Bertani (LB) and nutrient agar media. Total thirteen bacterial colonies were screen out by morphological identification i.e., color, shape, structure, etc. which were further reduced to 7 colonies e.g., IF001, IF002, F003, IF004, F005, IF006, and IF007. The bacterial isolates were also identified through molecular identification using 16S-rDNA sequencing. The genomic DNA was isolated and was sequenced using the Sanger® sequencing method. BLAST alignment results that IF001 and IF002 were members *Bacillus* sp. IF003 was a strain of *photobacterium damselae*, IF004 and IF006 were strains of *Rothia endophytica*, IF005 was a strain of *Acinetobacter bouvetiiand* IF007 was belonged to *Shewanella oneidensis*. The molecular identification confirmed the identification of bacterial isolates in the *Epinephelus* sp. obtained from the red sea.

**Keywords:** *Epinephelus* sp., morphological identification, molecular identification, bacterial microflora.

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## 1. INTRODUCTION

Aquatic organisms have a unique environment, which has different characteristics as compared to the terrestrial environment [1]. The marine environment is yet very different from the freshwater aquatic environment with different dynamics of food and energy. The marine animal also has adaptation and that particular environment. Microbes play a very important role in marine life especially bacterial microflora has a very significant role [2]. Normally it is extremely difficult to identify bacteria from deep sea; however, the bacteria present in the gut of the marine fish are accessible to get a profile of the marine bacteria [1]. These bacteria also show different types of bacteria present in marine animals and their effect can be analyzed in them [2].

It has been estimated that $4 \times 10^{30}$ cells of the microbes are present in marine water, which makes up to 91% of the total marine biomass [3]. The fish-microorganisms association can be either pathogenic or mutualistic [4]. Bacteria present in the gut of fish play a significant role in metabolic activities, food supply, and immune response same as bacteria play in humans and other organisms[3]. Fish has been an important source of food for a human being for millions of years and even now, up to millions of people all over the world use fish as a staple food. Up to 20% of the protein intake is fulfilled by fish consumption throughout the world[4].

Isolation of bacteria from the fish received significant attention at the beginning of the 20th century [5]. However, recently it drew utmost attention due to lots of studies in the field of microbiology which expanded the sector of aquaculture. Analysis of the microbes within the fish demonstrated that bacterial species vary in different types of fish [4]. Most pathogenic bacteria are present in the gut of the fish which came from environmental pollution and it deposited in the fish and cause foodborne diseases in the human being [5]. The bacterial consortium gets very query in the fish from species to species [6]. A different genus of bacteria are present in the different genus of the fish, such *Microbacterium oxydans*, *Staphylococcus Arlette*, *Staphylococcus warneri*, *Methylobacterium persicinum,* and *Achromobacter xylosoxidans* have been identified in fish[7]. Types of the bacteria depends on the environmental condition, types of food gene expression, temperature, and many other factors in each officially [6]. The profiling of the bacteria not only shows us the bacterial population within the fish but also gives us an idea about the geological conditions of that area. The bacterial profile also tells us the biological pollution in the form of pathogenic bacteria present in sea [7].

Overall, our current study was based on the screening and identification of bacteria from *Epinephelus* sp., fish commonly consumed in the Jeddah region. During this study, the *Epinephelus* sp. was collected from the market in Jeddah. The bacteria were isolated from the gut of the fish and identified morphologically as well as through DNA sequencing.

## 2. MATERIALS AND METHODS

### *2.1 Collection of sample and surface sterilization*

Ten fish samples of *Epinephelus* sp. were collected from Jeddah fish market, Jeddah Saudi arabia and was carried to the laboratory in an ice bucket. Each sample was surface sterilized by immersion in 70% ethanol for 2 min. The gut of the fish was aseptically dissected from the animal's musculature. The separated gut was cut into three parts e.g., upper, mid, and lower gut, and placed into a 20 mL sterile phosphate-buffered solution.

