Molecular and Biochemical Characterization of a Salt Tolerant α-Amylase Producing Isolate NH-25

Mahnaz Ahmad[1], Raheela Rahmat Zohra[1] and Shah Ali Ul Qader[2,*]

[1]Department of Biotechnology, University of Karachi, Karachi-75270, Pakistan

[2]Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan

Abstract: Halophiles are considered as important sources of extremozymes that are not only salt tolerant but can catalyze reactions efficiently under harsh conditions of various industrial processes. Among industrially important enzymes alpha amylase is used in many industries such as starch, pharmaceutical, detergent, paper, food, and textile. Due to wide applications there is a continuous need for novel sources of the enzyme to be discovered. One of the new variant from various amylase producing isolates of marine samples of Arabian Sea, an industrial dumping site and a rich natural source, particularly for microbes, a salt tolerant α-amylase producer was studied. This isolate based on cultural characteristics, biochemical tests and molecular characterization inferred from 16s rDNA sequencing identified as *Bacillus subtilis* NH-25.

Keywords: Salt tolerant, Amylase, Marine, Bacillus subtilis, 16s rDNA sequencing.

INTRODUCTION

Enzymes are biological catalyst that have assorted applications in numerous biotechnological products and processes. Among those, amyloglucosidases are one of the most important industrial enzymes used for the hydrolysis of starchy materials into oligosaccharides and, finally, into simple glucose units [1,2]. They belong to glycoside hydrolase family of enzymes (GH13 family) [3]. Amyloglucosidases are of three types, α-amylase (alternative names: 1,4-α-D-glucan glucanohydrolase; glycogenase) catalyze hydrolysis of α-1,4-glucosidic linkages in the interior of starch molecule in a random manner producing branched and linear oligosaccharides of different chain length, β-amylase (alternative names: 1,4-α-D-glucan maltohydrolase; glycogenase; saccharogen amylase) that hydrolyzes α-1,4-glucosidic linkages in polysaccharides so as to remove sequential maltose units from the non-reducing ends of the chains, and glucoamylase (alternative names: Glucan 1,4-α-glucosidase; amyloglucosidase; Exo-1,4-α-glucosidase; lysosomal α-glucosidase; 1,4-α-D-glucan glucohydrolase) hydrolyzes α-1,4 glycosidic bonds from the non-reducing ends of starch, resulting in the production of glucose and to a lesser extent, it also has the ability to hydrolyze α-1,6 linkages, also resulting in glucose as the end-product [4-6]. As one of the major stakeholder, amylase constitute a class of industrial enzymes having around 25%-33% of the enzyme share in the market [7-9]. They have impending applications in industries like food, pharmaceuticals, detergents, leather, starch conversion, alcohol production, textile and paper [10-13].

Amylases are obtained from animals, plants and microorganisms [14,15] however, for commercial production of enzymes microbial sources are preferred over others. Interestingly, there has been a lot of research on amylases obtained from various sources but not much work is done for obtaining amylases from marine environment, especially from microorganisms in deep sea environment [16]. Some marine microorganisms are reported to produce enzymes with industrially-important properties, such as stability at elevated temperature and alkaline pH conditions [17, 18].

In the present study, the taxonomic position of *Bacillus spp.* is determined through biochemical testing as well as 16S rDNA phylogenetic identification which is a useful technique for bacterial identification [19] so that the right taxonomic position of this isolate could be accurately assigned to identify its novelty.

MATERIALS AND METHODS

The marine water samples were collected from various sites of Arabian Sea and were screened for strains having amylogenetic activity [20]. Selected strain was inoculated onto Luria Agar media and the plates were incubated at 37°C for 24 h and were preserved on Luria Agar slants for additional analysis.

Morphological Characteristics

Organisms were observed through a phase-contrast light microscope (Optiphot XF-NT; Nikon) to determine

*Address correspondence to this author at the Department of Biochemistry, University of Karachi, Karachi-75270, Pakistan;
E-mail: saqader@uok.edu.pk

ISSN: 1814-8085 / E-ISSN: 1927-5129/18 © 2018 Lifescience Global

the Gram-staining and endospore-forming characteristics.

