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RESEARCH ARTICLE

Bio Prospecting of Novel Moderate Halophilic Bacterium *Marinobacter* spp. JS17 Recovered from the West Coast of Karnataka, India

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Abstract

Background: Halophilic bacteria are known for their bioactive molecule production and their unique characteristic features, especially broader salinity, pH and temperature tolerant abilities. **Aim:** To bioprospect the novel moderate halophilic bacterium *Marinobacter* spp. JS17 recovered from the west coast of Karnataka, India. **Methods:** A moderate halophilic bacteria was isolated from the Arabian Sea in Karnataka, India and selected for further studies. As part of the bioprospection, we have carried out the all required morphological, biochemical, enzyme production, and antibiotic resistance potential activities. Molecular identification especially 16S rRNA gene sequencing and phylogenetic studies were done. **Results:** The strain can able to grow in various concentrations of salts ranging between 0-20% (w/v), the optimum salt required is 10%(w/v), pH ranges from 5-10, and a broad range of temperatures 20-50°C, evident that the halophilic bacterium belonged to an extreme tolerant group. It was discovered that the strain was capable of producing extracellular enzymes like amylase, lipases, esterase, and proteases. Antibiogram investigations have revealed the strain's ability for multidrug resistance, which establishes the significance of its prospective medical application. Interestingly the strain showed considerable resistance against broad-spectrum antibiotics such as ceftazidime (30mcg/disc), cefotaxime (30mcg/disc), colistin (10mcg/disc) and neomycic. **Conclusion:** *Marinobacter* has indicated its multidrug resistance and multi-extracellular enzyme production capabilities. The isolated bacterium was identified as *Marinobacter* spp. JS17 and the accession of HQ834852were obtained after the gene sequence was deposited in the NCBI platform. 16S rRNA gene sequence examination along with the phenotypic, biochemical, and genotypic data have revealed that the isolated bacterium possesses versatile characteristic features and novel taxon within the *Marinobacter* genus with only 95% BLAST sequence similarity.

Keywords: Antibiotic Sensitivity, Biotechnological Application, Biomedical Applications, Exopolysaccharides, Extracellular Enzymes

1. Introduction

It is a well-known truth that most of the earth's surface is occupied by the ocean (70%), and the sea generally comprises an enormous number of microbes. Such surroundings always acquire distinguished environmental conditions such as higher salinity, alkalinity, higher-lower temperatures, low light, and higher pressure. However, in recent years the isolation of potential novel organisms is one of the fascinating areas of research. Therefore, these new types of microbes inherently carry novel industrially essential compounds. Due to the high adaptability ability of marine bacteria to the hostile environmental

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conditions of pH, temperature, salinity, alkalinity, and pressure, they are gaining a particular focus[1]. These hostile ecological conditions are known as extreme environments.

These halophiles usually develop two crucial strategies to endure well under the adverse conditions of higher salinity[2-4]. These bacteria accumulate a significant quantity of inorganic ions such as K^+, $Na^{+,}$ and Cl^- ions within their cytoplasm, and these ions help to maintain the osmotic pressure exerted because of excess salinity. In addition to this, halophiles can accumulate important osmolytes, mainly osmoprotectants (ectoine, glycine betaine, *etc.*) inside their cytoplasm. These osmolytes play a vital role in balancing the osmotic imbalance that occurs during the healthy metabolism of the cells in the presence of high salts[5].

The extreme environmental conditions are expected to produce potential novel consortia of microbes concerning the unacquainted functional genes possessing biocatalytic activities[6]. These enzymes that were recovered using extremophiles are extremozymes that have advantages over mesophilic organisms-derived enzymes. They can sustain harsh industrial processes such as elevated pH, higher salt concentration, temperature, and activity at non-aqueous medium[7,8]. Many researchers have revealed the diversity, ecological importance, and systematics studies on halophiles. Although there is a vast biotechnological potential of halophiles, they have been underutilized. It has created an enormous gap in understanding and harnessing these organisms for extracellular hydrolytic enzyme production. Subsequently, the hydrolysis of various biopolymeric constituents carried out through the catalytic processes of these enzymes plays a crucial role in the geochemical recycling of organic nutrients in the saltern environments.