### *2.2 Isolation of Bacteria from the gut*

Bacterial samples were isolated from the upper, mid, and lower gut, of the *Epinephelus* fish. Two types of media were used The Luria-Bertani (LB) agar: NaCl (1 g), yeast extract (0.5 g), tryptone (1 g), and agar (1.5 g) in 100 ml $H_2O$ was prepared as autoclaved. The nutrient agar: Peptone (0.5 g), NaCl (0.5 g), Yeast extract (0.1 g) and agar (1.5) was prepared in 100 mL for bacterial isolation. The upper, mid, and lower gut were surface sterilized using 70% methanol for 2 min. An autoclaved cotton bud was inserted into the guts and spread over the LB agar plate. The plates were then incubated at 37°C until the colonies of mixed cultures bacteria appeared. The colony-forming unit (CFU) was determined from the colonies that appeared on plates. The number of bacterial colonies was noted as CFUs from each swab and expressed as CFU.

### *2.3 Purification of bacteria*

The most common method to separate the single colony of the bacteria from the mixed culture is the streaking plate method. The mixed plates were carefully observed and the colonies which had to be separated were marked on isolation plates. The bacterial colonies were morphologically identified by shape, size, color, structure, texture of the colonies. Different colonies were streaked separately on LB agar plates. The plates were then incubated at 37°C until the homogenous colonies of bacteria appeared on the media.

### *2.4 Polymerase chain reaction*

Total genomic DNA was extracted from the isolates as described by Ullah et al. [8] and was subjected to PCR reaction. Universal PCR primers (27b F and 1492u R) were used to amplify the 16s-rDNA. The 50 ul PCR reaction mixture was made according to the protocol described by Ullah at al [8]. The PCR conditions were optimized for 40 cycles (95°C for 1 min, at 55°C for 30 s and 72°C for 1 min). The PCR purification kit was used to purify the PCR product and the product was sent to Macrogen, South Korea for sequencing.

### *2.5 Bioinformatical and phylogenetic analysis*

Sequences of the 16S-rDNA were aligned through BLAST using NCBI database [7]. E-value obtained from BLAST provides a base to identify the genus as well as species of the bacterial isolates. The phylogenetic trees of the identified strains were constructed by pairwise alignment tools. Genetic analyzes of isolates were performed using genetics using tools such as for multiple-sequence alignments and Gblocks to care for sequences [4, 8, 9].

## 3. RESULTS AND DISCUSSIONS

### *3.1 Bacterial colony counts on different media*

The bacterial isolates from the gut of the *Epinephelus* fish have grown two different media; nutrient agar media and LB media. The bacterial colonies grown on LB agar plats were noted. The results showed that sample 1 to sample 3 produced colonies$10^8$cfu. Sample 4, 5 8 produced colonies$10^9$ CFU, and samples 7, 9, and 10 produced $10^5$ CFU.

The bacterial isolates were also grown on nutrient agar plates. The bacterial colonies in the were counted on nutrient agar plates and the results showed that samples 1, 4, 5, and 6 produced $10^6$cfu. Sample 2, 3, and 10 produced $10^7$ CFU, and samples 7, 8, and 9 produced $10^5$ CFU.

**Table 1.** The CFU produced by bacteria isolated from the gut of *Epinephelus* sp. on LB agar and nutrient agar plate

| No. of Samples | CFU in LB Agar plates | CFU in Nutrient Agar plates |
|---|---|---|
| S 01 | $221 \times 10^8$ | $721 \times 10^6$ |
| S 02 | $201 \times 10^8$ | $415 \times 10^7$ |
| S 03 | $321 \times 10^8$ | $415 \times 10^7$ |
| S 04 | $512 \times 10^9$ | $721 \times 10^6$ |
| S 05 | $512 \times 10^9$ | $721 \times 10^6$ |
| S 06 | $256 \times 10^5$ | $721 \times 10^6$ |
| S 07 | $256 \times 10^5$ | $256 \times 10^5$ |
| S 08 | $512 \times 10^9$ | $256 \times 10^5$ |
| S 09 | $256 \times 10^5$ | $256 \times 10^5$ |
| S10 | $256 \times 10^5$ | $415 \times 10^7$ |

The results showed that bacterial growth was higher on LB agar plates instead of nutrient agar plates (Fig. 1). Large amounts of microorganisms in fish guts provide a basis for evaluating dangerous levels of pollution in marine waters [9]. It also gives us an idea of the possible health risks of seafood that can be dangerous to

human health. The bacterial community is very complex, so it is very difficult to isolate bacteria in a single medium [10]. Due to the complexity of the culture mechanism, very few bacteria could be cultured in the laboratory and taxonomically identified [11].

