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Isolation and molecular identification of moderate halophilic enzyme-producing bacteria

Gehad M. Abdelal [a], Nada F. Hemeda [a], Eissa, A. Eissa [a], Gamal M. Hassan[a,*]

[a] *Genetics Department, Faculty of Agriculture, Fayoum University, El Fayoum 63514, Egypt.*

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ABSTRACT

The production of industrially relevant monomeric subunits from the hydrolysis of 1-4linkages in cellulose is catalyzed by cellulase applications for cellulases can be found in the food and feed textile brewing washing and pulp and paper industries the need for cellulases that can endure extreme circumstances related to pH, temperature and salinity is constant in these sectors. Thus, newly identified halophilic bacteria that produce halotolerant cellulose was presented in this study. cellulase 10 isolates of moderate halophiles bacteria were isolated from different samples collected from Qarun lake Fayoum Egypt. However, only five isolates S1, S2, S3, S4 and S5 showed highest cellulolytic activity were chosen for further analysis. The optimum conditions of maximum cellulase production were following 35ºC, pH 9, 15 NaCl and 3 days culture age respectively. The isolates exhibiting the greatest level of cellulase activity ranged in enzyme activity from 1.039 to 1.398 U/ml. It came to light that the majority of the halophilic isolates were Gram-negative and that their coloring varied from creamy white to yellow. Based on their 16S rRNA gene sequences, the S4 isolate was identified as *Halomonas eurihaalin*.

1. Introduction

Extreme, moderate, minor, or nonhalophilic prefer 5 to 5.2 M.The most extreme moderate slight or non-halophilic extreme halophiles prefer 5 to 5.2 M (20–30%), where they exhibit optimal development in media comprises no less than 15% NaCl (w/v). They are capable of growing to saturation of salts about 32% (w/v) of NaCl. Halophiles are categorized according to the level of their halotolerance [1]. The ideal growth conditions for moderate halophiles are medium containing 3 and 15% NaCl (w/v), or around 0.5 to 2.5 M. The ideal range for slight to mild halophile development is 1 to 3% (p/v) of NaCl, or roughly 0.2 to 0.5 M [2]. In media containing less than 1% (w/v) NaCl, non-halophilic microorganisms are capable of developing (about 0.2 M). Because halophiles require salt (NaCl) to proliferate, moderately halophilic bacteria, or the products they produce, can be used in a wide range of manufacturing processes. Moderately halophilic enzymes are currently being further investigated for their application in biotechnology. On the contrary, researchers can develop enzymes that are more active at medium and high salt concentrations while being stable and active in the absence of salt. This varied category of microorganisms encompasses species from many genera, among which are H. marinococcus, H. flavobacterium, and C. paracoccus. These species have traits such explosive development, minimal needs for nutrition, and the ability to use a variety of materials as their sole source of carbon and energy [3]. Since halophiles exhibit strong stability and robust activity despite extremely high pH pressure, temperature, dryness, or lack of water activity, they were selected as the perfect option for further investigation into enzymes used in industries [4].Therefore, in order to make extremozymes on an industrial level and overcome the obstacles of biology at a fair cost, it is crucial to locate new sources and learn everything there is to know about these enzymes, including their structure producers and innovative capabilities. Halophilic bacteria are a diverse collection of bacteria with a varied physiological makeup. They can thrive outside of this range, but their ideal growth range is between 3 and 15% NaCl concentration [3]. The present requirement for commercially stable enzymes in various processes may be met by the isolation and characterization of a novel, industrially significant enzyme from the halophilic bacteria with special qualities of salt thermal alkaline and solvent-based organic stability [5]. In order to prevent unwinding and preserve protein solubility, halophilic enzymes have a considerably negative charge on their surfaces and hydrated carboxyl groups shielded by high levels of salt [6]. Nevertheless, little is known about able to tolerate salt enzymes. Multiple strains of halophilic enzyme-producing microorganisms were previously employed in the fish and soy sauce industries [7]. The present investigation aims to identify possible sources

* Corresponding author.
E-mail address: gmh01@fayoum.edu.eg (Gamal. Hassen); Tel.: +201002332803

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of stable hydrolytic cellulase enzyme by isolating moderate halophilic bacteria, screening them, characterizing them using morphological ones such, biochemical processes and molecular techniques.

2. Materials and methods

2.1. Collection of samples

Samples of water taken from the salty Qarun Lake in Fayoum, Egypt, and soil samples from Qarun Lake sediments were the two types of samples that were taken in July 2021. After being gathered in sterile plastic bags, the samples were brought to the lab and placed on ice to be processed further synthesis of the LNMO was previously reported [20, 22]. For the dielectric measurements, the as-prepared LNMO powder sample is pressed using a hydraulic press at about 3 tons into a round pellet of diameter and thickness of 1 cm and 2.7 mm, respectively. The pellet is then sintered at 950 °C for 12 h in an argon atmosphere. Then, the two round sides of the pellet were coated with silver paste.

2.2. Isolation of halophilic bacteria from collected samples

The Sehgal and Gibbons (S-G) medium was used to isolate moderately halophilic bacteria in accordance with the Sehgal and Gibbons [8] method. It was added with varying concentrations of salt (10% and 15%, w/v) NaCl. 100 µl of every sample that was collected, then for a maximum duration of seven days, the inoculation dishes had been incubated at 35°C with daily checks. Once the colonies were retrieved, they were placed on fresh agar plates and repeatedly sub cultured until pure cultures were produced. Bacterial isolates were preserved by preparing stocks of 20% glycerol of pure cultures and storing them at -80°C.

