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*Original Article*

# Molecular characterization of gene encoding halo tolerant amylase of *Bacillus paralicheniformis* isolated from Jazan region

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## ABSTRACT

**Objectives:** This study aims to characterize the gene encoding halo tolerant amylase of bacteria isolated from Jazan region.

**Materials and Methods:** Soil samples were collected from several area of Jazan region, KSA. The samples were serially diluted and plateted on starch agar plates. The amylase producing bacteria were detected by iodine test. To determine the halophilic amylase producing bacteria, several colonies were tested for their ability to grow at higher concentrations of NaCl ranging from 7 to 16%. The bacteria was identified by 16S rRNA and the full length amylase gene was fully identified by sequencing using specific primers.

**Results:** One bacterial halophilic isolate was able to grow on starch agar medium up to 14% NaCl. The Gram stain of the isolate indicated that it is Gram-positive, bacilli. The 16S rRNA gene homology study showed that the bacterial isolate was identified as *Bacillus paralicheniformis*. Two specific primers were designed named S1F, S1R, to amplify the amylase gene (AMY) region using PCR and the PCR product was sequenced. The sequencing results showed that the full-length amy gene of *B. paralicheniformis* was of 1452 encoding 483 amino acids. The expected M.Wt. of the protein expressed is of 55 KDa.

**Conclusion:** We report the isolation, identification, and characterization of an isolate of halophilic bacterium isolated from Jazan region. Based on molecular identification, this isolate was identified as *Bacillus paralicheniformis*. This bacterial strain has an α-amylase gene in its genome and is able to produce extracellular α-amylase. Based on the findings of this work we propose that *Bacillus paralicheniformis* amy gene could be cloned into expression vector for large scale production.

**Keywords:** *Bacillus paralicheniformis*, 16S rRNA, α-Amylase gene

## INTRODUCTION

Amylase (E.C.3.2.1.1) is a hydrolase enzyme that catalysis the hydrolysis of internal α-1, 4-glycosidic linkages in starch to yield products such as dextrin, maltose, and glucose. It is calcium metalo enzyme, depends on the presence of cofactor for its activity.[1] Amylase is a very common enzyme found in nature. Amylase obtained from different sources such as animals, plants, and microorganisms such as bacteria, fungi, yeast, and archaea.[2] The production of amylase from bacteria is cheaper and faster than other microorganisms. Furthermore, as mentioned-above genetic engineering studies are easier to perform with bacterial and they are also highly amenable for the production of recombinant enzymes.[3] A wide range of bacterial species isolated for amylase secretion. Most

of these species are *Bacillus* sp. (*Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus polymyxa*, *Bacillus mesentericus*, *Bacillus vulgaris*, *Bacillus cereus,* and *Bacillus* sp. *Ferdowsi scopus*), but amylase from *Rhodothermus gigantean, Chromohalobacter* sp., *Caldimonas taiwanensis*, *Geobacillus thermoleovorans,* and *Pseudomonas stutzeri* has also been isolated[4] *Halophilic* strains that produce amylase include *Haloarcula hispanica*, *Chromohalobacter* sp., and *Halomonas meridiana*. More studies the involving the isolation and improvement of novel strains will pave the way to creating important strains.[5]

Microbial production of amylase is more advantageous than other sources due to its economic costs; its production average is high and can be engineered to procure enzymes of required characteristics. Many industries such as medicinal, fine chemical, and paper industries may benefit from the microbial amylases; furthermore, microbial amylases contain a wide chain of industrial purposes because they are more stable with great genetic variation, great enzymatic effectiveness, and activity in a wide range of conditions (temperature, pressure, maximum pH, osmolarity. etc.), simple and cost effective production and easily standardized to obtain enzymes of required characteristics.[6,7]

## MATERIALS AND METHODS

### Collection of samples from soil

Soil samples were collected from various regions of Jazan, including Sabya, Baish, Shaqiq, and Farasan Island. Soil samples were taken from 3 to 5 cm depth after removing 5 cm from ground surface. These samples were collected into sterilized plastic bags and stored into iceboxes during their transport to the laboratory. In the laboratory, all samples were kept refrigerated until isolation.[8]

