The Secondary Structural Models (16S rRNA) of Alkaline Amylase Producing *Bacillus* species strain SP-RM2: Phylogenetics Analysis

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Abstract: *Bacterial isolates especially the Bacillus strains have gained an edge over other organisms across the globe. Enzymes derived from alkaliphilic Bacillus make up about half of the total industrial enzyme market. Detergents, textiles, starch, baking and animal feed are the major industries which utmost produce and use enzymes. Alkaline α amylases are among the most important enzymes and are of great significance for biotechnology. In the present investigation, one alkalitolerant bacterial isolate coded RM2 was characterized by partial 16S rRNA gene sequencing due to its ability to produce alkaline amylase at pH 8.0. The amplified consensus 16S rRNA gene sequence (RM2) was further searched against nucleotide database using Blast program to find out its potential homologs. Twelve closest bacterial spp. were identified and the phylogenetic analysis revealed the target bacteria (RM2) isolate belong to genus Bacillus. RM2 has shown relatedness to Bacillus flexus strain IFO15715 in sequence based divergence study whereas secondary structure of 16S r-RNA of Bacillus sp. strain SP-RM2 did not show any relatedness to Bacillus flexus strain IFO15715 even though they shared sequence based similarity.*

Keywords: *Alkaline α amylase, Bacillus, Phylogenetic analysis.*

Introduction

Extremophilic microbes have evolved several structural and chemical adaptations which allow them to survive and grow in extreme environments. The enzymes of these microbes, which function in extreme environments (extremozymes), have several biotechnological applications. Enzymes from extremophiles offer versatile tools for sustainable developments in a variety of industrial applications due to specific stability under extreme conditions, improved use of raw materials and decreased amount of waste products. Thus extremozymes offer new opportunities for biocatalysis and biotransformation [5].

Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes. Currently, only 1-2% of the microorganisms on the Earth have been commercially exploited and amongst these there are only a few examples of extremophiles [7].

Microbial enzymes are widely used in industrial processes due to their low cost, large productivity, chemical stability, environmental protection, plasticity and vast availability [2, 11]. Enzymes derived from alkaliphilic *Bacillus* make up about half of the total industrial enzyme market [16]. Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%) are the main industries, which use about 75% of industrially produced enzymes [4, 6, 9, 15]. Alkaline α amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25-30% of the world enzyme market [1, 13].

Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes [8]. These enzymes are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custards and chocolates to dextrins and other smaller oligosaccharides [12]. Amylases having activity at alkaline pH, maintain the necessary stability under detergent conditions [10].

16S rRNA gene sequencing is the widely used modern molecular approach for identifying bacteria as the genotypic methods are far more accurate than morpho-physiological or phenotypic identification [3].

The objective of the investigation was to characterize alkaline α amylase producing bacteria by morpho-physiological and 16S rRNA gene sequence analysis. The rRNA secondary structures of these bacteria were also predicted and evaluated for its divergence.

Materials and methods

Alkaline amylase producing bacterial strain

In the previous study alkalitolerant bacterial strains were isolated from red mud sample of Damanjodi district, Orissa, India. All the bacterial isolates were screened for extracellular enzymes. The isolate showing highest zone of clearance on an alkaline pH was selected for further characterization and molecular identification.

Evolutionary analysis using 16S rDNA sequences

Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S r-RNA partial sequence was deposited at GenBank of NCBI through BankIt on line submission tool for the accession number. Homologs 16S r-RNA sequences were identified for the isolate using BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) available at NCBI server. Multiple Sequence Alignment using ClustalW tool was carried out and the output of that is used for construction of phylogenetic tree in MEGA 6.0 (Molecular Genetics Evolutionary Analysis) software [18] using the Neighbor-joining (NJ) for RM2 [14]. The resultant phylogenetic tree topologies were evaluated by bootstrap analysis of data sets based on 1000 resamplings. Resampling methods allow reuse of sample data for drawing statistical inference.

