

Alpha-amylase Production by Extremely Halophilic Archaeon *Halococcus* Strain GUVSC8

Bhakti B. Salgaonkar,* Divya T. Sawant, Saranya Harinarayanan, and Judith M. Bragança

Starch hydrolyzing isolate GUVSC8 is obtained from commercial salt crystals of Vedaranyam, Tamil Nadu, India. GUVSC8 is categorized as an extreme halophile based on its growth on complex medium containing 25% (w/v) NaCl. Morphological, chemotaxonomic, and 16S rRNA gene sequencing reveal the cells to be coccus, producing bright orange C-50 bacterioruberin pigmentation with 95.95% similar to *Halococcus hamelinensis* DQ017835. The strain GUVSC8 grown in complex medium devoid of starch does not exhibit amyolytic activity, thereby confirming the induction of amylase production in presence of starch. On incubation of the starch with crude enzyme, numerous pores are observed on the starch granules after 24 h of incubation, indicating vigorous degradation and thereby confirming the amyolytic activity. Thin-layer chromatography (TLC) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis reveal that the major end product obtained after amyolytic activity are glucose, maltose, maltotriose, maltotetrose, maltopentose, and other maltooligosaccharides, thus confirming it to be an alpha-amylase. Effects of NaCl, pH, and temperature, on activity of partially purified amylase, reveal best amylase activity at 2M NaCl, pH6, and 45 °C. The amylase is stable and active in presence of divalent cations such as Mg²⁺, Ni²⁺, Zn²⁺, Ca²⁺, and Co²⁺. Enzyme activity significantly reduces with Cu²⁺ and increases in presence of Mn²⁺. The amylase is characterized as α-amylase enzyme as it lost 87% of its activity in presence of EDTA. To the best of the author's knowledge, this is the first report on alpha-amylase production by salt crystal isolate belonging to the genus *Halococcus*.

1. Introduction

Hypersaline regions such as the solar salterns, Great salt Lake, Dead Sea, etc are extreme environments with salinity

Dr. B. B. Salgaonkar, S. Harinarayanan, Dr. J. M. Bragança
Department of Biological Sciences, Birla Institute of Technology and
Science Pilani
K K Birla, Goa Campus
NH-17B, Zuarinagar 403 726 Goa, India
E-mail: salgaonkarbhakti@gmail.com

Dr. B. B. Salgaonkar, D. T. Sawant
Department of Microbiology, Goa University
Taleigao Plateau
403 206 Goa, India

DOI: 10.1002/star.201800018

approaching near saturation ($\geq 30\%$) and the microbial life inhabiting such environments are termed as extremophiles.^[1,2] Halophilic archaea are polyextremophilic microorganisms belonging to the domain Archaea which are able to endure and proliferate in extreme physical parameters such as radiation, temperature, pressure, and geochemical parameters such as pH, salinity, redox potential, etc. The ability of haloarchaea to thrive in extreme conditions demonstrates their great potential for novel biotechnological processes.^[3]

Extremozymes are novel hydrolytic enzymes obtained from extremophiles and studies on these enzymes are gaining momentum due to their catalytic activity under conditions usually unfavorable to mesophilic organisms.^[4,5] Despite the knowledge of vast diversity of halophilic archaea using culture-dependent/-independent approaches, there is limited knowledge on understanding their metabolic profile. The increasing demand for effective and stable biocatalysts can be met either by improving the properties of existing enzymes or identifying microorganisms producing novel enzymes with better activities. To date, enzymes employed by industrial process are mostly obtained from bacteria, fungi, and yeast.^[6,7] However, studies on the members of the third domain of life, Archaea, have provided evidence of the novelty and superiority of their biocatalysts as compared to other domains.^[8–10]