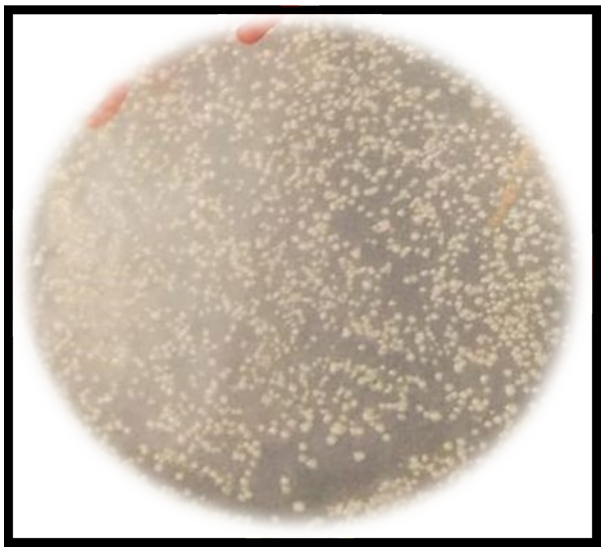

**Fig. 1.** Colonies of bacterial isolates from the gut of the fish

Multiple means were used to identify and isolate bacteria from fish, especially marine fish, but in the current study, we used different types of nutrient agar and LB agar plates. A bacterial load can be affected by several factors, including environmental and genetic factors. Different media contain different combinations of nutrients for salts and many other things that can affect bacterial growth and especially the concentration of NaCl has a significant effect on the bacterial population, but there is no prior record as there is comparative data with the study. A previous study measured the number of heterozygous bacteria in seawater between $10^5$-$10^6$ CFU and a total coliform count of $10^2$ CFU. [12] [10] (Turki and Madris, 2008). (From Culture-dependent bacteria in commercial fishes)

### *3.2 Morphological Screening of isolates*

To obtain a pure bacterial culture is the first step to bacterial identification. A pure culture is essential in the study of the morphology, physiology, biochemical characteristics, and susceptibility to antimicrobial agents of a particular bacterial strain. In the present study, bacterial isolates were grown upon the nutrient agar, and LB agar plates were examined. The examined for color, shape, structure, texture (Fig. 2A). One colony was selected and isolated from the mixed culture plate by the sterilized toothpick and stabbed on LB agar media (Fig. 2B). The plates were incubated at 37°C for 24 h. Thirteen colonies were screened in the first round. However, there was a duplication in the colonies, therefore; the second round of purification was carried out. One colony from the duplications was separated on a fresh plate.