RNA secondary structure folding

16s rRNA of bacterial sequences were predicted in Mfold web server [17, 19] to study and compare folding patterns among them. The minimum Gibb's free energy, ΔG was computed by the Mfold algorithm for each sequence, as lowest ΔG maps to evolutionary stability of RNA structures. During RNA structure prediction the temperature was fixed to 37 °C and assumed to be constant within the range of temperatures that might occur in vivo or in the laboratory. RNA sequences were taken as linear; the ionic conditions were fixed at $[Na^+]$ = 1 M and

[$Mg^{++}M$] = 0. The predicted secondary structures were compared according to the number of stems, loops (exterior/interior), multiple loops, hairpin loops and bulges to study the conservation at the structural level.

Results and discussion

Alkaline amylase producing bacterial strain

Among all the alkalitolerant bacterial isolates from red mud sample, one isolate (RM2) was found to be amylase positive at 37 °C in the starch agar plates at pH 8.0. It showed > 10 mm diameter zone at pH 8.0 as shown in Fig. 1.

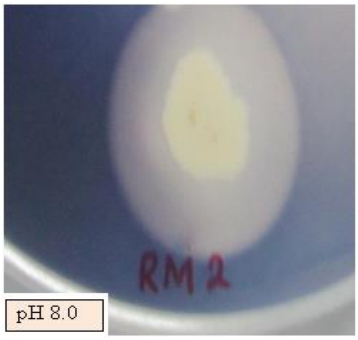


Fig. 1 Amylase plate assay of RM2 at pH 8.0

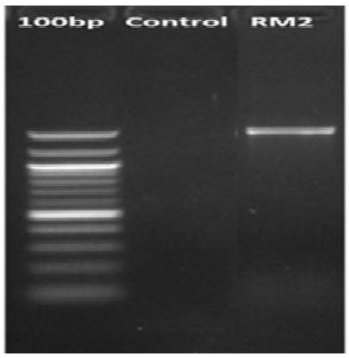


Fig. 2 Gel image of RM2 16S rDNA amplicon

Molecular identification of potent isolate (RM2)

Two 16S rDNA amplicons (one forward and the other reverse) of RM2 isolate were selected for constructing consensus sequence to minimize the amplification error. The amplified consensus 16S rDNA sequence of the alkaline amylase producing bacterial isolate RM2 bearing Accession No KR814820 was further searched against nucleotide database using Blast program to find out its potential homologs as presented in Figs. 2, 3 and 4, respectively. Twelve homologous bacterial spp. were identified which revealed that the target bacterial isolate belong to genus *Bacillus*. The detail about homologs, its identity and query coverage are presented in Table 1.

Phylogenetic tree construction of RM2 for divergence study

Evolutionary divergence studies among 16S rRNA gene sequences of selected bacterial species were carried out with RM2 and homologs of RM2. The uncultured Planctomycetes Sva0503 as an out-group has been taken (Fig. 6). Multiple sequence alignment and construction of tree were performed using Neighbor-joining algorithm (Figs. 5 and 6). The 13 *Bacillus* species are diverged into two major groups (I & II). Uncultured *Planctomycetes* Sva0503 diverged most from the two groups showing as an outgroup. In Group I, *Bacillus aryabhattai* strain B8W22 distantly related to *Bacillus flexus* strain IFO15715 but RM2 is closely related to *Bacillus flexus* strain IFO15715. But in another group i.e in Group-II, *Bacillus kyonggiensis* strain NB22 is distantly related to *Bacillus niacini* strain IFO15566.

RNA secondary structure analysis

Secondary structure of 16S r-RNA of *Bacillus* sp. strain SP-RM2 did not show any relatedness to *Bacillus flexus* strain IFO15715 even though they shared sequence based similarity. The structure of the outgroup showed complex 16S r-RNA folding pattern than the other *Bacillus* spp. (Fig. 7).