### Isolation of amylase producing bacterial isolates

One gram of soil was suspended in 9 ml sterile distilled water; diluted logarithmically up to $10^{-5}$ level. 1 ml of the dilution was seeded on starch agar plate (g/l) (peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, scratch 2.0, sodium chloride 5.0, and agar 15.0, HiMedia, India) and incubated at 30°C for 72 h. Bacterial isolates grown from the previous step were streaked on new starch agar plate and incubated at 30°C for 48 h. After the growth of colonies, iodine solution (g/100 ml water) (Iodine 0.34, Potassium iodide 0.66) was flooded with dropper for 30 s on the starch agar plate. The presence of blue color around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates positive result. The colonies were transferred and maintained on starch agar slants at 4°C.[9]

### Gram staining

The Gram stain was done according to[10] Gephardt *et al*., 1981.

### Screening of amylase producing halophilic bacterial isolates

To screen for halophilic amylase producing bacteria, the iodine positive bacterial isolates were grown on starch agar plate (g/l) (peptic digest of animal tissue 5.0, yeast extract 1.5, beef extract 1.5, strach 2.0, sodium chloride 5.0, and agar 15.0 from HiMedia company, India) supplemented with different NaCl concentrations including (7%, 10%, 12%, 14%, and 16%).[11]

### 16S rRNA Identification of halophilic bacterial isolates

The highest amylase producing halophilic bacteria were identified by using 16S rRNA method according to Abada *et al*., 2018.[12] Total genomic DNA was isolated from the bacterial cells using phenol–chloroform method as described by Ruzzante *et al.*, 1996.[13] Isolated DNA was checked for its quality and concentration by agarose gel electrophoresis using UV transilluminator. 16S rRNA region was amplified with universal primers named 27F 5'(AGAGTTTGATCMTGGCTCAG)3' and 1492R 5' (TACGGYTACCTTGTTACGACTT)3'. The PCR amplification was performed using a Thermal cycler (Bio-Rad), with a primary heating step for 2 min at 95°C, followed by 30 cycles of denaturation for 20 s at 95 C, annealing for 60 s at 55 C, and extension for 2 min at 72°C, then followed by a final extension step for 7 min at 72°C. Each 25 µl reaction mixture contained 2 µl of genomic DNA, 14.25 µl of MilliQ water, 2.5 µl of 10X buffer (100 mM Tris–HCl, pH 8.3; 500 mM KCl), 1.5 µl of $MgCl_2$ (25 mM), 2.5 µl of dNTPs mixture (dATP, dCTP, dGTP, and dTTP 10 mM), 1.0 µl of each primer (20 pmol/ml), and 0.25 µl of Taq DNA polymerase (Thermo). The amplified fragments were purified from agarose gels using (Gene JET TM PCR purification Kit) (Thermo). Finally, the PCR product was sequenced by GATC Company (GATC Company, South Korea) by the use of ABI 3730 xl DNA sequencer. After sequencing, sequence was retrieved from public databases using BLAST at the NCBI server.

### Molecular identification of amylase gene (AMY) from the bacterial isolates

#### *Design of specific primers*

Primers for PCR were designed based on highly conserved region from complete amylase sequences from already known *Bacillus sp*. To identify the AMY of *Bacillus paralicheniformis*, specific primers were designed named S1F 5' (GATTGCTGCCGCTGTTATTT) 3', S1R 5'(TATCTTTGAACATAGATCGAAACCGA)3', respectively (Macrogen Company, South Korea).

#### *Amplification and sequencing of Amy gene by PCR*

Total genomic DNA of the bacterial isolates prepared for 16S rRNA was used to amplify the amylase gene region. The PCR

mixture was prepared as previously mentioned. The PCR protocol was 5 min initial melting at 95°C, 30 s 95°C, 30 s 60°C, and 1 min at 72°C.[14] The PCR products were electrophorized on 1% agarose. The DNA was visualized under the UV-transilluminator (Prasad, 2014). The PCR products of Amy gene were purified from agarose gels using (Gene JET TM PCR purification Kit) (Thermo). The purified PCR products were sequenced by GATC Company (GATC Company, South Korea) by the use of ABI 3730 xl DNA sequencer.

### *Bioinformatics analysis*

The cleaned 16S rRNA and Amy gene sequences were compared to different bacteria in the reference to 16S rRNA and Amy gene on database of NCBI Nucleotide BLAST website using Blastn tool (https://blast.ncbi.nlm.nih.gov/Blast).[15]

## RESULTS

### Isolation and purification of amylase producing bacterial isolates

Soil samples were collected from various regions of Jazan, including Sabya, Baish, Al-Shuqaiq, and Farasan Island as shown in [Figure 1].