Starch rich food is an important source of energy and is an integral part of our dietary carbohydrate. Due to the increasing demand for starch-rich food and rapid development in the food/starch-processing industries, there is an increasing pressure for establishment of sophisticated technologies that employ amyolytic enzymes for starch processing. The amyolytic enzymes are placed into three groups, α-amylase, β-amylase, and glucoamylase based on the difference in their amino acid sequences, reaction/catalytic mechanisms, and structures (3-D).^[6] Alpha-amylase (E.C. 3.2.1.1) also known as 1, 4-α-D-glucan glucanohydrolase is part of glycoside hydrolase family 13 (GH-13) and catalyzes hydrolysis of α-1, 4-glycosidic linkages in starch and related carbohydrates made up of glucose. GH has been

classified into ≥ 150 families on the basis of amino acid sequence similarity and are part of the carbohydrate-active enzymes (CAZy) web-server (<http://www.cazy.org/Glycoside-Hydrolases.html>).^[11] Amylases are widely distributed in living organisms, and are produced by members of all domains of life, thereby playing a crucial role in the field of biotechnology. The subfamilies GH13_7 represent α -amylases mainly from hyperthermophilic archaeons belonging to two genera, *Pyrococcus* (*P. furiosus*, *P. woesei*)^[12,13] and *Thermococcus* (*T. hydrothermalis*, *T. onnurineus*).^[14,15] These enzymes tend to be active and stable at temperatures ≥ 80 °C and are characterized by determining their 3-D structures. Subfamily GH13_36 represent α -amylases AmyA a polyextremophilic α -amylase from the thermophilic halophile *Haloferax* *Halothermothrix orenii* whose 3-D structure has been determined.^[16]

The amylase from haloarchaeon, *Natronococcus amylolyticus* Ah-36, from Lake Magadi (soda lake) in Kenya, was the first one to be purified and documented.^[17] Furthermore, α -amylase are reported from *Halobacterium halobium*,^[18] *Haloferax mediterranei*, *Haloferax* sp.HA10 from Spain and India,^[19,20] *Haloarcula* strain S1 from France^[21] and *Halorubrum xinjiangense* from Iran,^[23] and *Haloterrigena turkmenica* DSM-5511.^[24] Haloarchaea have unique metabolic pathways and their enzymes exhibit optimum activities and stability at polyextremophilic conditions such as fluctuating temperatures, salt concentrations and pH, thereby acting as a very powerful tool in industrial processes operating at extreme conditions.^[10] The targeted application of halophilic/tolerant amylase could be starch hydrolysis in industrial processes in saline media such as treatment of agricultural waste in production of biofuel/chemicals.^[25–27]

Among the 48 genera and 177 species, of halophilic archaea, of the class *Halobacteria* reported till date,^[28,29] the ability to hydrolyze starch is reported only in six genera, namely, *Natronococcus*, *Halobacterium*, *Haloferax*, *Haloarcula*, *Halorubrum*, and *Haloterrigena*. To date, to the best of our knowledge, there is no report on genus *Halococcus* to hydrolyze starch. Exploring other haloarchaeal genera might help us to discover novel enzyme with superior biotechnological and industrial potential. In this study, we have isolated an extremely halophilic archaeon, GUVSC8 from the commercial salt crystals of Vedaranyam, Tamil Nadu, India. The strain GUVSC8 exhibited excellent starch hydrolysis ability as compared to the other haloarchaeal strains indicating its preeminent amylase activity. The isolate was identified as *Halococcus* strain GUVSC8, and to the best of our knowledge, this is the first report on alpha-amylase from salt crystal isolate belonging to the genus *Halococcus*.

2. Experimental Section

2.1. Halophilic Microorganism and Culture Conditions

Halophilic isolate GUVSC strain 8 (Goa University Vedaranyam Salt Crystals) was isolated from commercial salt crystals of Vedaranyam, Tamil Nadu, India. The isolate was maintained on EHM (Extremely Halophilic Medium) comprising of (g L^{-1}) NaCl 250, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 20, KCl 2, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.36, NaHCO_3 0.06, NaBr 0.23, Peptone 5, Yeast Extract 10, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.05,

Agar 18. The plates were incubated at 37 °C in self-sealing plastic bags and sub-cultured every 30–45 days. Starter culture was prepared by inoculating loopful of the pure culture from agar plates in 25 mL of EHM contained in 100 mL Erlenmeyer flask. The flasks were incubated at 37 °C, 110 rpm on a rotary shaker (Skylab Instruments, India) for 4–5 days.

2.2. Screening of the Halophilic Isolate for Extracellular Hydrolytic Enzymes

The extracellular hydrolytic enzymes production were screened by agar plate assay, using NH (Norberg and Hofstein) medium comprising of (g L^{-1}) NaCl 200, $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ 10, KCl 5, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.2, Yeast Extract 1, Agar 18, and supplemented with various substrates as sole source of carbon.^[30] The pH of all media was adjusted to 7.2–7.4 using 1M NaOH. Ten microlitres of log phase starter cultures (4 days) was spot inoculated followed by incubation of plates at 37 °C for 15 days.