Cultural Characterization

For colonial morphology culture was streaked on Blood Agar plates and incubated at 37°C for 24 h. Motility test was performed by inoculating the culture in SIM Agar and monitoring the growth pattern after 18 h.

Biochemical Characterization

Biochemical Characteristic Analysis was performed according to Bergey's Manual of Systematic Bacteriology [21].

Overnight culture in Luria Broth and on Luria Agar was used to inoculate various media for biochemical tests. IMViC test, propionate utilization, nitrate reduction and tyrosine utilization tests were performed. Glucose, maltose, lactose xylose and mannitol fermentation was performed along with catalase, gelatinase, lecithinase production test. Organism was also checked for its ability to grow at pH 5.6 and in 6.5% sodium chloride.

Molecular Characterization

To obtain genomic DNA cells were lysed using lysozyme and protinase K. DNA was isolated by the method of chloroform isoamyl alcohol and its concentration and purity was monitored at 260nm and 280nm UV. Purified DNA was resolved on 2.0% Agarose Gel in 0.5X TBE buffer. Amplification of fragment of 16s rDNA gene was done by using MA3F 5'- CAGCMGCCGCGGTAATAC-3' & MA4R 5'-TGACGGGCGGTGTGTACAAG-3' primers. The conditions for reaction mixture for PCR were as follows: 5X PCR Buffer II, 2.5 U AmpliTaq Gold DNA polymerase, 25mM $MgCl_2$, 12.5 mM dNTP's, 5 µl DNA, Reverse and Forward Primer 2 µl each. Ultra-pure DEPC water was mixed to make up final volume of 50 µl. The program applied was 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 62°C for 45 seconds, 72°C for 45 seconds and extension at 72°C for 10 minutes

Purification of amplicon to remove contaminants was done by Promega Wizard SV Gel and PCR Clean Up system. Concentration of the purified product was measured spectrophotometrically. Both forward and reverse sequencing of PCR amplicons were carried out with reverse and forward primers using Big Dye Terminator V3.1 sequencing system. Electrophoresis was done by 2% Agarose gel using 0.5 X TBE buffer to monitor the amplification.

Unwanted terminator dye was removed by CENTRI –SEP Columns. Samples were eluted with Dideoxy Terminator Reaction Mixture and desiccated. Lyophilized samples were then loaded with Hi Di Formamide on 50 cm long capillary Array having 4.5% Acrylamide Gel and POP 6 polymer on ABI 3130 Genetic Analyzer. Consensus Sequence of the data was displayed by using Sequence Analysis Software. BLAST search was done for further Identification

RESULTS AND DISCUSSION

The marine isolate, obtained from the sea waters of Arabian Sea, was Gram-positive rods 0.5–1.0 µm in diameter and 5–8 µm in length in the exponential growth phase. These organisms showed chained structures and produced oval endospores located at sub-terminal positions (Figure **1A**); on Blood Agar showed large, circular, smooth, off white, opaque colonies with β- hemolysis (Figure **1B**). Organisms exhibited diffused growth showing existence of flagella (Figure **1C**). Isolate NH-25 is able to produce citrase, nitrate reductase and produces neutral end products after glucose utilization but it lacks tryptophanase (Figure **1D**). Isolate is able to grow at pH 5.6 (Figure **1E**) and catalase (Figure **1F**) and lecithinase producer (Figure **1G**) whereas unable to degrade tyrosine (Figure **1H**).

16S ribosomal DNA (rDNA)-based molecular characterization could attain identification, due to its presence of species-specific variable regions and universal distribution among bacteria. This molecular approach has been widely used for bacterial phylogeny, leading to the establishment of huge public-domain databases and their use in bacterial identification, as well as that of unique environmental and clinical microorganisms, novel or unusual isolates and collections of phenotypically identified isolates [22].