AAGGTTACCCCACTCGACTTCGGGCTGATTACAAACTCTCGTGGTGTGACGGGCGGTGTG
TACAAGGCCCGGGACACGTATTCACCGCGGCATGCTGATCCGCGATTACTACGCGATTCC
GGAGCTTCATGTAGGCGAGCATTGCAGCCTACATATCCGAACATTGAGAGATGGTTTCGG
CTATGGGATTGGCTTGACCTCGCGGTCTTGCAGCCCTTTGGTGAGGTACCATCTCATTGT
AGCAACGATGTGTAGCCCAGGTCATAAGGGGCATGATGACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGTCACCGGCAGTCACTCTTCCGCAATGGCCAACTAAATGCTGAC
GGAGCAACGCCGCGTGAGTGATGAAGGCTTTGCGGGACTTAACCCAACATCTCACGACAC
GGAAGAACAAGTACAAGAGTACACCACCTGTCACTCTGTCCCTACCTAACCAGAAAGCCA
CGGCTAACTACGTGCCAGCAGCCGCGGTCAAGACCTAGGTGGCAAGCGTTATCCGGAATT
ATTGGGCGTAAAGCGCGCGCAGGACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTT
CAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAAGG
GCGGAAACCCTCTAACACTTAGCACTCAAATGCGTAGACGGCGTGGACTACCAGGAGTGG
CGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGTTACAGACCA
AAAAGCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTT
AGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTAC
GGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCCTTACCAGCTACGTCTTGACATCCTCTGACAAC
TCGTAGAGATAGAGC

Fig. 3 16S rDNA-RM2 isolate (consensus sequence)