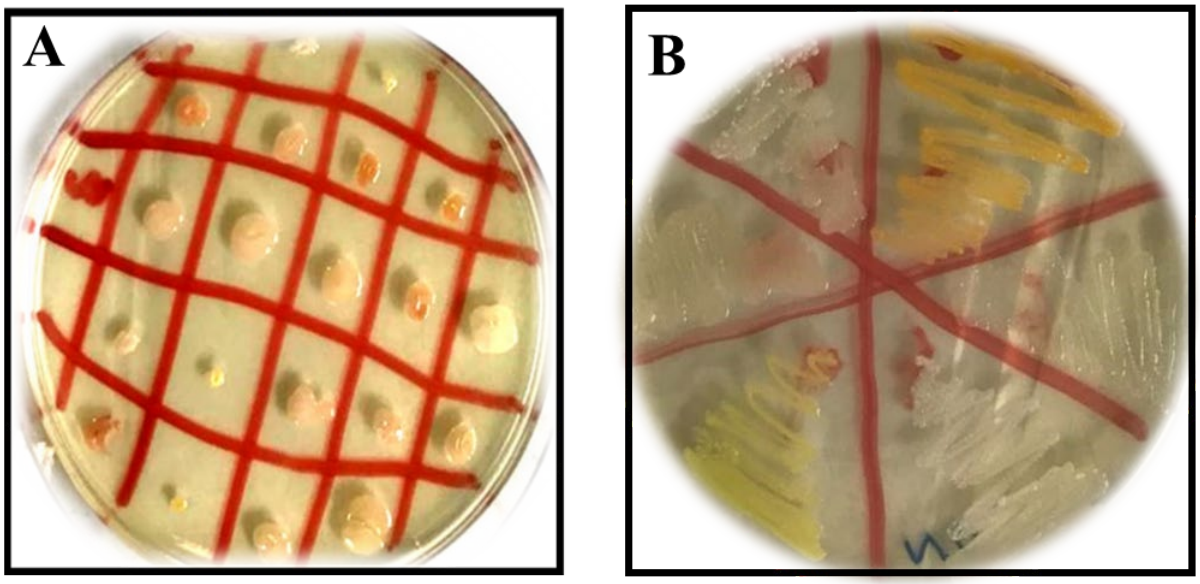


**Fig. 2.** Purification of bacterial colonies. (A) Screening of colonies based on shape, structure, texture. (B) Streaking of a single colony

The identification of a bacterial pathogen is important in fish diagnosis [11]. Treatment could be implemented only after the causative agent or the bacterium has been identified. Bacterial species differ in morphological, physiological, and biochemical characteristics and those can be used when coding or labeling them [13].

### *3.3 Molecular identification and phylogenetic analysis*

Sequences of the bacteria isolates were aligned with the available sequences in the NCBI database using BLAST [14]. The phylogenetic trees were constructed using neighbor-joining. Results of the BLAST sequences search indicated the isolate IF001 and IF002 had 98% sequences similarities with *Bacillus* bacteria. Based on the results of sequence homology and the phylogenetic analysis, bacterial isolate IF001 and had IF002 were identified as *Bacillus* safeness-IU001 and *Bacillus* sp. IF002 respectively (Fig.3).

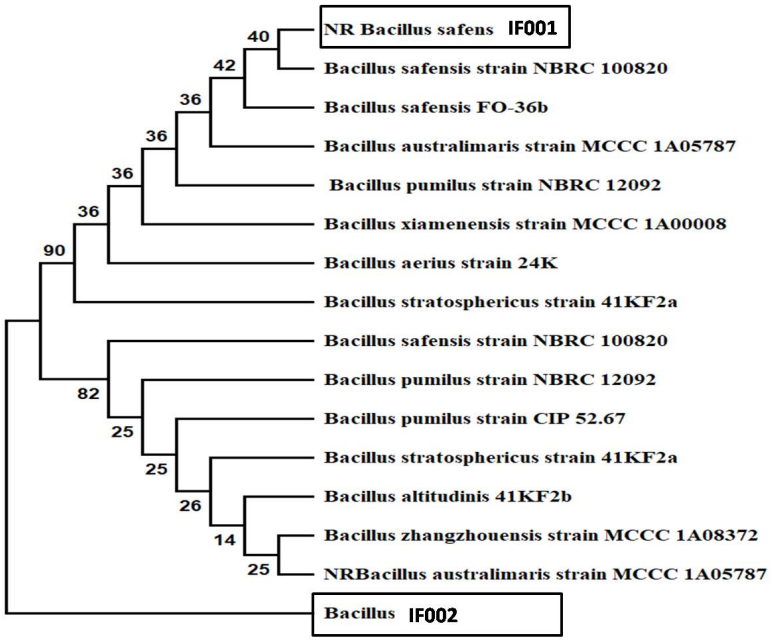


**Fig. 3:** Phylogenic analysis for identification of IF001 and had IF002, the tree was constructed based on percent similarity with related species

Members of the *Bacillus* bacteria are very important as because they offer immunostimulatory abilities to the host. They also produce different types of metabolites including antibiotics that are beneficent against pathogens.[15 8 16]

The third bacteria (IF003) isolated from the fish gut was identified as a member strain of *photobacterium damselae* because it showed 99-97% nucleotide homology. As shown in the result (Fig. 4) a neighborhood-linked phylogenetic tree was generated based on the 16S-rDNA gene sequence. *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damsela*) is a pathogen of a variety of marine animals including fish, crustaceans, mollusks, and cetaceans.