Soil samples were taken from 3 to 5 cm depth after removing 5 cm from ground surface. Ten-gram representative soil sample was suspended in 90 ml of sterile tap water and shaken thoroughly for 10 min. The samples were grown on starch agar plates and incubated at 30°C for 24–48 h. The growth of bacterial colonies was observed and studied morphologically. The total bacterial count was in a range of $4 \times 10^{-3}$ CFU. All bacterial isolates were white in color, circular with entire edge.

### Amylase screening test

Amylolytic activities of the soil bacterial isolates were screened on starch agar plates. After incubation at 30°C for 72 h, the zone of clearance was determined by flooding the plates with iodine solution. The potential amylase producers were selected based on zone of clearance diameter. Our results showed that the average of clear zone was between 0.5 and 2 cm [Figure 2]. Twenty isolates were selected for further studies.

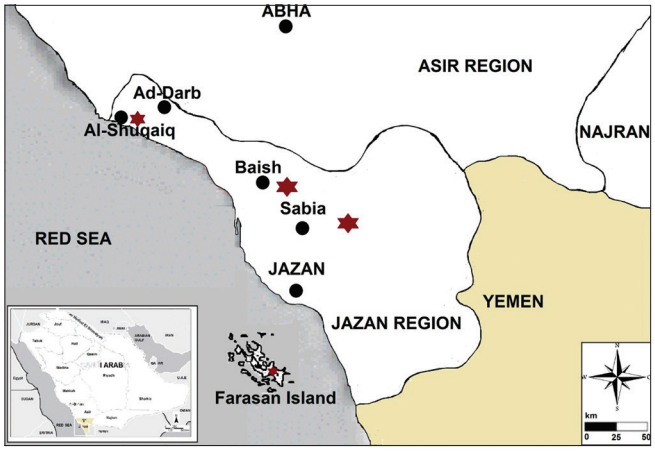


**Figure 1:** Area of the soil samples collection from Jazan region.

### Gram stain test

The Gram stain test showed that all isolates were Gram-positive bacilli.

### Selection of halophilic amylase producing bacteria

The twenty-amylase producing bacterial isolates were growing in different concentration of NaCl starch agar medium ranging from (7–16%), respectively. Only one sample named S1 was able to hydrolyze starch up to 14 % NaCl with clear zone of 1.4 ± 0.1 but there was no growth on 16 % NaCl.

### Molecular identification of bacterial strains by 16S rRNA

Our results showed that there are PCR products of around 1500 bp visualized [Figure 3]. The PCR products were purified using PCR purification kit (Qiagen). The purified PCR were sequenced using ABI Prism 3730XL DNA analyzer (Applied BioSystems). The sequence results of the PCR product using gene bank homology public databases BLAST at the NCBI server (http://www.ncbi.nlm.nih.gov/blast/). Our results showed that sample S1 identified as *B. paralicheniformis* [Figures 3 and 4].

### Molecular identification of AMY of *B. paralicheniformis*

*B. paralicheniformis* bacteria were chosen to identify their Amy gene since they can grow up to 14% NaCl. To identify the Amy of *B. paralicheniformis* specific primers were

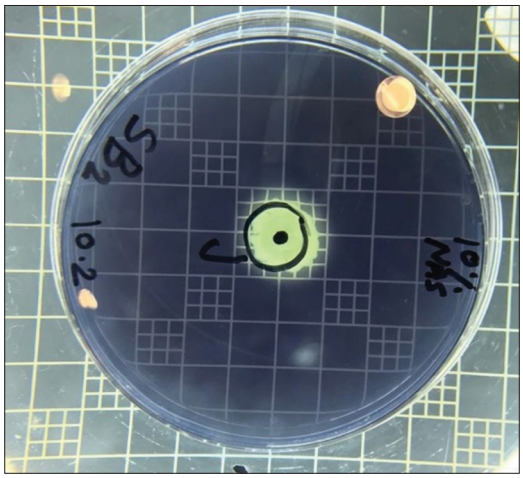

**Figure 2:** Amylase test activity for the amylase potent bacterial isolate from soil.

designed named S1F 3'(GATTGCTGCCGCTGTTATTT)5', and S1R 3'(TATCTTTGAACATAGATCGAAACCGA)5'; respectively. The specific primers were used to amplify the Amy gene using the PCR technique. The PCR protocol was 5 min initial melting at 95°C, 30 s 95°C, 30 s 60°C, and 1 min at 72°C. Our results showed that we have two PCR products of about 1500 bp when the *Amy* gene of *B. paralicheniformis* was amplified by S1F and S1R primers, respectively [Figure 5].