To determine the phylogenetic positions of this novel bacteria, the 16s RNA gene was PCR-amplified (Figure **2**) and sequenced (Figure **3**). A comparison of the obtained fragment with the sequences of all established Bacillus species indicated that strain clustered in the genus Bacillus, and 16S rDNA sequence similarities with the genus Bacillus were above 99%. The highest 16S rDNA sequence similarity was with *Bacillus subtilis* subsp. spizizenii str. W23, *Bacillus subtilis* B5n5, *Bacillus subtilis* subsp. subtilis

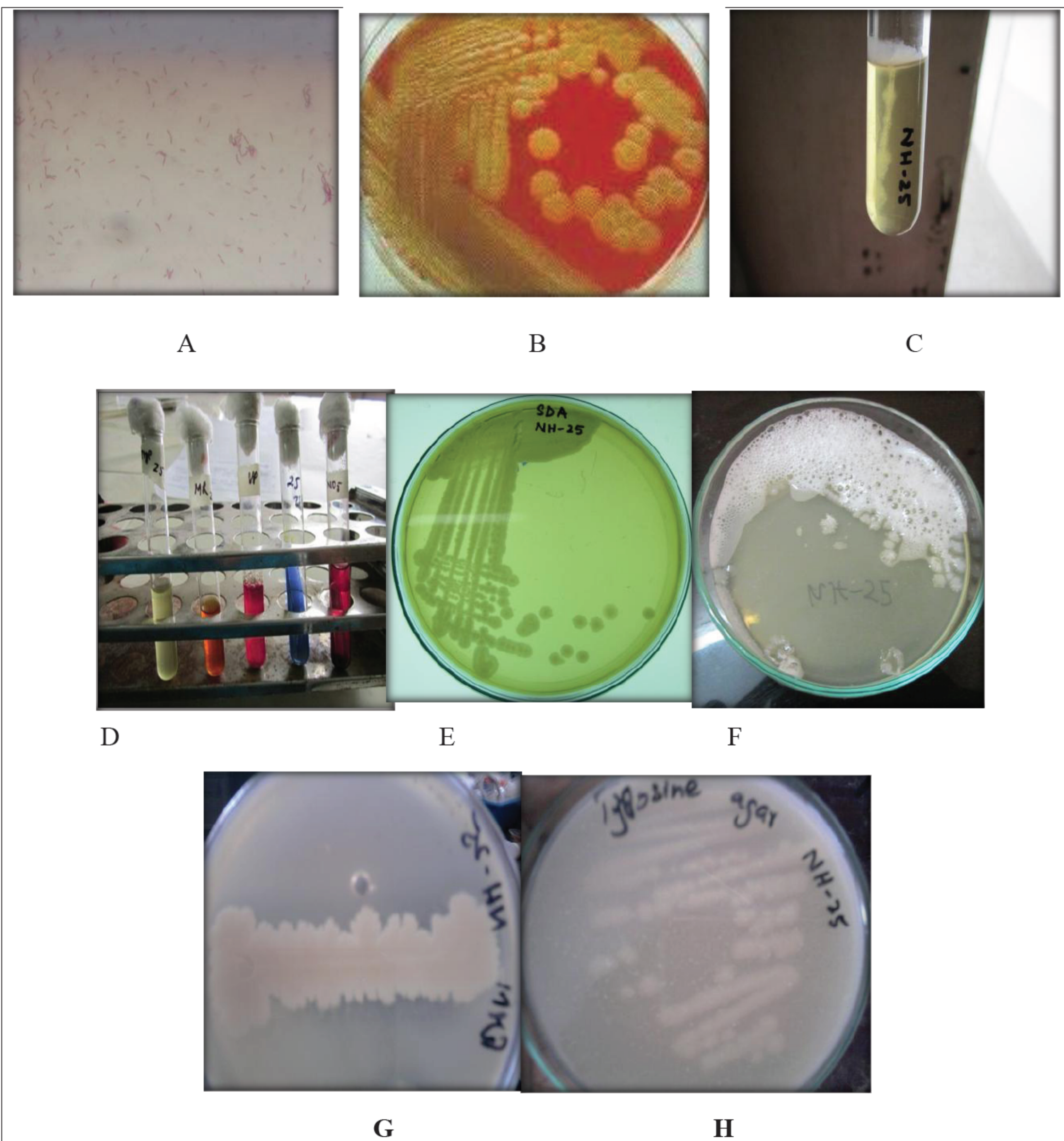

Figure 1: (**A**) Morphological characteristics shown by NH-25 after Gram's staining, (**B**): β –Hemolysis shown by NH-25 on blood agar plate, (**C**): the picture shows spreading growth of NH-25 in Sulfur Indole Motility medium confirm it is a motile organism, (**D**): After treatment with their respective reagents, tubes show Indole, M R and Citrate negative, whereas V P and nitrate test were positive. (**E**): Growth at pH 5.6 was monitored by growing the culture on SDA, (**F**): by flooding the plate with hydrogen peroxide, effervescence