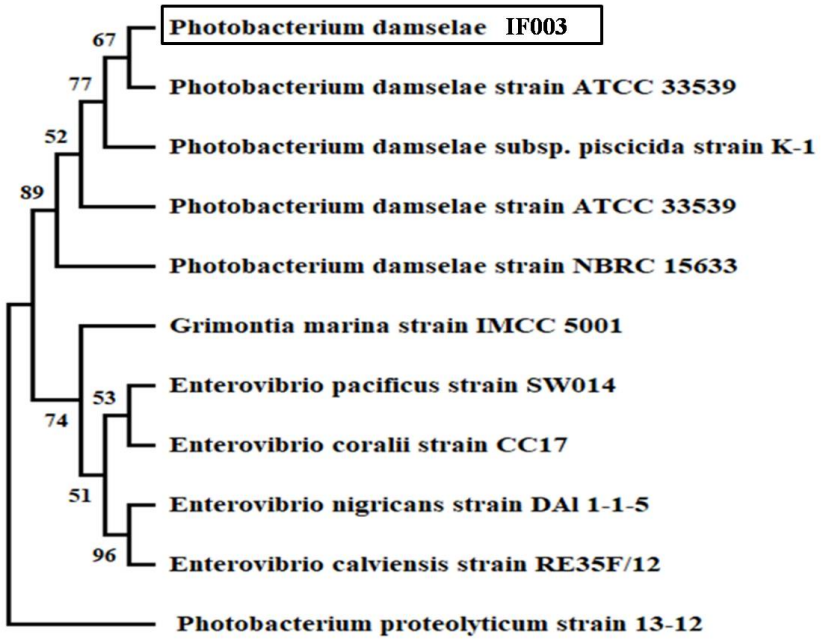


**Fig. 4:** Neighborhood-linked phylogenetic tree of IF003 was generated based on the 16S-rDNA gene

The *Photobacterium damselae* subsp. *damselae* has been identified as the primary pathogen of several species of the fish. These fish are economical and ecologically very important. It causes hemorrhagic septicemia infections in wild fish. Besides the wild species, cultivated species have also been reported to be infected by *P. damselae* [5 7].

The 16S-rDNA nucleotide sequences of IF004 and IF006 showed the highest sequence similarities with *Rothia endophytica* (<98%), followed by *R. terrae and R. mucilaginosa* (Fig. 5A and 5B). Bacteria such as *R. mucilaginosa* and *R. dentocariosa* have been reported to be consisted of about 2.6% and 3.4% in saliva respectively [11]. In addition, *R. aeria* has also been part of the normal flora in the human oral cavity indicated. These studies indicated that *Rothia* sp. Are normal flora of human oral cavity [13 11].