### *Sequencing the full length of amy gene of the strains B. paralicheniformis*

The PCR products of the amy genes of *B. paralicheniformis* were purified from the agarose gel by Multiscreen filter plate (Millipore Crop.). To get the full length of the Amy gene, the purified PCR products of the Amy genes were sequenced using S1F and S1R specific primers. The BigDye (R) terminator v3.1 cycle sequencing kit was used and ABI prism 3730XL analyzer with variant reporter version 2.1 as a software for variant analysis. The full length of Amy gene of B. paralicheniformis showed 93.73% homology with Amy gene of Bacillus licheniformis DSM 13 (https://blast.ncbi.nlm.nih.gov/Blast) and 79.8% homology with Amy gene of *Bacillus glycinifermentans* BGLY [Figure 6]. The DNA analysis of the full-length Amy gene was of 1452 bp encoding 483 amino acids. The expected M.Wt. of the protein expressed is of 55 KDa.

## DISCUSSION

### Isolation and molecular identification of halophilic amylase producing bacteria from Jazan region

Enzymes are not only essential for carrying out biochemical reactions within an organism but also their high specificity and catalytic characteristics have enabled them to be used in various industrial sectors for the production of a wide range of products. Amylase is one of those enzymes. Globally, amylase is being frequently used in food, textile, detergents, and paper industries. In addition, pharmaceutical and chemical industries use amylase on a regular basis to yield their products.[16] However, there is no local production of commercial amylase and thus lot of money is spent on the process of enzyme import. Harmful chemicals are also used as an alternative to amylase.[5] For this reason, it is necessary that a process is established to generate commercial amylase. Since industrial amylase is usually extracted from bacteria and fungi, it is mandatory to isolate a local high amylase producing strain. In this study, the main aim was to isolate halophilic amylase producing bacterial strain from different habitat in Jazan region and to identify the strain through genotypic methods. Furthermore, the full length sequence of the amylase gene was to identify.

Due to the availability of various types of bacteria in soil of Jazan, it was chosen as the source of bacterial isolation. The soil collected for this study was from Sabia, Baish, Al-Shuqaiq, and Farasan Island. In primary screening of the bacterial strains, it was observed that 20 out of hundreds of isolates were halophilic amylase producers. This was determined by growing the isolates on starch agar medium supplemented by different concentration of NaCl ranging from 7 to 16% and detecting any clear zone production around the bacterial colonies by adding Gram's iodine. The clear zones produced