2.3. Impact of salt content on halophilic bacterial isolates' growth, colony characteristics and generation of pigments

By cultivating the halophilic isolates in SG agar plates with varying sodium chloride concentrations ranging from 0% to 25%, the isolates' capacity to withstand NaCl was examined. Freshly generated isolate cultures were streaked over the NaCl incorporated plates, which were then incubated for seven days at 35°C. To determine and describe the optimal salt content, Oren's classification of halotolerant, mild halophiles, moderate halophiles, and nonhalophiles was employed [9].

2.4. Determine the optimal pH and temperature for growth of moderate halophiles isolates

The broth culture of each isolate was streaked on SG medium containing 15% NaCl at temperatures of 35ºC using different pH values of 4–12 at intervals of 1 pH unit in order to find the ideal pH for development of moderate halophiles isolates. The ideal temperature for the growth of moderate halophile isolates was also ascertained; each isolate's broth culture was streaked over SG medium containing 15% NaCl at pH 8, and it was then incubated at 20–50 ºC intervals of 5 ºC. The optical density at OD600 was used to track the growth of bacteria using a spectroscopic approach (model UV_160 A; Shimadzu, Japan).

2.5. The morphological description of selected moderate halophiles bacterial isolates

Light microscopy was used to investigate the morphology and movement of cells on a recently constructed exponentially increasing liquid culture. Gram staining was performed. After drying the slides, the bacterial colony was produced as a thin smear and stained using Dussault's procedure [10].

2.6. Biochemical characterization of selected moderate halophiles bacterial isolates

The five moderately halophilic isolates were subjected to a variety of metabolic profile tests using biochemical testing (methyl red test, KOH test, catalase test, and urease test). A stock (10% w/v) of sucrose, dextrose, fructose, maltose, and lactose was made using carbohydrates, and it was separately sterilized for 15 minutes at 121°C. Acid generation from the consumption of carbohydrates as stated by Leifson [11]. Results were recorded after five to seven days of development at 35°C. Yellow light appeared on the red phenol red indicator. Potassium phosphate, Urea and phenol red were added to urea broth before it was autoclaved in order to measure the urease activity.

2.7. Qualitative cellulase screening of the four moderate halophilic isolates

The selected isolates were tested for the production of cellulase enzyme using a plate assay procedure. The assay for the plates was conducted using SG agar medium supplemented with 1% (w/v) carboxymethyl cellulose (CMC). Discrete colonies with distinct morphologies were selected from the plates, streaked on a different CMC plate, and then incubated for ninety-six hours at 35°C. Additionally, the replica plates underwent independent staining preparation. For twenty minutes, 0.3% Congo red was flooded onto the replica plates. After the stain was drained out, 1M NaCl was used to wash the plates. Following washing, only the isolates from the master plate that had distinct, clear zones surrounding the colonies that indicated the generation of cellulase were chosen and picked for the liquid medium enzyme production [13].

2.8. Extracellular cellulase quantitative screening

After 900 µL of 1% carboxymethyl cellulose (CMC) was dissolved in sodium citrate buffer (50 mM, pH 6.5) with 100 µL of crude enzyme extract, the reaction mixture was heated at 40°C for 30 minutes and then boiled for 10 minutes[14].The procedure was terminated by adding 1 milliliter of 3, 5-dinitrosalicylic acid (DNS). Following cooling, the developing color was ascertained using a spectrophotometer and the absorbance at 540. The free

reducing sugars were tested with glucose serving as a reference. The amount of enzyme required to release one mole of glucose equivalent (reducing sugar) every minute under the experiment's conditions is known as one unit of cellulose [15].

2.9. Optimization of cellulase production at various parameters

Different culture conditions, pH levels (4, 5, 6, 7, 8, 9, 10, 11, and 12), and incubation times (1, 3, 5, and 7 days) were all used to the generation of cellulase. At 35°C and 130 rpm, the cellulase was generated in SG medium with 15% NaCl. The culture broth was kept at -20°C until additional analysis was performed after centrifuging it for 20 minutes at 10,000 rpm at 4°C. The crude enzyme-containing cell-free culture was used to estimate the activity of CMCase.

2.10. Molecular identification of the four selected moderate halophile bacterial isolates

2.10.1. Genomic DNA isolation from moderate halophile bacteria

The isolates' genomic DNA was isolated using the technique outlined by Tillett and Neilan [16].

2.10.2. Amplification of 16SrRNA gene by specific PCR reaction

The 16S rRNA genes of one isolate, S3, were amplified by PCR using the universal forward primer (27F) and reverse primer (1492R) using the extracted genomic DNA as a template [17] in PCR (Thermo Fisher, GeneAmp 9700) in order to confirm the species of bacterial isolates at the molecular level. Each PCR reaction mixture contained 10 μM of each primer, 2 U Taq Polymerase, 10 mM dNTPs (Sigma), 10x Taq buffer, and 1 μl of template DNA. A total of 35 cycles of denaturation (5 min at 94 °C), annealing (40 s at 56 °C), and extension (1.2 min at 72 °C) were performed, culminating in a final extension for 12 min at 72 °C to terminate the reaction. By using electrophoresis on a 1% agarose gel, the amplified product's size was estimated to be around 1.4 kb. Electrophoretic separation was carried out at 100 V for 30 minutes. A UV-VIS Spectrophotometer was employed to measure the resulting fragments of DNA. To evaluate the PCR product, the around 1400 bp PCR products were sequenced using Sanger's DNA sequencing method (Macrogen, Inc., South Korea).