shows the releases of Oxygen gas as the result of catalase, (**G**): egg Yolk agar was utilized to check zone of precipitation formed by the action of lecithinase on egg yolk, (**H**): Tyrosine utilization was observed by the zone of clearing around the colony.

str. RO-NN-1, *Bacillus subtilis* subsp. subtilis str. 168, *Bacillus subtilis* subsp. spizizenii str. TU-B- 10, *Bacillus subtilis* BE517003, *Bacillus subtilis* BE517613, *Bacillus amyloliquefaciens* Y2, *Bacillus amyloliquefaciens* subsp. plantarum A543.3, *Bacillus amyloliquefaciens* subsp. plantarum YAU B9601-Y2, *Bacillus amyloliquefaciens* subsp. plantarum CAU B945, *Bacillus amyloliquefaciens* FZB42.

A phylogenetic tree based on an almost complete 16S rDNA sequence revealed the relationships between the isolated strain and established Bacillus

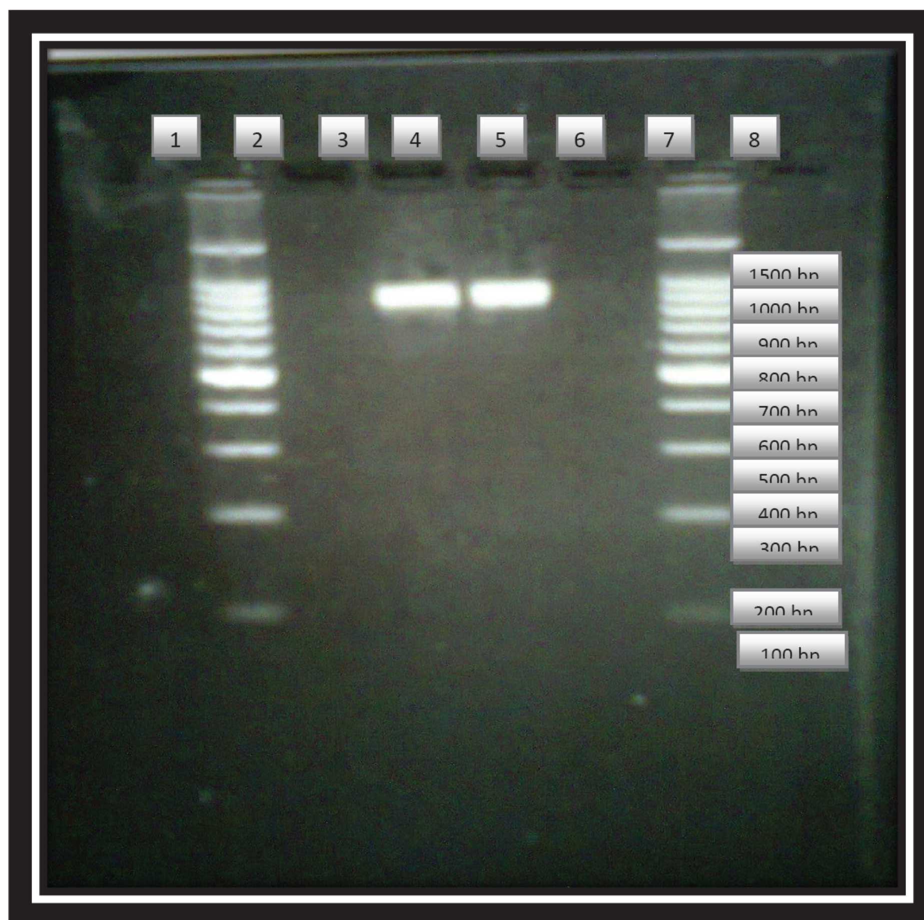

Figure 2: 2% Agarose Gel Electrophoresis of gene of 16S rRNA PCR product amplified for molecular characterization. Lane2 and 7 contain 100 bp ladder, lane 3 and 6 contains negative controls while 4 and 5 contains amplified product.