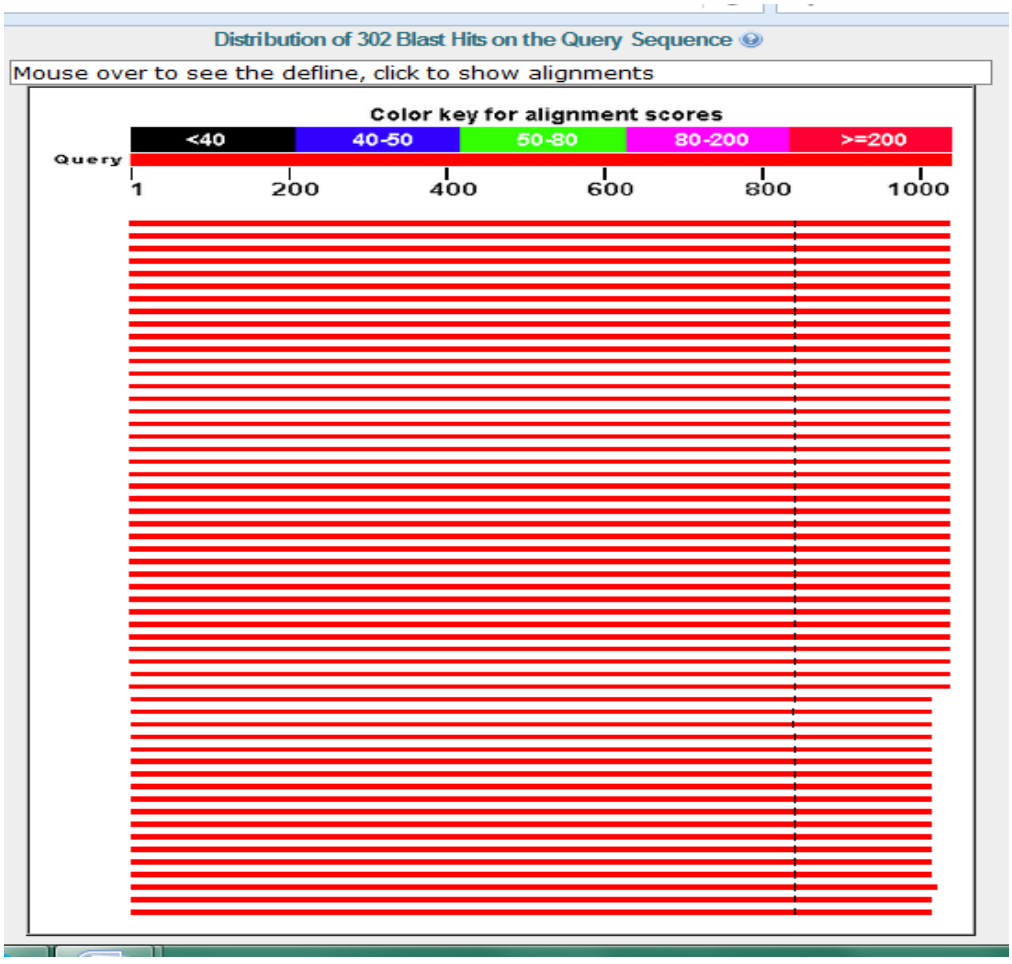


Fig. 4 Homologs identification of RM2
using Basic Local Alignment Search Tool (BLAST)

Table 1. List of closest homologs of RM2 isolate

Description	Max score	Total score	Query cover	Identity	Accession No.
Bacillus flexus NBRC 15715	619	1281	100%	81%	NR_113800.1
Bacillus flexus IFO15715	619	1281	100%	81%	NR_024691.1
Bacillus megaterium IAM 13418	610	1272	99%	81%	NR_043401.1
Bacillus aryabhattai B8W22	610	1260	99%	81%	NR_115953.1
Bacillus megaterium NBRC 15308	610	1272	99%	81%	NR_112636.1
Bacillus flexus SBMP3	608	1242	100%	81%	NR_118382.1
Bacillus megaterium ATCC 14581	606	1268	99%	81%	NR_117473.1
Bacillus megaterium QM B1551	599	1244	99%	81%	NR_074290.1
Bacillus niacini NBRC 15566	582	1183	99%	81%	NR_113777.1
Bacillus ginsengisoli strain DCY53	582	1166	99%	81%	NR_109068.1
Bacillus niacini strain IFO15566	582	1183	99%	81%	NR_024695.1
Bacillus kyonggiensis strain NB22	579	1174	99%	81%	NR_132682.1