2.10.3. Phylogenetic tree construction using computational analysis (BlAST)

The 16S rRNA gene sequences were uploaded to GenBank [18], and the results of the BLAST search were received from the National Center for Biotechnological Information (NCBI) (https://www.ncbi.nlm.nih.gov/gene/). Using 1000 repeats and the neighbor-joining approach, MEGA 6 was used to create a phylogenetic tree that was shown for 100 generations [19].

3. Results and discussion

3.1. Isolation of halophilic bacteria from collected samples

After the samples were plated out, visible cultures were regularly streaked over the suitable medium to filter them. They started to show after 7 days of acclimating to the growing media. Ten distinct areas of Qarun Lake in the Egyptian province of Fayoum yielded bacterial isolates. The majority of the isolates had circular colonies with uneven, complete, and filiform margins. Since all of the isolates could grow on SG agar media with varying salt concentrations, more research will be conducted on this medium. Halophilic salt-tolerant bacterial strains were identified and tested from different regions of Iran by Rohban et al. [20]. Halophilic bacteria were obtained by Cojoc et al. [21] from a subsurface rock salt crystal in Romania. Additionally, certain investigations [22, 23] describe the segregation of different bacteria that can resist high salt concentrations. *Dielectric properties*

3.2. Impact of salt content on halophilic bacterial isolates' growth, colony characteristics and generation of pigments

The findings displayed in Table (1) demonstrate the halophilic bacteria's resistance to NaCl. It was observed that the isolates' cell development varied as the concentration of salt increased. Every isolate was grown on SG agar plates with 5–20% NaCl, with the exception of isolate S9, which did not develop on SG medium with 15–20% NaCl and then showed minimal or no growth when the salt concentration was raised to 25% (Table1). The isolates (S1, S2, S3, S6, S7, S8 and S10) demonstrated their ability to grow until 25%, whereas the isolates (S4 and S5) can grow until 20% and the isolates (S9) until 10%. The results also demonstrated the isolates' ability to grow at different concentrations of sodium chloride. It suggests that some of the isolates have salt tolerance to a suitable degree. Based on the maximum tolerable salt concentration (MTSC) results, eight isolates (slightly halophiles) grew to a maximum of 5%, three isolates grew to 10%, five isolates grew to 15% (moderately halophilic), and two isolates grew to 20% in a medium containing NaCl and without salt (halotolerant). The isolates S1, S2, S3, S4 and S5 grew at their best between 10% and 15% (Table 1). This conclusion is consistent with that of Mudryk and Donderski [24], who discovered that halophiles' metabolic activity increases when the amount of sodium chloride in the culture medium increases. Increased enzyme activity may be the cause of the enhanced metabolic activity observed in cells cultivated in higher salt concentrations, as suggested by Forsyth and Kushner's [25] discovery that Na+ ions promote the passage of carbohydrates and amino acids across the cytoplasmic membrane. However, the isolate (R2) indicated that the growth rate decreased as the salt level increased [26]. Similar findings were reported by Buckmire and Macleod [27] and Mudryk and Donderski [24], who found that the metabolic activity of the moderately halophilic bacteria decreases as the salt level increases. The rate of normal metabolism may be slowed down in the presence of high extreme salt concentrations because halophilic bacteria typically have lower salt concentrations inside their habitat than outside. This is because maintaining an appropriate salt balance between the interior and exterior environments requires a significant amount of energy. However, due to the suppression of specific enzymes, large concentrations of salt can occasionally induce disruptions in the metabolism of halophilic bacteria.

Table 1. The effect of different concentrations of sodium chloride on the growth of bacterial isolates grown on SG agar medium at 35ºC for 7 days.

Isolates	0 %	5 %	10%	15%	20%	25%
S1	++	++	+++	+++	++	++
S2	++	++	++	+++	++	++
S3	++	++	++	+++	++	++
S4	-	++	++	+++	+++	-
S5	-	++	++	+++	+++	-
S6	++	++	++	+	+	+
S7	+	+	++	++	+	+
S8	+	+	++	++	+	+
S9	++	++	+++	-	-	-
S10	++	++	+++	++	+	+

+++ = High growth, ++ = Moderate growth, + = Low growth, - = No growth

3.3. Determine the optimal temperature and pH for growth of moderate halophiles isolates

The purpose of the study was to determine whether the isolates could grow at various temperatures and whether temperature had an impact on the growth of isolates that were moderate halophiles. The isolates also needed to know how to tolerate salinity based on several critical development conditions, including pH and temperature. The isolates (S4 and S5) may grow at 45ºC, according to Table (2) data. The isolates grew best at a temperature of 35ºC. Since there was no growth at 50ºC and 50ºC was the maximum temperature at which growth could occur, the isolates in this investigation are thought to be moderately thermophilic. This result is consistent with the findings of Hochstein et al. [28], who hypothesized that the majority of isolates of halophiles originate from warm areas and that many of them are moderate thermophiles. Halophilic bacteria thrive best at a temperature of 37ºC, while reports have indicated that they can grow at temperatures as high as 50ºC. All examined isolates were able to develop in the pH range of 4–10, with a pH of 7-9 being the ideal range, according to the results displayed in Table (3). This result is consistent with recent research showing that neutrophiles make up the majority of halophiles and halotolerant microorganisms reported to date. These bacteria thrive best in media with a pH between 6.8 and 7.5 [28, 29].

Table 2. The effect of different temperature degrees on the growth of moderate halophiles isolates grown on SG medium supplemented with 15% NaCl at 35 ºC for 7 days.