Screening of the extracellular hydrolytic enzyme production was determined using the methods described by Rohban et al.^[31] and the diameter of the zone of clearance/precipitation (devoid of the culture growth diameter) was directly proportional to amount of hydrolysis. Briefly; (i) amyolytic activity was determined using 0.2% soluble starch (HiMedia Lab, Mumbai, India) and detected by I_2 (0.3% I_2 + 0.6% KI) solution, (ii) lipolytic activity was determined using 0.5% Tween 80 (Thomas Baker, Mumbai, India), (iii) Proteolytic activity was screened using 0.5% (w/v) skimmed milk (Nestle slim milk, India), (iv) xylanase and cellulase activity was determined using 0.2% Xylan (HiMedia Lab) and 0.2% carboxy methyl cellulose (CMC) sodium salt (Thomas Baker) and detected with 0.1% congo red solution, followed by destaining with 1N NaCl, (v) Gelatinase activity by 0.5% gelatin (Merck, India) and detected by 15% mercuric chloride, acidified with 20% (v/v) concentrated HCl solution,^[32] and (vi) phosphatase activity was detected using 0.5% Para Nitro phenol (pNP) phosphate (HiMedia Lab).

2.3. Parameter Optimization for Growth and Amylase Production

Halophilic isolate GUVSC8 exhibited excellent starch hydrolysis and was studied further. Various parameters were optimized and the amylase activity was qualitatively estimated using starch agar plate assay. Briefly, 10 μL of the GUVSC8 starter culture was spot inoculated on NH medium containing soluble starch as the carbon source. For all the parameters except temperature optimization, plates were incubated at 37 °C for 15 days. The following parameters were optimized for growth and amylase production; (i) NaCl concentrations: NH agar plates containing various concentration of NaCl (w/v), that is, 0%, 5%, 10%, 15%, 20%, 25%, and 30%, (ii) Starch concentration: NH agar plates containing various concentrations of starch (w/v), that is, 0.5%, 1.0%, 1.5%, 2.0%, 3.0%, and 4.0%, (iii) Temperatures: NH agar plates containing 20% NaCl and 0.2% Starch were incubated at various temperatures such as 4, 27, 37, and 45 °C, and (iv) NH agar plates with a range of pH from acidic to alkaline, that is, 4, 5,

6, 7, 8, 9, and 10. The plate showing the best culture growth and zone of clearance was determined as optimum NaCl, starch, temperature, and pH conditions for growth and amylase activity.

2.4. Determination of Mode of Amylase Production: Constitutive or Induced

The agar well diffusion assay (AWDA) was performed to confirm whether starch was required for α -amylase gene expression in *Halococcus* strain GUVSC8. A 1 mL of the Log phase culture of strain GUVSC8 grown in (a) nutrient rich complex medium (EHM) and (b) minimal medium (NH) containing 0.5% starch, was centrifuged at 10 000 rpm, for 5 min. A 100 microlitre of the cell free supernatant (CFS) was introduced in wells (0.5 mm diameter) bored in NH agar medium supplemented with 0.5% (w/v) starch. The CFS was allowed to diffuse completely by incubating the plates at 37 °C for 24 h, followed by flooding the plates with iodine solution. Zone of clearance against blue-black background around the agar well indicated amylolytic activity.

2.5. Starch Hydrolysis and End Product Analysis

2.5.1. Scanning Electron Microscopy

Cell-free supernatants obtained from halophilic isolate GUVSC8 grown in NH medium supplemented with starch, were incubated with 1% (w/v) starch granules (final concentration is 10 mg mL⁻¹) at 37 °C, with agitation at 200 rpm for various time intervals. After incubation, starch granules were separated from the CFS by centrifugation at 13 000 rpm for 10 min, and washed with water. The samples were subjected to a series of increasing gradient of ethanol-water, corresponding to 30%, 50%, 70%, and 90% for 10 min, and finally to 100% ethanol, for 30 min followed by air drying. The samples were mounted on stubs with double-sided adhesive tape and coated with gold/palladium using sputter coater model Leica EM ACE200 (Leica, USA). Samples were analyzed using field emission scanning electron microscopy (FESEM) model Quanta FEG250 (FEI, the Netherlands). The magnification and other details are represented on the micrographs.