The present research effort towards the isolation of novel *Marinobacter* spp. and screened mainly for various extracellular enzymes such as amylase, lipase, esterase, proteases, and inulinase. The listed enzymes have proven biotechnological applications, as many researchers and Izadpanah *et al.*,[9] reports have endorsed it. Therefore, the enzymes can be utilized in many industrial applications, such as in producing food, feed, detergents, leather, biofuel, and pharmaceuticals.

High molecular weight carbohydrate biopolymers that are frequently secreted by cells into the extracellular milieu are known as bacterial exopolysaccharides or EPSs. EPSs serve a variety of beneficial purposes for bacteria. Biomedical applications have previously made use of some bacterial EPSs with useful physicochemical features. For example, among all the EPSs that have been discovered, Bacterial Cellulose (BC) has been the subject of the most research as a material for wound dressings, dental implants, and temporary skin substitutes, which has resulted in the commercialization of biomedical products[10]. By the end of 2025, the global market for microbes and BC is expected to have grown from its 2017 valuation of US$ 250 million to US$ 680 million[11]. An additional EPS that is exhibiting a rising market value is xanthan gum, whose global market was estimated to be worth approximately US$ 1 billion. The morphological, biochemical, molecular analysis and phylogenetic studies have revealed the isolated bacteria belonged to the novel species within the *Marinobacter* genera and there is a huge scope of such organisms in biomedical applications.

2. Materials and Methods

2.1 Isolation of the Bacterial Culture

The isolation halobacterial culture was executed[12]. The medium used in the separation and maintenance of the strains consists of 10g Yeast extract, 5g Tryptone, 5g Casaminoacids, 3g sodium citrate, 2g KCl, 20 g Magnesium sulfate ($MgSO_4.7H_2O$) a final volume of 1L was maintained by adding distilled water, pH adjusted[2] to 7.2. All the experiments concerning the morphological (grams staining, pigmentation, motility, spore-forming tests), biochemical (fermentation of various sugars) and hydrolytic enzyme activities (amylase, protease, lipases, esterases, inulinases) were carried out in a medium containing 10% NaCl and at 37°C.

2.2 Identification of *Marinobacter* spp. JS17

Genomic DNA extraction was done[1] and PCR experiment was carried out using an Applied Biosystems model 9800 and sequencing analysis was achieved by the other publications[12,13].

2.3 Extracellular Enzymatic Activities

Later stage, the isolate *Marinobacter* undergone to screen various extracellular enzymes mainly protease, amylase, lipase, inulinase, and esterase with the help of listed plate assay methods as detailed.

2.3.1 Proteolytic Activity

The production medium consisted of skim milk agar; The protease activity of *Marinobacter* spp. was tested using a medium containing skim milk (10%), agar (2%), and NaCl (10%). Inoculation was done and the Petri plates were incubated in temperatures ranging from 20-50ºC. A zone of hydrolysis around the microbial colony can ensure proteolysis activity[12,13].

2.3.2 Extracellular Amylase Production

Determination of amylolytic activity was performed according to Yaradoddi e*t al*[2]. The strain was inoculated to grow on the medium consisting of 10% salts and starch (1%). A zone of hydrolysis around the inoculation after 168 h of incubation at temperature 37ºC, after flooding with 0.3% I_2- 0.6% KI solution can be considered a positive test for amylase production.

2.3.3 Extracellular Lipolytic Activity

The qualitative plate assay of lipolytic activity was detected by growing the strain in plates containing saline medium supplemented by 1% Tween-80, inoculated plates were incubated for about 48 h. The assay for lipolytic activity cell supernatant was used to determine by the spectrophotometer, with p-nitrophenol esters as the main substrate. Separation in the p-nitrophenol esters was estimated at temperature 37ºC and by adding 0.1 M phosphate buffer (pH 7.5), as per the reports of Winkler and Struckmann[14].