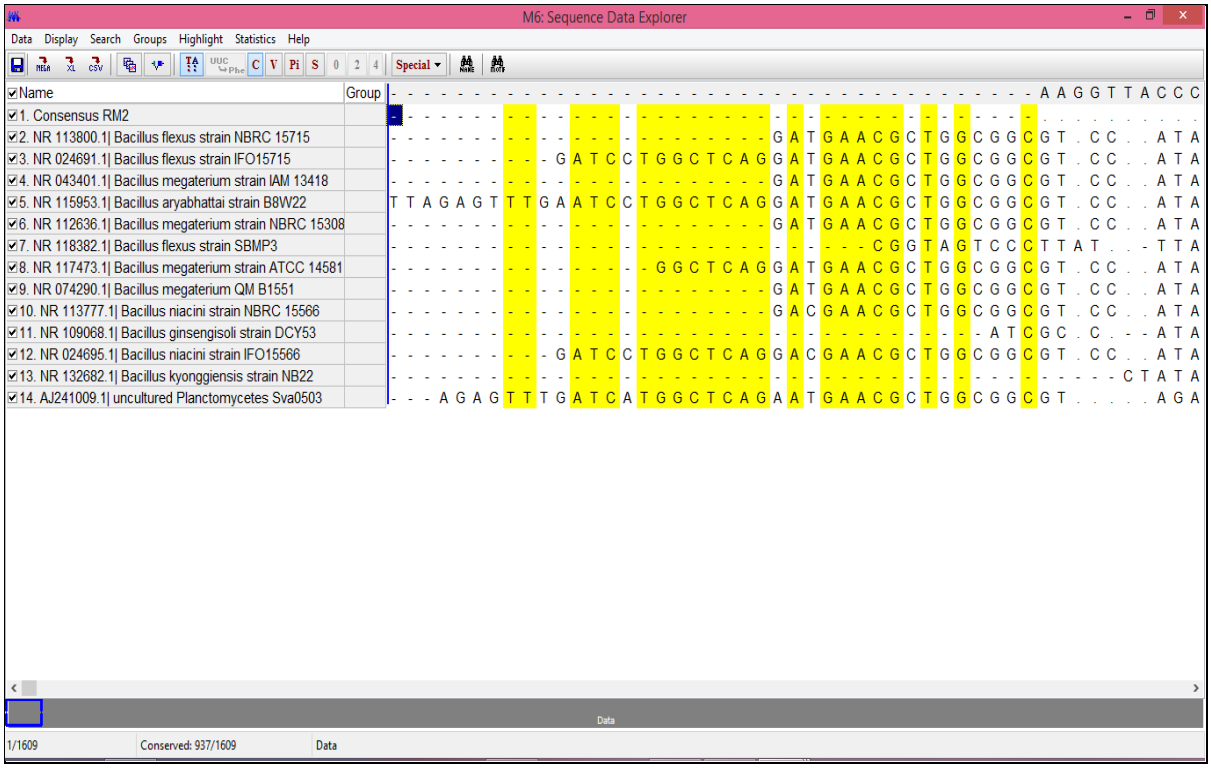


Fig. 5 Multiple Sequence Alignment of RM2
showing 937 conserved sites out of 1609 total sites