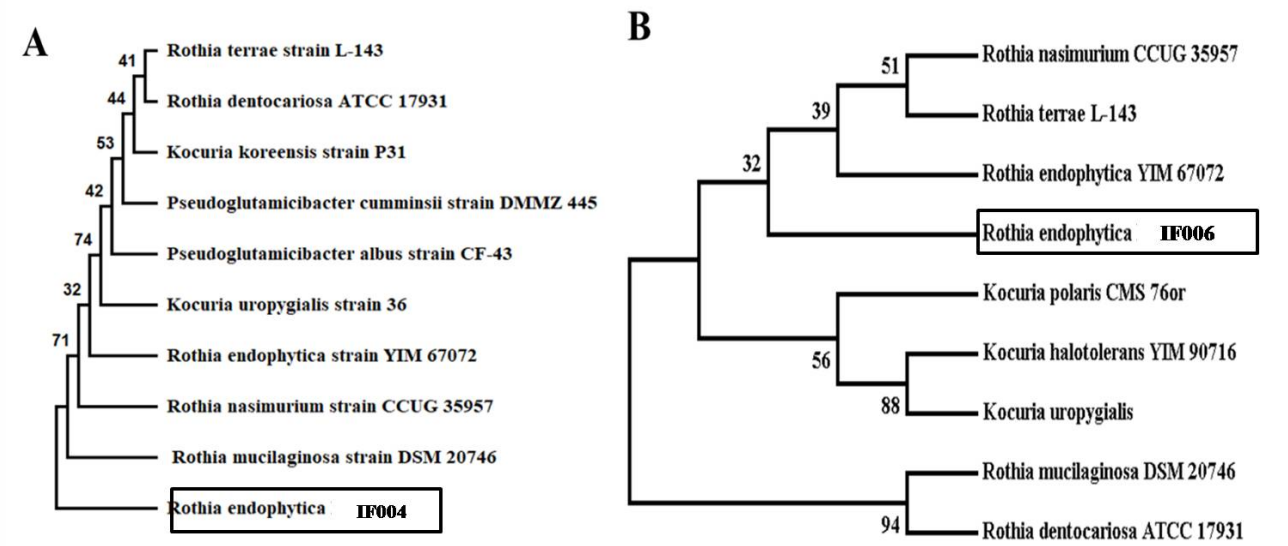


**Fig. 5:** Phylogenic analysis (A) Identification of IF004 (B) Identification of IF006. The tree was constructed based on percent similarity with related species.

Similarly, IF005 showed nucleotide sequence similarity with *Acinetobacter bouvetii* (98%) followed by other species of the *Acinetobacter* genus [1].

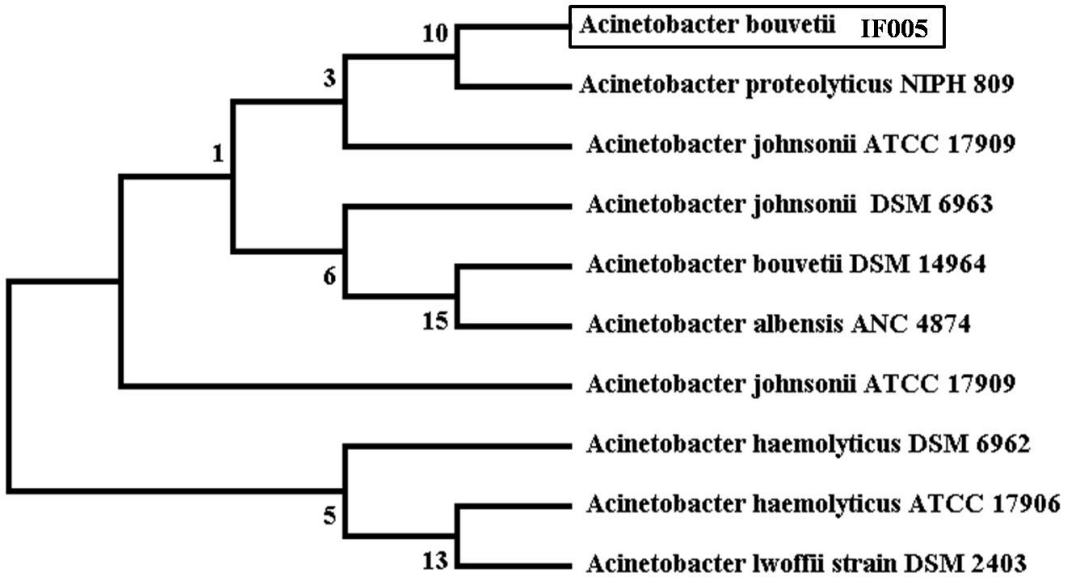


**Fig. 6:** Phylogenic analysis of IF005, identified as a member of *Acinetobacter*. The tree was constructed based on percent similarity with related species.

The nucleotide sequence of IF007 was subjected to BLAST, which showed similarity with species of *Shewanella*. The maximum similarity was shown with *Shewanella oneidensis* so the strain was named *Shewanella oneidensis* IF007 (Fig. 7). *Shewanella oneidensis* was first isolated from the deep-sea; however, it is present in seashore and marine animals including fish [17]. The entire genome of *Shewanella oneidensis* has been sequenced by The Institute for Genomic Research (TIGR) in Rockville, Maryland, and La Jolla, California [9].