Isolates	Temperature						
	20ºC	25ºC	30ºC	35ºC	40ºC	45ºC	50ºC
S1	++	+++	+++	+++	+++	-	-
S2	++	++	+++	+++	++	-	-
S3	++	+++	+++	+++	+++	-	-
S4	-	-	++	+++	+	+	-
S5	++	++	++	+++	+++	+	-

+++ = High growth, ++ = Moderate growth, + = Low growth, - = No growth, ND. Not detactabl

Table 3. The effect of different pH levels on the growth of moderate halophiles isolates grown on SG medium supplemented with 15% NaCl at 35 ºC for 7 days.

+++ = High growth, ++ = Moderate growth, + = Low growth, - = No growth, ND. Not detactable

Isolates	pH								
	4	5	6	7	8	9	10	11	12
S1	ND	+	+++	+++	++	+	++	ND	ND
S2	-	+	++	+++	++	++	++	ND	ND
S3	-	-	++	+++	++	+	++	ND	ND
S4	-	ND	++	++	+++	+++	ND	-	-
S5	-	+++	+++	+++	+++	+++	++	-	-

3.4. Morphological and biochemical Identification of the moderate halophilic isolates

Most of the halophilic isolates were Gram-negaitive and had cocci and cocci shape when seen under a light microscope (Table 4). By using microscopic imaging, it was discovered that the halobacterial cells were arranged as single, double, cluster, rod, rod to cocci, or cocci (Fig. 1). The isolates were then exposed to additional biochemical examination. Urease was produced by each of the five isolates that were chosen. Because urease activity produces ammonia, which has a basic pH by nature, the environment's pH rises. It was found that the isolates used a range of carbohydrates. Only three isolates metabolized sucrose, whilst the other five generated cellulase. Four of the five isolates used lactose, and all five used fructose. Only the isolate S5 used mannitol. Four isolates used lactose, maltose, and dextrose (Table 4). Several researchers also produced results of a similar kind [30, 31]. The H_2S production, indole test, and methyl red test results for any of the examined bacterial isolates were all negative. According to Bergey's manual of systematic bacteriology, these biochemical tests are used to identify bacteria as well as display their various features.

Table 4. Morphological and biochemical characters of the moderate halophilic isolates

+ = Positive - = Negative

Morphological characterization	S1	S2	S3	S4	S5
Colony pigmentation	White creamy	White creamy	White creamy	Creamy	Yellow
Gram staining	+	+	-	-	-
Cell shape	Cocci	Cocci	Cocci	Short rod	Short rod
Motility	+	-	-	+	-
Biochemical characterization	S1	S2	S3	S4	S5
Catalase	+	+	+	+	+
Methyl red	-	-	-	+	+
KOH	-	-	+	+	+
Urea activity	+	+	+	+	+
Hydrolysis of:					
Tween 80	-	+	-	+	+
Casein	-	-	-	-	-
Starch	-	-	-	-	-
cellulase	+	+	+	+	+
Acid production					
Mannitol	+	-	-	-	+
Sucrose	+	+	+	-	-
Fructose	+	+	+	+	+
Lactose	+	+	+	-	+
Maltose	+	+	+	-	+
Dextrose	+	+	+	-	+

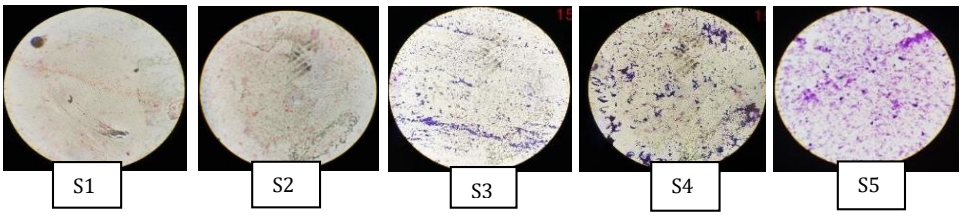

Fig. 1. The image represents the microscopical observation of halophilic isolates.

3.5. Qualitative cellulase screening of the five moderate halophilic isolates

Five halophilic isolates were chosen using plate tests with media containing 15% NaCl in order to produce cellulase. Using Congo red dye as an indicator and CMC as a carbon source to look at the zone of inhibition brought on by the hydrolysis of cellulose, these isolates were screened for their capacity to generate cellulase. Out of ten moderate halophile bacterial isolates, only five demonstrated positive cellulase production tests on CMC agar plates with Congo red pigment. Isolates (S1, S2, S3, S4, and S5) that showed the greatest ratio of clear zone diameter to colony diameter on Congo red agar plates and the formation of a clear zone on the screening medium were chosen, and it was discovered that the five isolates had cellulolytic activity as indicated in Figure (2). Five moderate halophiles were isolated for this investigation from several locations around Qarun Lake in Fayoum, Egypt. These isolates were chosen from a group of ten isolates based on their capacity to generate more active cellulase. Moderate halophiles are highly desirable for development and screening of novel enzymes with peculiar features since they produce enzymes throughout a very wide range of salinities [32]. A class of intriguing biotechnological enzymes known as cellulases finds use in the food, beverage, textile, distilling, and pharmaceutical sectors as well as in starch scarification [33]. Because the five isolates showed the most pronounced zones of CMC hydrolysis surrounding their colonies, they were chosen based on their cellulase activity. Cellulase producers was the classification given to these five isolates. Similar to this, Cojoc et al. [21] identified three amylases, twelve lipases, and two cellulases from subsurface rock salt crystal (Romania) that produced halophilic bacteria that produced enzymes. The various enzymatic activities of these bacteria are further supported by their study. Similar research was conducted by Rohban et al. [20], who discovered 177 amylase, 195 lipase, and 65 cellulase-producing bacteria after testing the various hydrolytic activities of isolated halophiles from Howz Soltan Lake, Iran. Clearing zones surrounding microbial growth colonies that show the presence of cellulase after an adequate amount of incubation. When used for plate flooding, Gram's iodine produced a more noticeable and faster result than hexadecyltrimethyl ammonium bromide or Congo red, according to Kasana et al. [34]. Additionally, cellulase-producing microorganisms, such as bacteria, Sreedevi et al., [36] and fungi, Shahriarinour et al., [35] were screened using gram iodine. However, due to the weak link between halo size and enzyme activity, plate-screening techniques using dyes are not quantitative approaches.