2.3.4 Extracellular Esterase Activity

The esterase activity of the bacteria was determined using a plate assay method. The plate containing 10g/l of Yeast extract, 20g/L of $MgS0_4.7H_2O$, 2g/L of KCl, 0.1g/L $CaCl_2.H_2O$, 10% of NaCl, 20g/L of agar, and the medium supplemented with 0.1% Tween-80 as the primary substrate. Plates were inoculated and incubated at 20-50ºC temperature. The occurrence of a hydrolytic zone surrounding the inoculation determines a positive test for esterase activity[12].

2.3.5 Inulinase Activity

Screening of *Marinobacter* species for the production of inulinase enzyme was analyzed by growing the isolated strain in the medium consisting of: NH_42SO_4 0.5 g l^{-1}, $MgSO_4$. $7H_20$ 0.2 g l^{-1}, inulin 2g l^{-1}, KH_2PO_4 3 g l^{-1} and agar 20 g l^{-1}, which was supplied with 20% of NaCl as the organism is halophile. However, the medium's sole carbon source is the substrate inulin, therefore growth of the bacteria after 48 hours at 37°C will demonstrate their inulinase activity.

2.4 Antibiotic Sensitivity Test

The antibiotic susceptibility of the isolated bacterial culture was examined via the spread plate method, where the suspension culture of the *Marinobacter* species grown on Halobacterium medium (ATCC medium 213) with 10% salts (NaCl), further antibiotic discs were inserted onto the solid media. The strain was allowed to grow at 30°C temperature for about 24 h. Further, we have observed plates and recorded the results in terms of sensitivity or resistance based on the zone of inhibition with a minimum measurement of about 2 mm surrounding the pre-inserted discs containing antibiotics[15]. The different antibiotics used and their concentration are given in Table 2.

3. Results

3.1 Bacterial Identification

Isolated *Marinobacter* was observed to be a Gram-negative, non-spore-forming cocci, anaerobe, catalase-positive, catalase, oxidase-positive, and citrate utilization was found to be negative (Table 1). This morphological, biochemical and molecular systematic indicated the marine microbe related to the *Marinobacter* and later it was deposited as *Marinobacter* spp. JS17. Specifically, which was showing 95% similarity within a reported genus, possibly novel species under the same genus. However, this needs to be confirmed by the DNA-DNA hybridization method. Meanwhile, the nucleotide gene sequence obtained was deposited in the GenBank under the accession number HQ834852.

3.1.1 Screening for Protease, Amylase, Lipase, Esterase and Inulinase Activities

The saline medium was supplemented with a specific substrate like skim milk powder, starch, tween-80/

rhodamine, tween-80, and inulin, respectively, in the determination of hydrolytic activities. Throughout the process, the isolated strain has shown potential enzymatic activities of protease, amylase, lipase, and esterase and confirmed by observing the zone of hydrolysis around the microbial colonies (Figure 1, Table 3). However, the isolate could not be able to produce inulinase. Most importantly, the isolated organism could withstand up to 50°C temperature, and despite the harsh conditions, the strain has shown multiple enzymatic activities. Moreover, the strain can also grow well and able to produce enzymes even at 10% salts (NaCl).Itis a much-desired result due to utmost industrial techniques generally held at extreme environments such as in higher temperatures, pH, salinity, heavy metals, and solvents[16]. Whereas most of the organisms fail to sustain their lives, and the production of metabolites is too far away.

Despite the substantial biotechnological potential of these halobacteria, they have been least explored until today. The west coast of the Arabian Sea, India inhabited by a wide variety of halophilic bacteria and these bacteria are not only able to produce hydrolytic enzymes, but they are also important sources of bioactive compounds such as antibiotics, which could have versatile applications against various pathogenic microbes.