ACAGGGGGTCGGGATATTGGGCGTAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGT
GAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAG
AAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCT
CTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
TTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCAC
AAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGCATCGC
TAGTAATCGCGGATCAGCATGCCGCGGATGAATACGTTTCCCGGCCTTACC

Figure 3: Sequence of the16S rDNA gene of strain NH-25.

species, and supported the view that the isolated strain is members of the genus Bacillus (Figure **4**). The tree was created using the CLUSTAL W software [23] and phylogenetic distance was calculated by using the neighbor joining method [24,25].

Stackebrandt & Goebel (1994) stated that levels of similarity between 16S rDNA sequences that are less than 97% suggest that the strains do not belong to the same species [26].

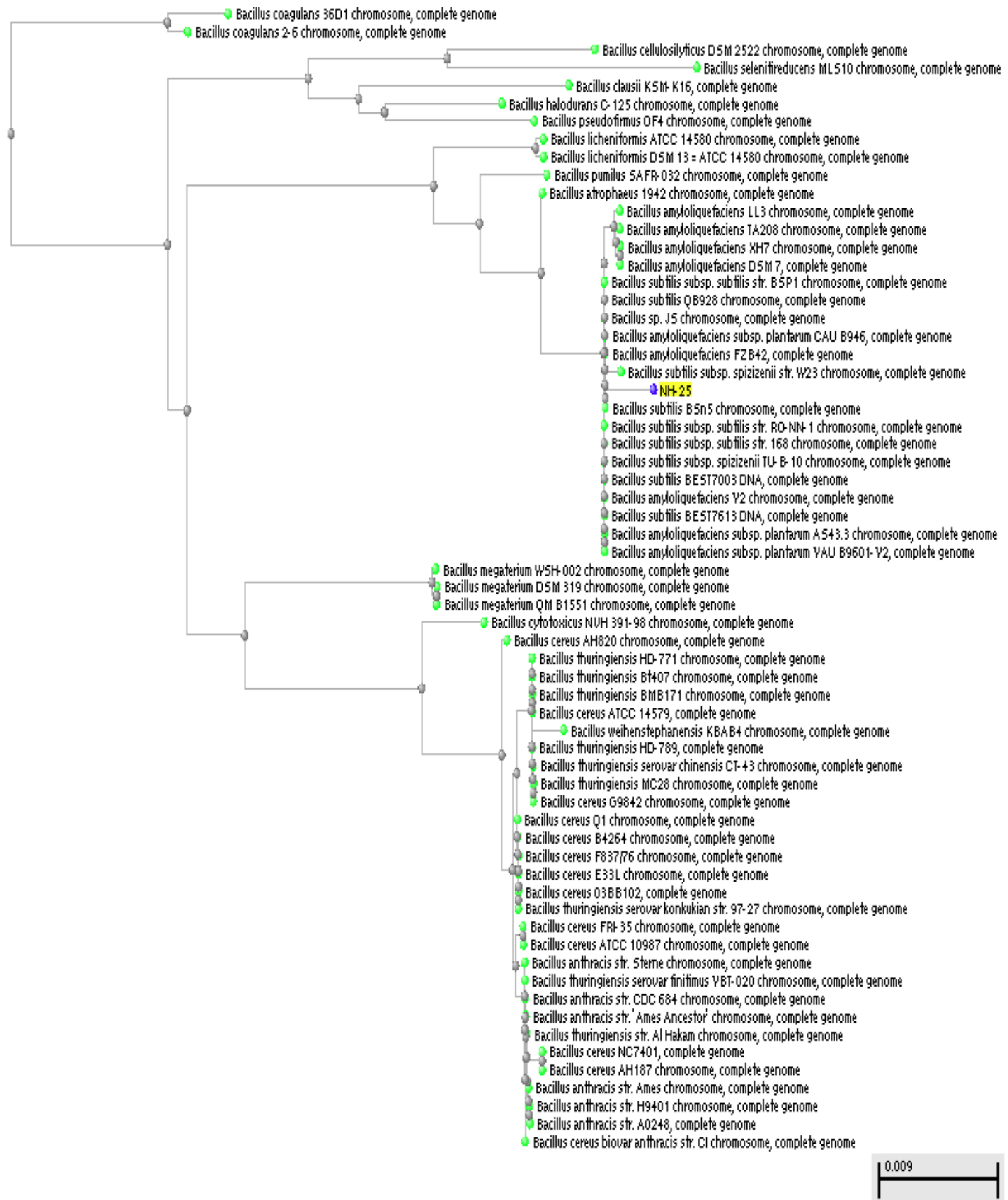

Figure 4: Phylogenetic tree of *Bacillus subtilis* NH-25 on the basis of 16S rDNA sequence.

The isolates can also be distinguished from established Bacillus species on the basis of a combination of the physiological and phenotypic properties.

The obtained fragment of 16 sDNA showed homology with Bacillus subtilis. The precise position of this isolate was recognized via phylogenetic identification. DNA-DNA relatedness showed relevance to *Bacillus subtilis.* Therefore, this bacterium was assigned as *Bacillus subtilis NH-25.*

ACKNOWLEDGEMENTS

The authors acknowledge Professor Dr. Tashmeem F. Razzaki, Professor Dr. Rana Muzzaffar and Dr.

Salma Batool, SIUT Karachi, Pakistan, for their guidance, support and for allowing to use one of the finest Molecular Biology setup in Pakistan.