2.5.2. Thin-Layer Chromatography (TLC)

For qualitative determination of saccharolytic breakdown products produced by *Halococcus* strain GUVSC8, cell-free supernatant was incubated with 1% starch in 50 mM TrisHCl buffer (pH 8.0) at 55 °C for 48 h. The samples were analyzed by thin-layer chromatography (TLC) as described by Ryan et al.^[33] An aliquot (3 μ L) of the reaction products of a sample was spotted onto a Silica Gel 60 plate (10/10 cm; Merck) and the chromatogram was developed with a butanol:acetic acid:water (5:5:3, v/v/v) solvent system in a vertical developing chamber at room temperature. The plate was allowed to air dry in a hood and developed by spraying it evenly with sulfuric acid:ethanol (1:4, v/v), followed by heating at 100 °C for 10–15 min to visualize the sugar-containing spots. Standard sugars such as glucose,

maltose, maltotriose, maltotetraose, maltopentose, and maltohexose, were used as molecular weight standards and were purchased from SISCO Research Laboratories and Sigma.

2.5.3. Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis

The Agilent 1290 Infinity II LC System was used for analysis. Chromatographic separations were performed on ZORBAX Eclipse Plus C18, Rapid Resolution HT 1.8-Micron column (2.1 \times 100 mm column) (Agilent, USA) at the temperature of 35 °C. The mobile phase A consisted of Acetonitrile (ACN)/H₂O (80/20) and B, ACN/H₂O (30/70, v/v) each with 0.1% (v/v) NH₄OH solution pumped at gradient elution. The running gradient was 40–60% of B for 5 min, followed by resetting and equilibrating with 60–40% of B for 2 min. Flow rate was 170 μ L min⁻¹ with total run time of 7 min. Mass spectrometric detection was performed on an Agilent 6460 Triple Quadrupole LC/MS System (Agilent, Santa Clara, CA, USA), equipped with positive electrospray ionization (+ESI). High purity nitrogen was used as nebulizer, gas flow rate was 10 L min⁻¹ and ionization source temperature was 350 °C. Capillary voltage was set at 4000 V. The instrumentation parameters were, fragmenter voltage and cell acceleration voltage were set at 110 and 7.

2.6. Partial Purification of Amylase

Five percent (25 mL) of the actively grown GUVSC8 culture in EHM was used as starter culture to inoculate 500 mL of NH medium contained in 1000 mL Erlenmeyer flask, supplemented with 0.5% soluble starch. The flask was incubated at 37 °C, 110 rpm and after every 24 h interval the growth was measured by recording the absorbance of culture broth at 600 nm using UV-Visible spectrophotometer (SHIMADZU UV-2450). After 8 days, the culture was centrifuged at 8000 rpm for 15 min at 4 °C and the cell free supernatant (CFS) was used for amylase purification. Pre-chilled ethanol was added slowly to the CFS up to 80% (v/v) saturation with constant stirring. This ethanol-CFS mixture was centrifuged at 10 000 rpm for 30 min at 4 °C using cooling centrifuge (REMI C-24 PLUS). The aqueous layer was discarded and the precipitate was dissolved in sterile Tris-HCl buffer^[19] of pH 8.0 and 3.4M NaCl.

2.7. Determination of α -Amylase Activity

The amylase activity was determined using Bernfeld method with slight modification.^[34] Total reaction mixture (2.5 mL volume) consisting of 1.0 mL of 1% (w/v) soluble starch (Merck) solution in Tris-HCl buffer and 0.5 mL of partially purified enzyme was incubated at 37 °C for 10 min. After incubation the reaction was terminated/stopped by addition of 1 mL of 3,5-dinitrosalicylic acid (DNSA) reagent to the enzyme substrate mixture. A control was maintained simultaneously with 1 mL of 1% soluble starch without enzyme. The mixture was placed in a boiling water bath (100 °C) for 10 min, cooled to room temperature (28 °C), followed by vortexing for proper mixing

of the contents. The amount of reducing sugar released by the enzyme was determined spectrophotometrically at 540 nm (SHIMADZU UV-2450) and quantified using DNSA with maltose as standard. The amylase activity was determined in terms of U mL^{-1} ; that is, 1 unit of α -amylase activity is defined as the amount of enzyme that releases 1 μmol of reducing sugar as maltose per minute under assay conditions.