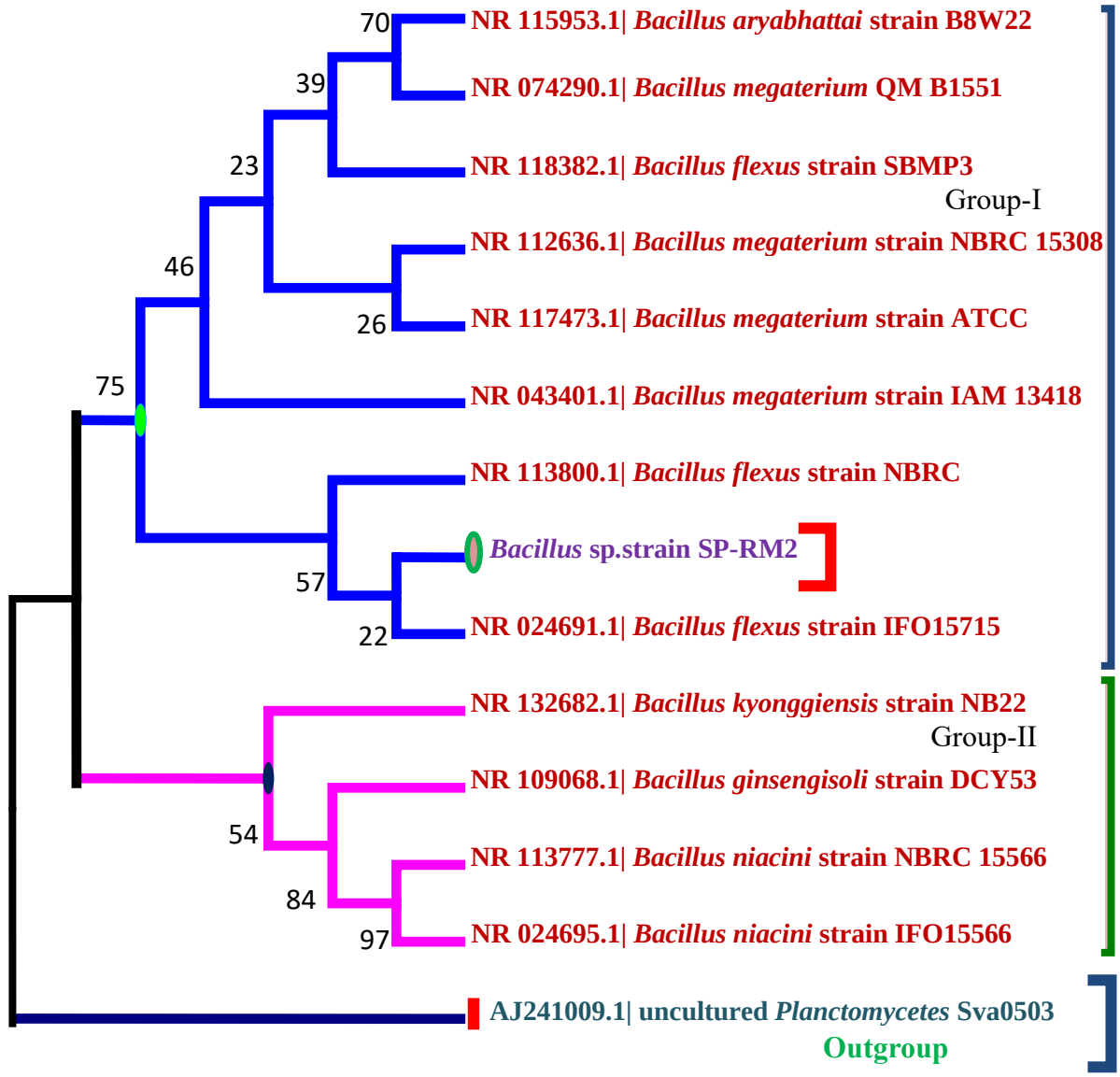
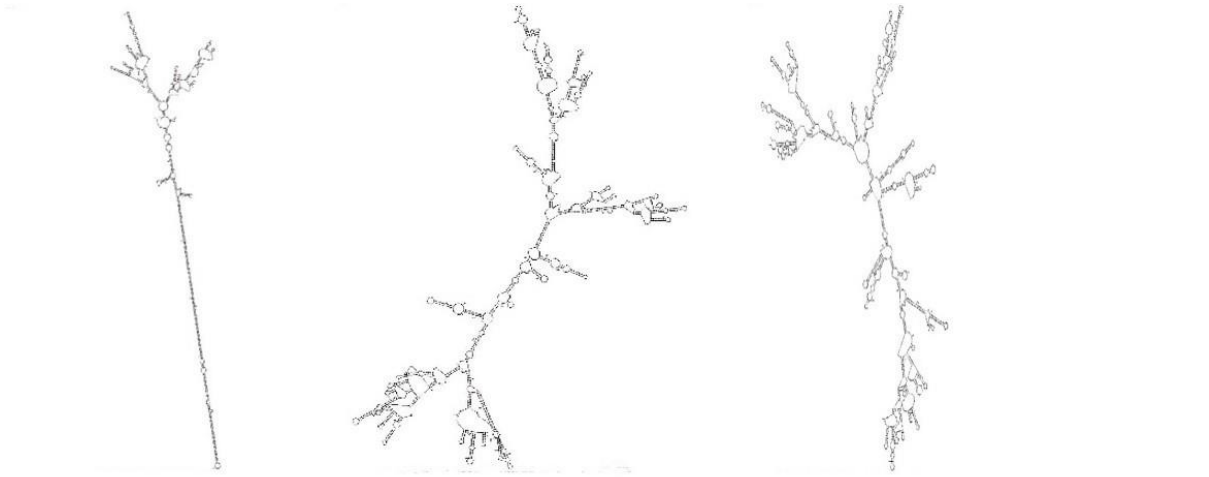


Fig. 6 Phylogenetic Neighbor Joining Tree of RM2
showing divergence among different group of *Bacillus* species

Characterization of Bacillus sp. strain SP-RM2 isolate

The result of cultural, biochemical, physiological and enzymatic tests of *Bacillus* sp. SP-RM2 isolate is presented in Table 2. *Bacillus* sp. strain SP-RM2 is Gram positive rod, motile and facultative anaerobe. On solid medium it showed creamy white, medium size, margin irregular and flat elevation. Biochemically it was positive for VP, citrate utilization, Catalase, Esculin hydrolysis, urease and nitrate reductase and negative for MR, Oxidase and Indole production. Among the different sugar tests, the isolate was found to utilize only mannitol, fructose, cellobiose and salicin. The organism was able to grow at 37 and 50 °C and it was able to grow up to 10% NaCl concentration. No growth was seen at 15% NaCl concentration on nutrient agar plate. Enzymatic study revealed that the isolate was positive for amylase with zone of clearance >10 mm diameter followed by lipase and protease (<10 mm diameter) at pH 8.0.