REFERENCES

[1] Diler G, Chevallier S, Pöhlmann I, Guyon C, Guilloux M, Le-Bail A. Assessment of amyloglucosidase activity during production and storage of laminated pie dough. Impact on raw dough properties and sweetness after baking. J Cereal Sci 2015; 61: 63-70.
https://doi.org/10.1016/j.jcs.2014.10.003

[2] Malik S, Iftikhar T, Haq I, Khattak MI. Process optimization for amyloglucosidase by a mutant strain of *Aspergillus niger* in stirred fermenter. Pak J Bot 2013; 45: 663-666.

[3] Bordbar AK, Omidiyan K, Hosseinzadeh R. Study on interaction of α-amylase from *Bacillus subtilis* with cetyl trimethylammonium Bromide. Colloids Surf. B: Biointerfaces 2005; 40: 67-71.
https://doi.org/10.1016/j.colsurfb.2004.10.002

[4] Malik SK, Mehta S, Sihag K. Isolation and screening of Amylase Producing Fungi. Int J Curr Microbiolo App Sci 2017; 6(4): 783-788.
https://doi.org/10.20546/ijcmas.2017.604.098

[5] Khan JA, Priya R. A study on partial purification and characterization of extracellular amylases from Bacillus subtilis. Adv Appl Sci Res 2011; 2(3): 509- 519.

[6] Mertens JA, Skory CD. Isolation and characterization of a second glucoamylase gene without a starch binding domain from *Rhizopus oryzae*. Enzy Microbia Technol 2006; 40: 874-880.
https://doi.org/10.1016/j.enzmictec.2006.07.003

[7] Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B. Microbial α-amylases: A biotechnological perspective. Process Biochem 2003; 38: 1599-1616.
https://doi.org/10.1016/S0032-9592(03)00053-0

[8] Ahmad M, Zohra RR. Isolation, characterization and partial purification of α-amylase from a marine bacillus NH-25. Pak J Biochem Mol Biol 2012; 45(3): 175-177.