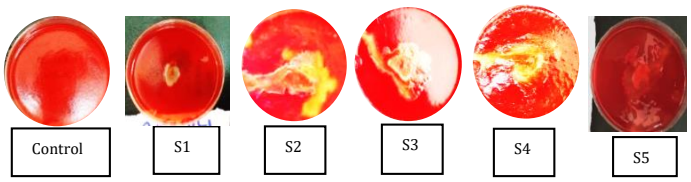

Fig. 2. The method of screening petri dishes for isolates of halophilic cellulolytic bacteria is facilitated by the application of Congo red dye. The clearing zone that surrounds the colonies indicates that carboxymethyl cellulose (CMC) is being hydrolyzed by secreted CMCase.

3.6. Optimization of cellulase production at various parameters

Using SG media containing 15% NaCl at various pH values ranging from 4 to 12 and different incubation times at 35°C, the five selective bacterial isolates exhibiting cellulase-positive were tested for quantitative CMCase production from the previous experiment. The isolates S1, S2, S3, S4, and S5 showed the highest CMCase production with corresponding activities. 1.02, 1.46, 1.1, 1.39 and 1.13 U/ml, pH 4 for(S1,S2,S3,S4,S5) , S4 at 1day (0.806 U/ml) , S4 at 3 day(1.039U/ml) ,S4 at 5 day(0.797), S3 at 5 day(0.807 U/ml),S1 at 9 day(1.020 U/ml), pH 5 for(S1,S2,S3,S4,S5), S4 at 1day (0.924U/ml), S4 at day(1.169U/ml), S4 at 5 day(0.775 U/ml), S4 at 7 day(0.706 U/ml),S3 at 9 days(0.708 U/ml) , pH 6 for(S1,S2,S3,S4,S5), S3 at 1 day(0.824 U/ml),S4 at day(1.297U/ml),S4 at 5 day (0.749 U/ml), S2 at 7 day (1.077 U/ml), S2 at 9 day (1.463U/ml), pH 7 for(S1,S2,S3,S4,S5),S4 at 1 day (0.867 U/ml) , S4 at 3 day (1.088 U/ml), S5 at 5 day (0.742 U/ml),S3 at 7 day(0.862U/ml),S3 at 9 day (0.816U/ml), pH 8 for(S1,S2,S3,S4,S5), S3 at 1 day (0.920 U/ml), S4 at 3 day (1.334 U/ml), S5 at 5 day (0.832 U/ml), S2 at 7 day (0.894 U/ml), S1 at 9 day (0.872 U/ml), pH 9 for(S1,S2,S3,S4,S5), S3 at 1 day (0.783 U/ml), S4 at 3 day (1.398 U/ml), S5 at 5 day (0.902 U/ml), S1 at 7 day (0.954 U/ml), S3 at 9 day (0.927 U/ml), pH 10 for(S1,S2,S3,S4,S5), S1 at 1 day (0.656U/ml), S4 at 3 day (1.398 U/ml), S5 at 5 day (0.902 U/ml), S5 at 7 day (0.951 U/ml), S1 at 9 day (0.857U/ml), pH 11 for(S1,S2,S3,S4,S5), S5 at 1 day (0.872 U/ml), S3 at 3 day (1.1U/ml), S5 at 5 day (0.785 U/ml), S5 at 7 day (0.832 U/ml), S1 at 9 day (0.760 U/ml), pH 12 for(S1,S2,S3,S4,S5), S3 at 1 day (0.759 U/ml), S4 at 3 day (0.666 U/ml), S4 at 5 day (0.802 U/ml), S5 at 7 day (1.136 U/ml), S1 at 9 day (0.768 U/ml) respectively, (Table 5 and Table 6). Among the isolates, isolate S4 exhibited the highest expression of cellulases. The generation of CMCase was optimized using these isolates. The effects of pH on cellulase activity at a range of pH values, from 4 to 12 were studied. All of the select pH ranges showed activity for cellulase; however, pH 9 was shown to

be the optimum pH. The activity gradually declined until pH 11 over the optimum pH, and then it abruptly fell at pH 12. The optimum pH and incubation period are two crucial variables that affect how much cellulase can be produced at maximal output. Thus, for the best synthesis of this hydrolytic enzyme, the effects of pH and incubation duration should be studied. Cellulase derived from a different isolates showed excellent alkali stability over a wide pH range (6–12), indicating that it is alkali stable.

Although significantly lower than that of *Halomonas* sp. PV1 reported b.y Benit et al. [39], the cellulase activity of the isolates belonging to the *Halomonas* species was found to correspond with the report of Shivanand et al. [38]. Nonetheless, compared to isolates from *Halomonas* species, the cellulase activity of *Bacillus* species isolates was at least ten times higher. These results suggest that *Bacillus* species are more capable of producing cellulase than those *Halomonas* species. However, compared to *Oceanobacillus profundus*, the isolate from *Oceanobacillus oncorhynchi* had a cellulase activity that was almost 10 times lower, as reported by Gbenro et al. [40].