The recent investigation also has revealed the morphological, biochemical, partial 16S rRNA gene sequences; and phylogenetic features of the isolated bacterium. The tree indicated the phylogenetic position of *Marinobacter* spp. since it belonged novel group of bacterium so form the out-group among the compared bacteria (Figure 2). These *Marinobacter* species and halophiles have greater potential in various biotechnological applications as they can be employed in bioplastic production, bioremediation, bioaugmentation and biodegradation of complex polymers[17-26], *etc.*

Table1. Morphological and biochemical features of *Marinobacter* spp. JS17

S. No.	Attributes	*Marinobacter* spp. JS17
1	Pigment production	Light green
2	Spore feature	Non sporulating
3	Gram's staining	Gram "-"ve, cocci
4	Bacterial motility	Motile
5	Temperature range	20-50°C
6	Salt tolerance	0-20%
7	pH	5-10
8	Citrate permease	-
9	Oxidase	-
10	Catalase	+
11	Sugar utilization	
	Mannose	+
	Sucrose	+
	Glucose	+
	Raffinose	-
	Fructose	+
	Galactose	-
12	Malonate utilization	+

Table 2. Resistance exhibited by *Marinobacter* spp. JS17 against the various antibiotics used

S. No.	Antibiotics Disc	Conc. of antibiotic (mcg/disc)	Results
1	Ceftazidine (CA)	30	R
2	Cephotaxime (CC)	30	R
3	Ampicillin (A)	10	S
4	Chloramphenicol (CP)	30	S
5	Cefepime (CPM)	30	R
6	Colistin (CL)	10	R
7	Neomycin (N)	30	R
8	Ciprofloxacin (CF)	10	S

3.2 Antibiogram

The antibiotic sensitivity profile was carried out to check the multidrug-resistant capacity of isolated strains and which has revealed some interesting facts.

Table 3. Hydrolytic enzyme activities of **(A)**amylase, **(B)**protease, **(C)**esterase, **(D)**lipase by the halophilic bacterium *Marinobacter* spp. JS17

Hydrolytic activities of enzymes	*Marinobacter spp. JS17*
Protease	Positive (B)
Amylase	Positive (A)
Lipases	Positive (C)
Esterase	Positive (D)
Inulinase	Negative (-)