[9] NZBIO. Global Alpha-Amylase (α-Amylase) Market Research Report 2017 [Updated on June 30 2017, cited on March 23, 2018] Available from http: //www.nzbio.org.nz/global-alpha-amylase-%CE%B1-amylase-market-research-report-2017

[10] Rajagopalan G, Krishnan C. Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresour Technol 2008; 99: 3044-3050.
https://doi.org/10.1016/j.biortech.2007.06.001

[11] Sivaramakrishnan S, Gangadharan D, Nampoothiri KM, Soccol CR, Pandey, A. α-amylases from microbial sources—An overview on recent developments. Food Technol Biotechnol 2006; 44: 173-184.

[12] De Souza PM, Magalhães PO. Application of microbial amylase in industry-a review. Braz J Microbiol 2010; 41: 850-861.
https://doi.org/10.1590/S1517-83822010000400004

[13] Mukesh DJ, Jayanthi S, Amutha D, Monica D, Bala. Purification and Characterization of α-Amylase and -Galactosidase from *Bacillus* Sp. MNJ23 Produced in a Concomitant Medium. American-Eurasian. J Agric Environ Sci 2012; 12: 566-573.

[14] Andualem B. Isolation and screening of amylase producing thermophilic spore forming Bacilli from starch rich soil and characterization of their amylase activities using submerged fermentation. Int Food Res J 2014; 21: 831-837.

[15] Fentahun M, Kumari PV. Isolation and screening of amylase producing thermophilic spore forming Bacilli from starch rich soil and characterization of their amylase activity. Afr J Microbiol Res 2017; 11: 851-859.

[16] Chakraborty S, Khopade A, Kokare C, Mahadik K, Chopade B. Isolation and characterization of novel α-amylase from marine Streptomyces sp. D1. J Mol Catal B 2009; 58: 17-23.
https://doi.org/10.1016/j.molcatb.2008.10.011

[17] Ventosa A, Nieto J. Biotechnological applications and potentialities of halophilic microorganisms. World J Microbiol Biotechnol 1995; 11: 85-94.
https://doi.org/10.1007/BF00339138

[18] Simair AA, Khushk I, Qureshi AS, Bhutto MA, Chaudhry HA, Ansari K.A, Lu C. Amylase Production from Thermophilic *Bacillus* sp. BCC 021-50 Isolated from a Marine Environment. Fermentation 2017; 3: 25.
https://doi.org/10.3390/fermentation3020025

[19] Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, Nadarajan R, Brodie EL, Lynch SV. Use of 16S rRNA Gene for Identification of a Broad Range of Clinically Relevant Bacterial Pathogens. PLOS ONE 2015; 10(2): e0117617.
https://doi.org/10.1371/journal.pone.0117617

[20] Ahmad M, Zohra RR. Isolation, characterization and partial purification of α-amylase from a marine bacillus NH-25. Pak J Biochem Mol Biol 2012; 45(3): 175-177.

[21] Logan NA, De Vos P. Bacillus. In: De Vos P, Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, *et al.* editors. Bergey's manual of systematic bacteriology. 2nd ed. New York, NY, USA: Springer; 2009; pp. 21-128.

[22] Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult, D. 16S Ribosomal DNA Sequence Analysis of a Large Collection of Environmental and Clinical Unidentifiable Bacterial Isolates. J Clin Microbiol 2000; 38(10): 3623-3630.

[23] Thompson JD, Higgins DG. Gibson, TJ. CLUSTAL W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 1994; 22: 4673-4680.
https://doi.org/10.1093/nar/22.22.4673

[24] Wang W, Sum M. Phylogenetic relationships between *Bacillus* species and related genera inferred from 16s rDNA sequences. Braz J Microbiol 2009; 40: 505-521.
https://doi.org/10.1590/S1517-83822009000300013

[25] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4: 406-425.

[26] Stackebrandt E, Goebel BM. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994; 44: 846-849.
https://doi.org/10.1099/00207713-44-4-846

Received on 21-03-2018 Accepted on 21-04-2018 Published on 16-05-2018

https://doi.org/10.6000/1927-5129.2018.14.27

© 2018 Ahmad *et al.*; Licensee Lifescience Global.
This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.