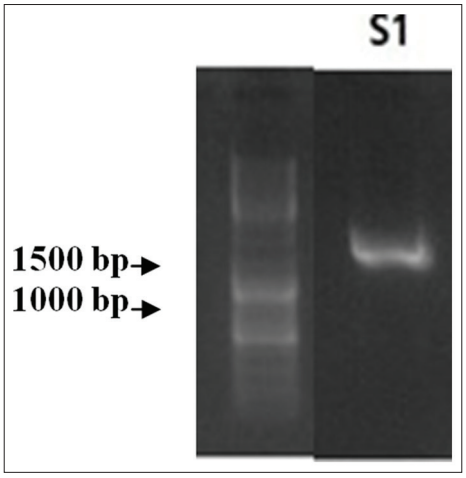


**Figure 3:** PCR amplified 16s rRNA of S1 isolate using primer 27F and 1492R

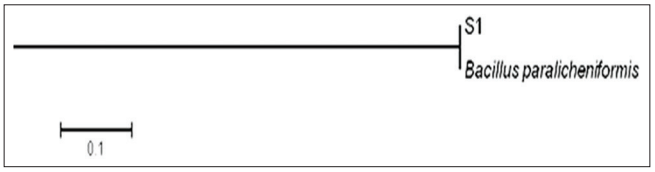


**Figure 4:** Phylogenetic tree analysis of the 16S rRNA gene sequence of the S1

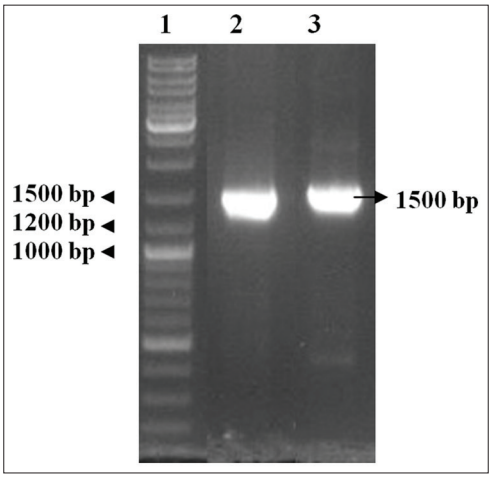


**Figure 5:** PCR product of the amy gene of the *Bacillus paralicheniformis*. Lane 1, DNA ladder marker, Lane 2, PCR product of primer S1F while Lane 3, PCR product of primer S1R.