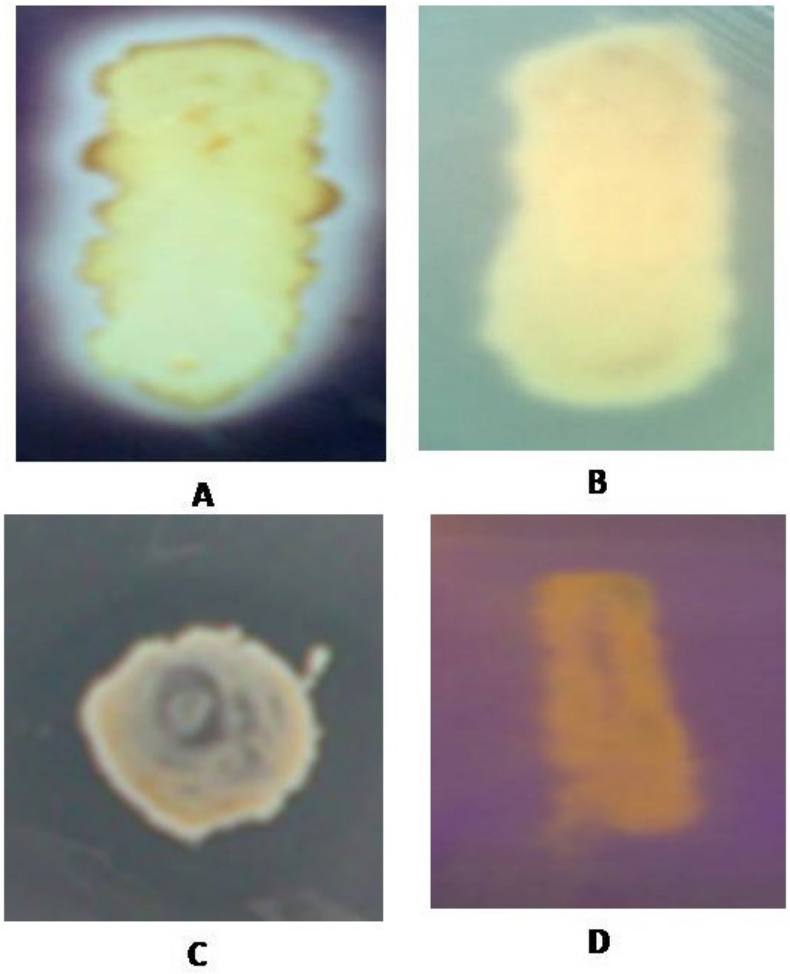


Figure1. Hydrolytic enzyme activities of amylase **(A).** protease; **(B).** esterase; **(C).** lipase; **(D)**. by the halophilic bacterium *Marinobacter* spp. JS17.

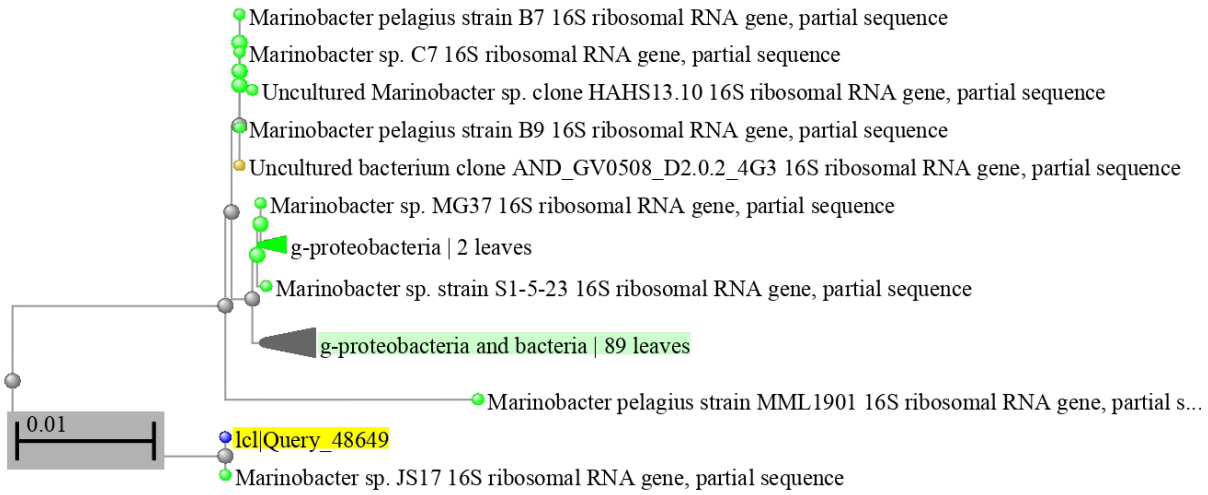


Figure 2. The phylogenetic tree indicated the relationship of *Marinobacter* spp., JS17 with gram-positive halo bacteria obtained in a tree constructed using the BLASTn tree platform. This has shown the outgroup among the compared BLASTn results, perhaps determining its novelty.