Bacillus sp. strain SP-RM2 *Bacillus flexus* strain IFO15715 Planctomycetes Sva0503(outgroup)

Fig. 7 Secondary structure analysis of *Bacillus* sp. strain SP-RM2, *Bacillus flexus* strain IFO15715 and Planctomycetes Sva0503(outgroup)

Table 2. Characteristics of *Bacillus* sp. SP-RM2

Characteristics			***Bacillus* sp. SP-RM2**
Cultural		Colony on agar	Irregular form, flat elevation
		Color	Creamy white
		Other	motile
Morphological		Gram staining	Gram positive rods
		Endospore staining	+
Biochemical		MR	-
		VP	+
		Indole production	-
		Esculin hydrolysis	+
		Citrate utilization	+
		Catalase	+
		Nitrate	+
		Urease	+
		Oxidase	-
Enzymatic		Amylase	+++
		Lipase	++
		Protease	++
		Pectinase	-
		Cellulase	-
Physical	Temperature, [°C]	37	+
		50	+
	NaCl tolerance, [%]	5	+
		10	+
		15	-

Conclusion

Alkaline amylase finds application in many industries such as starch, textile and especially in detergent industry where it can be used as an ingredient in detergent for laundry and dish-washing. Due to the wide application offered by these extremozymes, the researchers are giving priority to isolation, characterization and most importantly identification of microorganisms (mainly bacterial isolates) showing desired properties. For production of enzymes for industrial use, isolation and characterization of new strains is an essential step. Microbial identification is done to identify pathogens, food-spoilage linked species and bacterial pollutants of water or environmental prokaryotes expressing some properties which could be exploited. Estimating the phylogenetic position of bacterial isolates by genetic sequence comparisons is considered as the gold-standard in taxonomy. Thus conventional phenotypic tests used for species differentiation are nowadays coupled with16S ribosomal RNA sequences.

In the present study, the bacterial isolate (RM2) isolated from red mud sample was found to be positive for alkaline amylase on starch agar plate at pH 8.0. It was further characterized by various morphological, cultural, biochemical and enzymatic tests. In addition, analysis of molecular phylogenetic tree and RNA secondary structure catapulted better identification of the bacteria. The isolate (RM2) was identified based on phenotypic characteristics, phylogenetic positions based on 16S rRNA gene analysis and sequences submitted to Gen Bank. 16S rRNA gene analysis confirmed that the isolate belonged to the genus *Bacillus*. The phylogenetic approach revealed that our amylase producing isolate *Bacillus* sp. strain SP-RM2 is closely related to *Bacillus flexus* strain IFO15715 however the secondary structure analysis 16S r-RNA of *Bacillus* sp. strain SP-RM2 did not show any significant similarity with *Bacillus flexus* strain IFO15715. Uncultured Plantomycetes was taken as outgroup for the divergence study. In the present investigation, uncultured *Planctomycetes* Sva0503 has diverged the most from the two groups in the phylogenetic tree, aptly taken as an outgroup and its structure showed complex 16S r-RNA folding pattern than the other *Bacillus* spp.