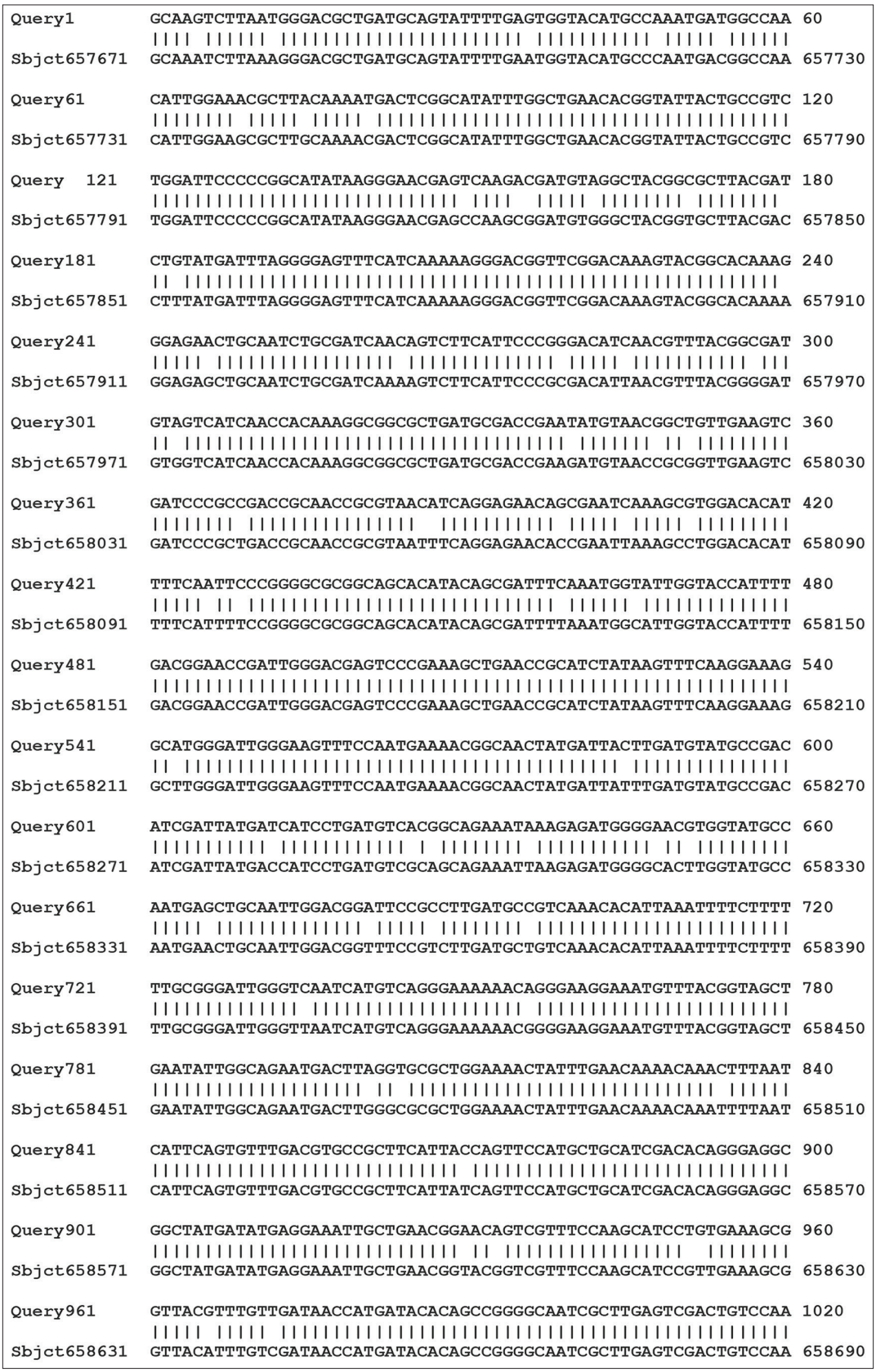


**Figure 6:** Alignment view of the amy *gene* of *Bacillus paralicheniformis* (Query) and the amy *gene* of the *Bacillus licheniformis* (Subject).

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Query1021    ACATGGTTTAAGCCGCTGGCTTACGCTTTTATTTTGACAAGAGAAGCAGGCTACCCGCAG 1080
             |||||||||||||||| |||||||||||||| | ||||| ||| | || ||||| |||
Sbjct658691  ACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGGATACCCTCAG 658750

Query1081    ATTTTCTACGGGGATATGTACGGGACGAAAGGAGCCTCGCAGCGCGAAATTCCTGCCCTG 1140
             |||||||||||||||||||||||||||||||| ||| ||||||||||||||||| ||
Sbjct658751  GTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAATTCCTGCCTTG 658810

Query1141    AAACACAAAATCGAACCGATCTTAAAAGCGAGAAAACAATATGCGTACGGAGCACAGCAT 1200
             |||||||||| |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct658811  AAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGGAGCACAGCAT 658870

Query1201    GATTATTTCGATCATCATAACATTGTCGGCTGGACGAGGGAAGGCGACAGCTCGGTTGCA 1260
             |||||||||| || ||| ||||||||||||||| ||||||||||||||||||||||||
Sbjct658871  GATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAGCTCGGTTGCA 658930

Query1261    AATTCTGGTTTGGCGGCGTTAATAACAGACGGACCCGGCGGGACAAAGCGAATGTATGTC 1320
             ||||| |||||||||| |||||||||||||||||| ||| ||||||||||||||||
Sbjct658931  AATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCGAATGTATGTC 658990

Query1321    GGCCGGCAAAACGCCGGTGAGACATGGCATGACATCACCGGAAACCGTTCCGATTCTGTT 1380
             |||||||||||||||||||||||||||||||||||| |||||||||||||| ||  | |||
Sbjct658991  GGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTCGGAGCCGGTT 659050

Query1381    GTCATCAATGCAGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGATCGGTTTCGATCTAT 1440
             ||||||||| | |||||||||||||||||||||||||||||||| |||||||| || |||
Sbjct659051  GTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGTTTCAATTTAT 659110

Query1441    GTTCAAAGATAG 1452
             ||||||||||||
Sbjct659111  GTTCAAAGATAG 659122
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**Figure 6:** (*Continued*)

were due to the absence of starch which was hydrolyzed by the amylase enzyme excreted by the bacteria. Our results showed that one sample was able to hydrolyze starch up to 14% NaCl but there was no growth on 16% NaCl. The S1 isolate was able to hydrolysis starch up to 14% NaCl with clear zone of 1.4. The 16S rRNA gene homology had revealed that S1 was identified as *B. paralicheniformis*, with 99% homology.

Spread plate technique was adopted to isolate discrete colonies.[17] Bacterial strains were screened for their ability to produce α-amylase by streaking them on starch agar plates. Screening and isolation of alpha-amylase producing fungi and bacteria from marine water and soil using enrichment techniques were reported by Medda and Chandra (1980) and Pretorius *et al.* (1986).[18,19] Different sources of fungi (seventy-eight strains of *Aspergillus oryzae*) were reported to be isolated from soils of different habitats by serial dilution method (Clark *et al.*, 1958).[20] The high yielding 17 isolates were partially identified using protocols given in Bergey's manual of determinative bacteriology. Similarly using protocols given in Bergey's manual of determinative bacteriology, the partial identification of bacteria was performed and identified as *Bacillus* spp.[21,22] Sneath *et al.* (1986); and Ajayi and Fagade (2003) have reported several bacterial isolates, based on the zone of starch hydrolysis. They were reported as *Bacillus* sp.1 and *Bacillus* sp.2, which produced more α-amylase enzyme. Bacterial identification was performed on randomly selected colonies using standard bacterial taxonomy procedures according to the 8th edition of Bergey's Manual of Determinative Bacteriology.