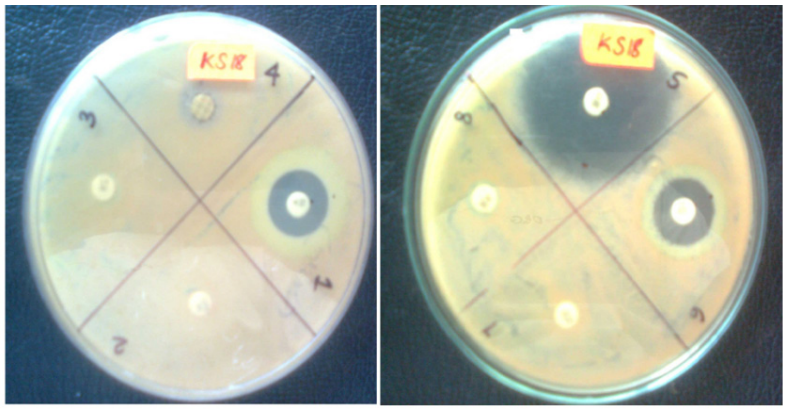


Figure 3. Indicating *Marinobacter* spp. strain JS17, **(A).** Showing susceptibility towards(1) Ciprofloxacin, (2) resistance against Ceftadizin, (3) Cephataxime and (4) Cefepime antibiotics, respectively; (B). (5) Susceptible to Ampicillin, (6) Chloromphenical, (7) Resistance to Colistin and (8) Neomycin, respectively against various generation antibiotics.

Out of the seven third-generation antibiotics used, the strain showed significant resistance towards higher concentrations ofcefepime (30mcg/disc), where most of the gram-negative bacteria are susceptible to the specific antibiotics of cefepime.Meanwhile, the strain is susceptible to the small concentrations of ampicillin (10mcg/disc) a moderately broad-spectrum antibiotic, chloramphenicol (30mcg/disc) broad-spectrum antibiotic, and Ciprofloxacin (10mcg/disc) another moderate spectrum antibiotics (Table 2).

4. Discussion

Previously many researchers[27] have revealed the production of hydrolytic enzymes from the marine origin and recognized as potential producers of other bioactive molecules. In recent studies, Castro *et al.*,[28], have described the isolation and identification of industrially important enzymes mainly, amylase, cellulase, protease, and esterases from the endophytes of the mangrove ecosystem. Recently Ming *et al.*,[29] and Xiao *et al.*,[30] have explored the marine actinobacteria for the recovery of industrially important enzymes. Similarly, Rachana *et al.*,[31] investigated the Indian Ocean to characterize marine bacterium-produced extracellular protease. Previously, the production and characterization of different extracellular hydrolytic enzymes were reported. Soares Junior *et al.*,[32] investigated the Exo and endo glucanases production by different groups of bacteria reported from the mangrove system. More recently, the cellulase enzyme recovered from the species belonged to *Bacillus* and *Brucella* was reported. Therefore, the present study aimed to isolate and screen industrially relevant bacteria from mangrove plants. In recent times, the diversity of enzyme-producing rhizospheric and endophytic bacteria from various mangrove systems of Thuwal, Jeddah, Saudi Arabia[33] was screened for antifungal activities.

Recently marine *Bacillus altitudinis* MSH2014 was discovered on, the Red Sea Coast of Egypt, they can able to produce glucose, mannuronic acid, and sulfate-heteropolysaccharide. All these isolates had the antibacterial effect against yeast, *S. aureus* (18.8 mm), *B. subtilis* (17.8 mm), *P. aeruginosa* (15.6 mm), *E. coli* (24.9 mm), also other microorganisms. At the optimal temperature of 25ºC and pH 9, *Halomonas saccharevitans* can synthesize extracellular polymeric substances (Extracellular polymeric substances) through malt extract and lactose as its nitrogen and carbon sources respectively, giving rise to increased EPS yields at the rate of 138 gL^{-133}. Contrary to earlier reports the presented isolate *Marinobacter* spp. JS7 can able to ferment different sugars such as fructose, sucrose, glucose and mannose.Astoundingly the strain showed considerable resistance against broad-spectrum antibiotics such as ceftazidime (30mcg/disc), cefotaxime (30mcg/disc), colistin (10mcg/disc) and neomycin and the *Marinobacter* had indicated its multidrug resistance potentiality (Figure 3). The

current research has exposed the enzyme-producing ability as well as potential producers of antibiotics.