Table 5. Cellulase production at different pH levels on the SG medium contained 15% NaCl.

pH	Cellulase production U/mL Mean ± S.E.				
	S1	S2	S3	S4	S5
4	0.642±0.207ab	0.637±0.153b	0.687±0.125abc	0.758±0.213a	0.607±0.114cd
5	0.665±0.260ab	0.516±0.136b	0.679±0.086abc	0.827±0.217a	0.586±0.104d
6	0.634±0.158ab	0.837±0.396a	0.674±0.125abc	0.795±0.286a	0.599±0.108d
7	0.664±0.137ab	0.598±0.066b	0.673±0.155abc	0.782±0.198a	0.665±0.114bcd
8	0.711±0.180ab	0.592±0.163b	0.772±0.114ab	0.790±0.284a	0.703±0.163d
9	0.676±0.187ab	0.669±0.145b	0.798±0.232a	0.724±0.244a	0.841±0.320 a
10	0.755±0.184a	0.641±0.168b	0.629±0.114bc	0.806±0.441a	0.675±0.238bcd
11	0.627±0.114ab	0.561±0.080b	0.693±0.361abc	0.733±0.077a	0.759±0.116 ab
12	0.602±0.121b	0.615±0.336b	0.613±0.123c	0.701±0.121a	0.754±0.224abc
Total	0.664±0.177	0.630±0.2217	0.691±0.182	0.768±0.247	0.688±0.195

Table 6. Cellulase production at different pH levels on the SG medium contained 15% NaCl

Period time (Day)	Cellulase production U/mL Mean ± S.E				
	S1	S2	S3	S4	S5
1	$0.609±0.092^{bc}$	$0.456±0.037^{c}$	$0.679±0.227^{ab}$	$0.726±0.145^{b}$	$0.656±0.153^{c}$
3	$0.563±0.064^{c}$	$0.653±0.101^{b}$	$0.690±0.262^{ab}$	$1.07±.0353^{a}$	$0.642±0.081^{c}$
5	$0.588±0.123^{bc}$	$0.613±0.240^{b}$	$0.601±0.064^{b}$	$0.738±0.087^{b}$	$0.750±0.094^{b}$
7	$0.662±0.211^{b}$	$0.775±0.177^{a}$	$0.715±0.110^{a}$	$0.705±0.123^{b}$	$0.873±0.27^{a}$
9	$0.898±0.11^{a}$	$0.652±0.310^{b}$	$0.769±0.141^{a}$	$0.598±0.107^{c}$	$0.518±0.108^{d}$
Total	0.664±0.177	0.630±0.221	0.691±0.182	0.768±0.247	0.688±0.195

3.7 Molecular identification of the four selected moderate halophile bacterial isolates

3.7.1. Amplification of 16S rRNA gene by specific PCR reaction

The 16S rRNA gene sequence was analyzed in order to molecularly identify the isolate S4, which was chosen (Figure 3). The sequence of the chosen isolate S4 was compared using the BLAST program and the sequence match tool in the GenBank database information from the National Center for Biotechnology Information (NCBI) to type strains obtained from the Ribosomal Database Project. There was a significant degree of sequence similarity (93.3%) between the 16S rRNA sequence of strain S4 and *Halomonas eurihalina*. The select moderate halophilic isolate S4 underwent phylogenic analysis using the 16S ribosomal RNA gene sequencing technique.

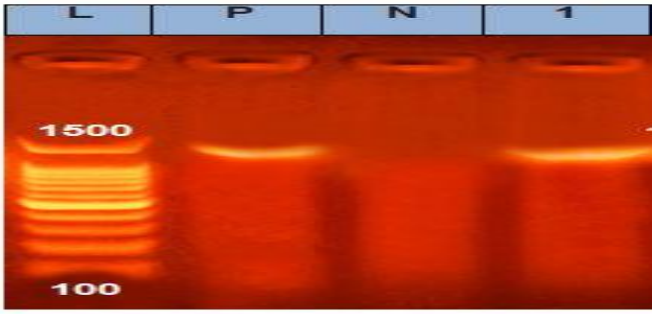

Fig. 3. Agarose gel of the 16S rRNA PCR product obtained from the *Halomonas* S4 isolate. Lane 1: 100 bp DNA leader, lan2: Positive control, Lane3: Negative control, lan4: Isolate S4.

3.7.2. Computational analysis (BlAST) construction of phylogenetic tree

Figure (4) shows the evolutionary distance as well as the isolate's phylogenetic relationship and sequence comparison using the Basic Local Alignment Search Tool (BLAST) with the GenBank database. Based on the gene sequences of 16S rRNA, phylogenetic analysis showed that strain S4 identified itself to the closest phylogenetic relatives of *Halomonas eurihalina*. Nonetheless, strain S4 phylogenetic assignment based on 16S ribosomal RNA gene analysis indicates that it differs from *Halomonas eurihalina*. A bacterial species is generally considered to be a collection of strains that show a high degree of overall similarity and differ considerably from related strain groups with respect to many independent characteristics, according to Colwell et al. [41] in this context. It should be mentioned that certain species have multiple copies of the 16S rRNA gene, and these copies might differ greatly, which complicates phylogenetic analyses of the family Halobacteriaceae based on 16S rRNA gene sequences [42].