2.8. Effect of Salt Concentration, Temperature, and pH on α -Amylase

The effect of various conditions on the stability of the partially purified α -amylase was determined in presence of 1.0% (w/v) soluble starch, followed by measuring the activity under the following conditions. (i) The effect of salt concentration on enzyme activity was determined by incubating the enzyme for 24 h at 37 °C in 50 mM Tris-HCl, pH 8.0, buffers containing various NaCl concentrations, that is, 0.5M, 1M, 2M, 3M, 4M, and 5M (highly saturated) (ii) The effect of pH on enzyme activity was determined by incubating the enzyme for 24 h at 37 °C in 50 mM Tris-HCl, buffer, containing 3.4M NaCl with pH in the range of 4, 5, 6, 7, 8, 9, and 10; and (iii) The effect of temperature on enzyme activity was determined by incubating the enzyme in a thermostatic bath for 30 min in 50 mM Tris-HCl, pH 8.0 buffer, containing 3.4M NaCl at various temperatures, ranging from 0, 4, 27, 37, 45, 55, 70, and 100 °C.

2.9. Effect of Metal Ions and/or Chelators on Amylase

For determining the effect of metal ions and/or chelators, on amylase activity, the enzyme was pre-incubating at 37 °C for 30 min, in Tris-Cl buffer containing 10 and 5 mM concentration, of various metal ions and EDTA (ethylenediamine tetraacetic acid), respectively. The various divalent metals tested were $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. The enzyme activity was determined in presence of 1.0% (w/v) soluble starch, as mentioned above and compared with the enzyme activity obtained without metals (control).

2.10. Identification of the Halophilic Isolate GUVSC8

Morphological characterization of 5 days old actively growing culture of halophilic isolate GUVSC8 was determined using Scanning Electron Microscopy (SEM) as described by Mani et al.^[35] The bright-orange pigment from the isolate GUVSC8 was characterized by extracting it in chloroform: methanol (2:2 v/v) and analyzing using UV-spectrophotometer (SHIMADZU UV-2450). Molecular characterization of the isolate was performed according to the protocol described in halohandbook^[36] and as modified by Mani et al.^[35] The extracted genomic DNA was amplified for 16S rRNA gene using archaea specific primers A109 (F) AC(G/T)GCTCAGTAACACGT and 1510(R) GGTTACCTTGTTACGACTT.^[37] The amplified PCR product was purified and sequenced using an automated DNA sequencer (Applied Biosystems, USA). The results obtained were subjected

to similarity search using EzTaxon server.^[38] Multiple sequence alignment was performed with MUSCLE and the Phylogenetic tree was constructed by the neighbor joining method of MEGA 5.0.^[39] The sequences obtained were deposited in GenBank and DDBJ (DNA Data Bank of Japan).

3. Results and Discussion

3.1. Halophilic Microorganism GUVSC8 and Extracellular Hydrolytic Enzymes

A bright orange pigmented, halophilic isolate GUVSC8 was obtained from commercial salt crystals of Vedaranyam in Tamil Nadu, India. Extremely halophilic microorganisms are a group of halophiles thriving at salt concentration from 15% upto saturation.^[40] The isolate GUVSC8 was categorized as extreme halophile based on its growth on EHM containing 25% NaCl.

Extreme halophilic isolate GUVSC8 could efficiently hydrolyze three substrates, starch, gelatin, and tween 80 with the zone size on agar plate measuring (cm) 5.2, 1.9, and 1.0, respectively, indicating its amylolytic, gelatinase and lipase activity (Figure 1). However, the isolate failed to hydrolyze skimmed milk, xylan, CMC, and pNP phosphate indicating absence of protease, xylanase, cellulase, and phosphatase activity. Among the seven substrates screened, culture GUVSC8 exhibited best starch hydrolysis indicating highest amylase production. Hence, further studies were carried out on amylase production by culture GUVSC8.