In the previous investigations, the synthesized EPS was used to test against the pathogenic fungus *A. niger* and bacteria *V. fluvialis*[34]. The HPLC examination of the afore mentioned EPS revealed the presence of raffinose carbohydrate, which had a notable peak at 3.910 retention seconds. Halobacterial cultures mainly *Halomonas*, *Psychrobacter*, *Bacillus,* and *Alcaligenes*can able to synthesize eight exopolysaccharide compounds along with antimicrobial efficacy, as per the report the E15 strains were significantly active against the *S. aureus*, *S. saprophyticus*, *B. cereus*, *Proteus mirabilis*, *Enterococcus faecalis*, *Streptococcus pneumonia*, *Acinetobacter* spp., *Enterobacter cloacae*, *Campylobacter jejuni* and MRSA, by using minimum inhibition concentrations between 250 and 500 µg/mL[35]. Therefore, by considering the potential antagonistic activities of various halophilic, the isolated and identified *Marinobacter* strain could also possess phenomenal antibiotics within its genome.

E37 also demonstrated broad antibacterial action, with MICs of 250, 62.5, 125, and 500 g/mL against the same organisms as before. In general, EPS made from halophilic isolates showed stronger antibacterial activity more effective than EPS produced from isolates of the genuses *Salinivibrio*, *Chromohalobacter*, *Halomonas*, *Brevibacterium*, *Nesiotobacter*, and *Salinicoccus* against *S. pasteuri*, *B. cereus*, *S. cerevisiae*, *M. luteus*, *P. aeruginosa*, and *E. coli*[36]. Many EPS were reportedly formed in saline environments, but only weak antibacterial action against microbiological infections was found.

4.1 Future Prospects for *Marinobacter* spp. in Pharmaceuticals: Applications

The pharmaceutical industry and healthcare are expected to utilize halophilic microbial products significantly[37]. Pharmaceutical drugs are created using proteolytic enzymes[38]. Bioactive chemicals are important but understudied and related to bioactivities, such as antiviral, anti-tumour and antibacterial agents produced by various halophilic bacteria. The development of medications like the now-being-tested curacin and cryptophycin by marine cyanobacteria has drawn considerable attention[39].

Regarding their antioxidant and anticancer properties, peptide compounds from marine diatoms have also been studied[40,41]. Biosurfactants produced by halophiles are getting more prominent roles for their antifungal, antiviral, antioxidant, anti-adhesive, antibacterial, immune modulator, anticancer, stimulating dermal fibroblasts, gene therapy, and vaccination properties[42]. Halophilic bacteria need to develop new medications, antioxidants, sunscreens, suitable solutes, and hydrolytic enzymes from uncharted locations to achieve a tipping point.

Brominated analogues exhibit a 32-fold increase in activity against MRSA and a 16–64-fold increase in activity against *P. aeruginosa* and *E. coli*, highlighting recent developments in the addition of halogenated chemicals to peptides (oligomers of N-substituted glycines), which enhance antibacterial effectiveness against multiple drug-resistant infections[43]. Drug-resistant bacteria may be more susceptible to halogenated medications in the future[44,45]. Due to recent discoveries of halophilic bacteria, even in terrestrial habitats, OMIC technologies now provide fresh promise for the future discovery of unique characteristics and/or novel biomolecules produced from halophiles[46,47].

5. Conclusion

The present research investigated the extracellular hydrolytic enzyme activities and featured bioactive metabolites, especially antibiotic resistance patterns.16S rRNA gene analysis revealed its gene similarity was 95%, so believed to be one of the potential novel taxon. One of the significant outcomes of the present work has revealed the extremophilic nature of *Marinobacter* spp. This is due to most of the industrial processes commonly held at relatively higher temperatures, pH, and salinity, etc. The *Marinobacter* spp. could tolerate temperatures of about 50°C, pH ranging between 5-10 and 20% of salts. Therefore, the described versatile bacteria interestingly have these all-characteristic features, and it has inherent biotechnological applications. Further, investigations are needed to be carried out for the extraction, characterization, and purification of various industrial necessary enzymes at commercial scale. The present article provides vital information on simple steps involved in the bioprospection of different bioactive compounds and insights on *Marinobacter* spp. Yet, the abundance and the relevant screening data

are despairing amongst the halophilic bacteria; there is a huge opportunity for their exploration both for health, environmental and industrial applications.

6. References

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