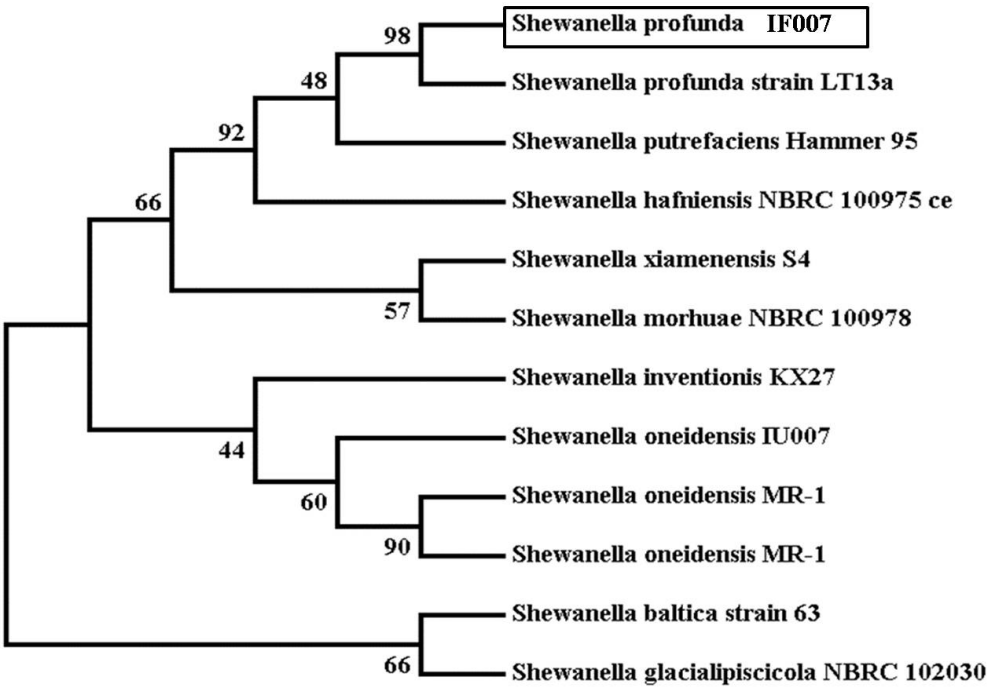


**Fig. 7:** The phylogenetic tree of IF007, was generated based on the 16S-rDNA gene.

Based on the previous results, 16S-rDNA genes were considered an effective method for obtaining an association between the determination of classification and the degradation of hydrocarbon capacity [9]. More light was added to our findings by Subathra et al. [16] applying both biochemical and evolutionary methods to identify 3 biodegradable crude oil bacteria such as *Bacillus subtilis* [13], *Pseudomonas aeruginosa* I5, and *P. putida* I8. Furthermore, our obtaining results for the application of biochemical tests and 16S-rDNA sequencing to identify oil-degrading bacteria was with the approval of [8], [15]. The bacteria such as *Brevibacillus* sp., *Microbacterium oxydans*, *Staphylococcus Arlette*, *Staphylococcus warneri*, *Methylobacterium persicinum,* and *Achromobacter xylosoxidans* were identified in the light of the results of biochemical and molecular identification [11].

## 4. CONCLUSIONS

Bacteria from the gut of *Epinephelus* sp. of the red sea were isolated and characterized. The bacteria were isolated on two different media, LB agar media, and nutrient agar media. Total thirteen bacterial colonies were screen out initially which were further reduced to 7 e.g., IF001, IF002, F003, IF004, F005, IF006, and IF007 by morphological identification. The molecular identification showed that the isolates were member *Bacillus* sp., *photobacterium damselae*, *Rothia endophytica, Acinetobacter bouvetii*and, and *Shewanella oneidensis.*

## ACKNOWLEDGMENT

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## CONFLICT OF INTEREST

There is no conflict of interest among the authors regarding this study.

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