### Molecular Identification of amy gene of *B. paralicheniformis*

Our results showed that the full length of Amy gene of *B. paralicheniformis* had 93.73% homology with Amy gene of *Bacillus licheniformis* DSM 13 (https://blast.ncbi.nlm.nih.gov/Blast) and 79.8% homology with Amy gene of *Bacillus glycinifermentans* BGLY. The DNA analysis of the full length Amy gene was of 1452 bp encoding 483 amino acids. The expected M.Wt. of the protein expressed is of 55 KDa. While, moreover, *Haloarcula japonica* amylase was of 1989 nucleotides encodes 663 amino acids.[23] Evidently, the halophilic enzymes have typical enzymatic properties with additional polyextremities, namely, salt requirement for activity and stability, alkaline inclination, and resistance to unfolding even with high concentration of chaotropic reagents. This raises interest in their structure and adaptive features at molecular level. It has been reported that the full length of the amylase gene of *Natronococcus* sp. was of 1512 bp with signal peptide of 43 amino acids.[24] *Halothermothrix orenii* amylase gene was of 1545 encoding 490 amino acids[25,26]. Identified the

amylase gene of *Exiguobacterium* sp. was 1545 bp with Open Reading Frame (ORF) encodes 514 amino acids. [27]. Cloned a novel gene (*amyZ*) encoding a salt-tolerant and cold-active α-amylase (AMYZ) from a marine bacterium *Zunongwangia profunda*, and the protein was expressed in *Escherichia coli*. The gene has 1785 bp length and encodes an α-amylase of 594 amino acids with an estimated molecular mass of 66 kDa. Choubane *et al*.[28] (2015) isolated α-amylase-producing *Bacillus* sp. R2 from Red Sea water at Hurghada, Egypt. They prepared readily available medium using pasta cooking water as basal medium for industrial amylase production Kanpiengjai *et al*., (2015).[29] Isolated and characterized a maltose-forming amylolytic lactic acid bacterium *Lactobacillus plantarum* S21. The main hydrolysis by-products from starch, amylose, amylopectin, as well as glycogen were maltose (60%) and glucose (38%). The amylase gene encodes a protein consist of 910 amino acids including a signal peptide sequence. Khusro and Aarti (2015)[30] isolated five hyper-amylase-producing *Bacillus* strains from poultry feces soil sample. A maltotriose-producing α-amylase, from *Microbulbifer thermotolerans* DAU221, a newly isolated bacterial strain was purified and characterized by Lee et al. (2015).[31] Liaquat *et al*. (2015 [32] isolated partial characterized, and purified α-amylase by *B. subtilis* (RAS-1) and *Clostridium perfringens* (RAS-4) from anaerobic digester carrying anaerobic co-digestion of cow manure with fruit-vegetable waste and agricultural residues in continuous stirred tank reactor. Their results suggested the cost-effective production of α-amylases enzyme from organic waste during biogas production. The purified α-amylase with a molecular weight of approximately 80 kDa shared a sequence motif characteristic of the glycoside hydrolase family 13. Purification of extracellular amylase produced by the bacterium, *Corynebacterium alkanolyticum* ATH3 isolated from the distal intestine of *Anabas testudineus*, a freshwater fish, was carried out using column chromatography [33]. Best to our knowledge, this is the 1st study to identify halophilic amylase producing bacteria from soil and water of Jazan region. Further plans will include expression of the Amy gene of *B. paralicheniformis* encoded by the 1452, respectively, using different expression system. The recombinant form of the amylase of both isolates will be evaluated before large-scale production.

## CONCLUSION

We report the isolation, identification, and characterization of an isolate of halophilic bacterium isolated from Jazan region. Based on molecular identification, this isolate was identified as *Bacillus paralicheniformis*. This bacterial strain has an α-amylase gene in its genome and is able to produce extracellular α-amylase. Based on the findings of this work we propose that *Bacillus paralicheniformis* amy gene could be cloned into expression vector for large scale production.

### Declaration of patient consent

Patient's consent not required as there are no patients in this study.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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