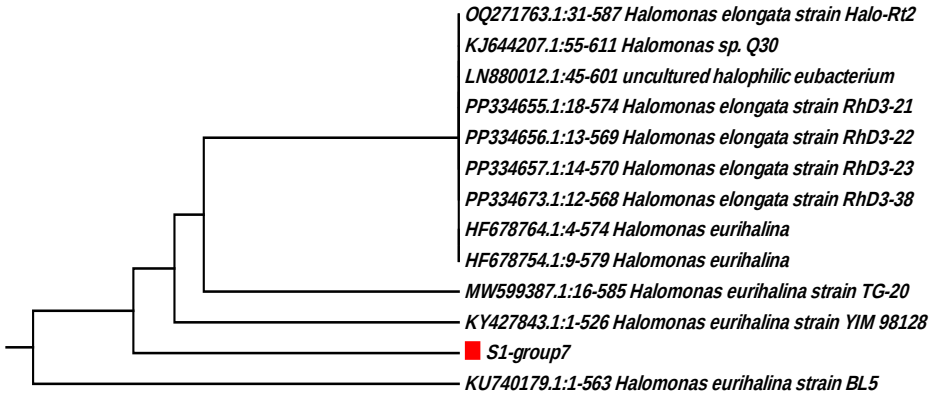

Fig. 4. The phylogenic tree of *Halomonas eurihalina* strain (S4)| constructed using the distance method and 16S rRNA gene sequence

4. Conclusions

Ten bacterial isolates with different salt concentrations were used in this investigation. Among the halophilic isolates, five of them exhibited cellulase activity at the designated concentrations of salt. High amounts of salt were causing these bacterial isolates to exhibit hydrolytic activity. As a result, the isolated halophilic bacteria have the potential to develop halophilic extracellular enzymes that have industrial and biotechnological applications in the future.

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Author Contributions

All authors contributed to this work. G. Abdelal cpllected the samples and isolated the isolates and completed the experimental measurements. Both G. Hassan and N. Hemeda shared writing and followed the performance of the experiments. G. Hassan completed the paper writing, analyzing the data, and validation. G. Hassan followed the revision and submission of the manuscript for publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

R eferences

[1] G.M. Marion, C.H. Fritsen, H. Eicken, M. C. Payne, The search for life on Europa: limiting environmental factors, potential habitats, and Earth analogues. Astrobiology. 3 (2003) 785–811.
[2] B. Ollivier, P. Caumette, J.L. Garcia, R.A. Mah, Anaerobic bacteria from hypersaline environments. Microbiol. Rev. 58 (1994) 27–38.
[3] A.Ventosa, J.J Nieto, Oren. A Biology of moderately halophilic aerobic bacteria. Microbiol. Mol. Biol. Rev 44(1998). 62:504.
[4] A.M. Kakhki, M.A. Amoozegar, E.M. Khaledi. Diversity of hydrolytic enzymes in haloarchaeal strains isolated from salt lake. Int. J. Environ. Sci. Technol., 8 (2011) pp. 705-714.
[5] P.M.D. Souza. Application of microbial α-amylase in industry-A review. Braz. J. Microbiol. 41(2010) (4): 850-861.
[6] W.A. Joo, C.W. Kim, Proteomics of *Halophilic archaea*. J. Chromatography B. (2005) 815: 237-250.
[7] A. Oren., Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. J. Ind. Microbiol. Biotechnol. (2002)28: 56-63.
[8] S.N. Shegal, N.E. Gibbons. Effect of metal ions on the growth of *Halobacterium cutirubrum*. Can. Journ. Microbiol. 6(1960) 165- 169.
[9] A. Oren, The order Halobacteriales. In: Dworkin M,Falkow S,Rosenberg E,Schleifer KH,Stackebrandt E,(eds) The prokaryotes. A handbook on the biology of bacteria. Vol. III. (Ed. 3) Springer,New York. (2006)113-164.
[10] H.P, Dussault An improved technique for staining red halophilic bacteria J. Bacteriol., 70 (1955), pp. 484-485.
[11] E. Leifson, Determination of carbohydrate metabolism of marine bacteria. J. Bacteriol, 85(5) (1963) 1183.
[12] A .K. Yagi, A. Nakamura, Sekine. Comparison between magnifying endoscopy and histological, culture and urease test findings from the gastric mucosa of the corpus. Endoscopy, 34 (05) (2002), pp. 376-381.
[13] L. Potprommanee, X. Q .Wang, Y. J. Han, D.Nyobe, Y. Peng , Q. Huang : Characterization of a thermophilic cellulase from Geobacillus sp. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass. PLoS One. (2017) 13; 12(4).
[14] Miller GL Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem31: (1959) 426-428
[15] T.M. Wood, K.M. Bhat Methods for measuring cellulase activities. Methods in enzymology, vol 160. Academic Press, Cambridge, (1988):pp87-112.
[16] D. Tillett, and B. A .Neilan: Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. Journal of Phycology. (2000) 36 251–258.
[17] B. Meral, C. Baris, M. Bulent , E. B. Rahel, O. Aharon , O. N. Mehmet, Ayse Ogan Extremely halophilic Archaea from Tuz Lake, Turkey, and the adjacent Kaldirim and Kayacik salterns . World J Microbiol Biotechnol 23(2007)309–316.
[18] T. Barrett, K. Clark, R. Gevorgyan, V. Gorelenkov, E. Gribov, I. Karsch-Mizrachi, M. Kimelman, K.D. Pruitt, S. Resenchuk, T. Tatusova. BioProject and BioSample databases at NCBI: facilitating capture and organization of metadata. Nucleic Acids Res; 40.(2012) D57–D63.
[19] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., 28 (2011) 2731-2739.
[20] R.Rohban, M .A. Amoozegar & A. Ventosa, Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran, J Ind Microbiol Biotechnol, 36(3) (2009) 333–340.
[21] R.Cojoc, S. Merciu, G. Popescu, L. Dumitru, M. Kamekura & M. Enache, Extracellular hydrolytic enzymes of halophilic bacteria isolated from a subterranean rock salt crystal, Rom Biotechnol Lett, 14 (2009) 4658–4664
[22] M.Khunt & N. Pandhi, Moderate halophilic bacterial community in excreta of wild ass (Equus hemionus Khur). Int J Biosci, 1(5) (2011) 31–37.
[23] S.Jayachandra, S. A. Kumar, Y. Shouche & M. Sulochana, Culturable diversity of extremely halophilic bacteria from west coast of Karnataka, India, Int J Biol Pharm Allied Sci, 2(2) (2013) 391–405.
[24] Z. Mudryk, W. Donderski, Effect of sodium chloride on the metabolic activity of halophilic bacteria isolated from the lake gardno estuary. Estuaries. 14 (4) (1991) 459-498.
[25] M.P.Forsyth, D.J. Kushner, Nutrition and distribution of salt response in populations of moderately halophilic bacteria. Can J Microbiol. 16 (1970) 253-261.
[26] R. Buckmire, R. A. Macleod, Nutration and metabolism of marine bactyeria. XIV. On the mechanism of lysis of marine bacterium. Canadian Journal of Microbiology. 11: (1965) 677-691.
[27] L. I Hochstein, The physiology and metabolism of the extremely halophilic bacteria. In: (Ed. Rodriguez-Valera F.) Halophilic Bacteria. 2 (1988) 67-84. CRC Press Inc.
[28] T.M. Caton, I.R. Caton, L.R. Witte , M.A. Schneegurt, Archaeal diversity at the Great Salt Plains of Oklahoma described by cultivation and molecular analyses. Microb. Ecol. 58 (2004) 519- 528.