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References

1. Azad M. A., J. H. Bae, J. S. Kim, J. K. Lim, K. S. Song, B. S. Shin, H. R. Kim (2009). Isolation and Characterization of a Novel Thermostable Alpha-amylase from Korean Pine Seeds, N Biotechnol, 26(3-4), 143-149.
2. Burhan A., U. Nisa, C. Gokhan, C. Omer, A. Ashabil, G. Osman (2003). Enzymatic Properties of a Novel Thermophilic, Alkaline and Chelator Resistant Amylase from an Alkalophilic *Bacillus sp*. Isolate ANT-6, Process Biochem, 38, 1397-1403.
3. Contreras A. R., M. Koller, M. M. D. Dias, M. Calafell-Monfort, G. Braunegg, M. S. Marques-Calvo (2013). High Production of Poly [3-hydroxybutyrate] from a Wild *Bacillus megaterium* Bolivian Strain, J Appl Microbiol, 114(5), 1378-1387.
4. Das S., S. Singh, V. Sharma, M. L. Soni (2011). Biotechnological Applications of Industrially Important Amylase Enzyme, Int J Pharma and Bio Sci, 2(1), 486-496.
5. Duckworth A. W., W. D. Grant, B. E. Jones, R. Van Steenbergen (1996). Phylogenetic Diversity of Soda Lake Alkaliphiles, FEMS Microbiol Ecol, 19, 181-191.

6. Ferrari E., A. S. Jarnagin, B. F. Schmidt (1993). Commercial Production of Extracellular Enzymes *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics, American Society for Microbiology Press, Washington DC, 917-939.
7. Gomes J., W. Steiner (2004). The Biocatalytic Potential of Extremophiles and Extremozymes, Food Technol Biotechnol, 42(4), 223-235.
8. Gupta R., P. Gigras, H. Mohapatra, V. K. Goswami, B. Chauhan (2003). Microbial α Amylases: A Biotechnological Perspective, Process Biochem, 38(11), 1599-1616.
9. Harwood C. R. (1992). *Bacillus subtilis* and Its Relatives: Molecular, Biological and Industrial Workhorses, Trends Biotechnol, 10(7), 247-256.
10. Kirk O., T. V. Borchert, C. C. Fuglsang (2002). Industrial Enzyme Applications, Curr Opin Biotechnol, 13, 345-351.
11. Mishra S., N. Behera (2008). Amylase Activity of Starch Degrading Bacteria Isolated from Soil Receiving Kitchen Wastes, Afr J Biotechnol, 7(18), 3326-3331.
12. Mukherjee A. K., M. Borah, S. K. Rai (2009). To Study the Influence of Different Components of Fermentable Substrates on Induction of Extracellular α Amylase Synthesis by *Bacillus subtilis* DM-03 in Solid State Fermentation and Exploration of Feasibility for Inclusion of α Amylase in Laundry Detergent Formulations, Biochemical Engineering Journal, 43, 149-156.
13. Rajagopalan G., C. Krishnan (2008). Alpha-amylase Production from Catabolite Derepressed *Bacillus subtilis* KCC103 Utilizing Sugarcane bagasse hydrolysate, Bioresource Technology, 99(8), 3044-3050.
14. Saitou N., M. Nei (1987). The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees, Molecular Biology and Evolution, 4, 406-425.
15. Schallmey M., A. Singh, O. P. Ward (2004). Developments in the Use of *Bacillus* Species for Industrial Production, Canadian Journal of Microbiology, 50, 1-17.
16. Simonen M., I. Palva (1993). Protein Secretion in *Bacillus* Species, Microbiol Rev, 57, 109-137.
17. Mohapatra S., S. N. Rath, S. K. Pradhan, D. P. Samantaray, C. C. Rath (2016). Secondary Structural Models (16S rRNA) of Polyhydroxyalkanoates Producing *Bacillus* Species Isolated from Different Rhizospheric Soil: Phylogenetics and Chemical Analysis, Int J Bioautomation, 20(3), 329-338.
18. Tamura K., G. Stecher, D. Peterson, A. Filipski, S. Kumar (2013). MEGA 6: Molecular Evolutionary Genetics Analysis version 6.0, Mol Biol Evol, 30(12), 2725-2729.
19. Zuker M. (2003). Mfold Web Server for Nucleic Folding and Hybridization Prediction, Nucleic Acid Research, 31(13), 3406-3415.

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