[29] B. M. Jothi, V. Indra, J. Hemapriya, S. Vijayanand, Bioprocess optimization of halophilic protease production by a halophilic bacterial Strain JS4. Int. J. Curr. Res. Aca. Rev. 3(4): (2015) 309-31.
[30] M. Khunt, N. Pandhi, Moderate halophilic bacterial community in excreta of wild ass (Equus hemionus Khur). Int J Biosci, 1(5) (2011) 31–37
[31] S.Gupta, P. Sharma, K. Dev, M. Srivastava & A. Sourirajan, A diverse group of halophilic bacteria exist in Lunsu, a natural salt water body of Himachal Pradesh, India, Springerplus, 4 (2015) 274.
[32] X.Li, and H. Yu, -Y, Characterization of a novel extracellular lipase from a halophilic isolate, *Chromohalobacte*r sp. LY7-8. Afr. J. Microbiol. Res., 6(14) (2012). 3516-3522.
[33] A. Pandey, C.R. Soccol, P. Nigam, V.T. Soccol Biotechnological potential of agro-industrial residues. I, sugarcane bagasse. Bioresour Technol 74 (2000) 69–80
[34] R.C. Kasana, R.Salawan, H. Dhar, S. Dutt, A. Gulati, A rapid and easy method for the detection of microbial celluloses on agar plates using gram's iodine. Curr Microbiol., 57(5) (2008). 53-507.
[35] M. Shahriarinour, M.N.A. Wahab, A. Ariff and R. Mohamad, Screening, isolation and selection of cellulosic fungi from oil palm empty fruit bunch fibre. Biotechnol. 10: (2011)108-11.
[36] P. Saravanan, R. Muthuvelayudham, T. Viruthagiri, Enhancedproduction of cellulose from pineapple waste by response surfacemethodogology, J. of Engineering. 8pages (2013).
[37] G. Fia, G. Giovani, I. Rosi. Study of ß-glucosidase production by wine-related yeasts during alcoholic fermentation. A new rapid fluorimetric method to determine enzymatic activity. J Appl Microbiol., 99: (2005) 509-517.
[38] P. Shivanand, G. Mugeraya, A. Kumar, Utilization of renewable agricultural residues for the production of extracellular halostable cellulase from newly isolated *Halomonas sp*. strain PS47. *Ann. Microbiol.* 63, (2013).1257–1263
[39] N. Benit, G. D. Biji, P. Vijayaraghavan, Optimization of carboxymethyl cellulase production from *Halomonas* sp. isolated from saltpan. *Int. J. Pharm. Biol. Sci.* **8**(3), 1025–1030 (2018).
[40]T. Gbenro, A. Adesanmi, M. Kamaraj, K. Saraswathi, F. P. Sindhu, Optimization of culture conditions for partially purified cellulase production by *Oceanobacillus* species isolated from wood industry soil in Chennai, India. *Int. J. Sci. Eng. Res.* **10**(2), 384–389 (2019).
[41] R. R. Colwell, R. A. Clayton, B. A. Ortiz-Conde, Jacobs, D. & Russek-Cohen, E. The microbial species concept and biodiversity. In: *Microbial Diversity & Ecosystem Function*, eds Allsopp, D., Colwell, R. and Hawksworth, L. pp. 3–15. Wallingford, UK (1995).
[42] A. Oren. Halophilic Microorganisms and Their Environments. Springer; Berlin/Heidelberg, Germany: 2002