Few haloarchaeal isolates from Tuzkoy Salt Mine, Turkey were reported positive for β -galactosidase, cellulase, gelatinase, caseinase and DNase activities, whereas oxidase, catalase, amylase and lipase activity was detected in all isolates mostly belonging to the genera, *Halobacterium*, *Haloarcula*, *Natrinema*,

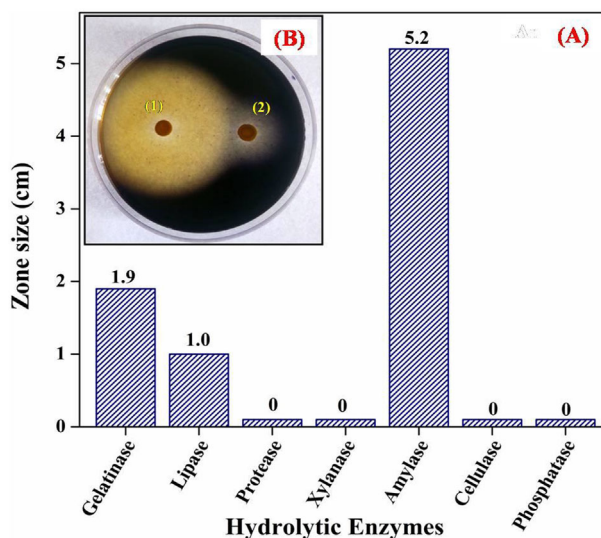


Figure 1. A) Graphical representation of the extracellular hydrolytic enzyme synthesis by halophilic isolate GUVSC8. B) Comparison of amylase produced by two halophilic isolates (1) GUVSC8 and (2) GUVSC6 on starch agar plates.

and *Halorubrum*.^[41] Study by Kakhki et al.^[42] on haloarchaeal strains obtained from Aran-Bidgol hypersaline lake, Iran, reported most hydrolytic activities among the isolates belonging to the genera *Halorubrum*, *Haloarcula*, and *Natrinema* with amylase activity being the most common among members of the Genus *Halorubrum*. Kharroub et al.^[43] reported, amylase, gelatinase, and lipase positive strains belonging to genera *Halorubrum*, *Haloferax*, *Haloterrigena*, *Halogeometricum*, *Halobacterium*, *Halomicrobium*, and *Haloarcula* from the Algerian hypersaline environments. To the best of our knowledge, this is the first study on hydrolytic enzymes from extremely halophilic isolates obtained from commercial salt crystal of Vedaranyam, India.

3.2. Optimization of Culture Growth and Amylase Production

The culture halophilic isolate GUVSC8 grew and exhibited amylase activity over a wide range of salt concentrations, that is, 10–30 % NaCl, 0.5–4% starch concentration, 5–10 pH and temperatures of 15–45 °C (Figure S1, Supporting Information), with maximum starch hydrolysis, indicating highest amylase activity at 20% NaCl ($\approx 3.4\text{M}$), 0.5 % starch, pH 8 and temperature of 45 °C. Stability and activity of the enzyme at extreme salt concentration, acidic to alkaline pH, thermophilic temperature, makes it a strong candidate as an industrial biocatalyst. Bajpai et al.^[20] optimized the culture conditions for extracellular amylase production by halophilic archaeon, *Haloferax* sp. HA10 on starch agar plate and reported maximum zone at 3 M (17.5%) NaCl, 1 % starch, pH7 and 37 °C indicating highest amylase activity.

3.3. Inductive Mode of Amylase Production

The agar well containing CFS of *Halococcus* strain GUVSC8 grown in minimal medium (NH) supplemented with starch exhibited zone of clearance against blue-black background indicating amylolytic activity (Figure S2, Supporting Information). Whereas the culture supernatant from complex medium devoid of starch did not exhibit amylolytic activity, thereby confirming the induction of amylase production in presence of starch by *Halococcus* strain GUVSC8.

Amylases from halophilic microorganisms are mainly inducible and starch has been reported as the best inducer for significant increase amylase production in both halophilic archaea and bacteria. The amylase production in the presence of soluble starch has been reported to accelerate in extremely halophilic archaea, *Halobacterium salinarum* (formerly *Halobacterium halobium*), and *Natronococcus* sp. strain Ah-36, indicating starch to be the best inducer.^[44,17] Similarly, moderately halophilic bacteria *Halomonas meridian* and *Micrococcus* sp. are reported to produce amylase only in the presence of starch.^[45,46] Interestingly, Amoozegar et al.^[47] reported constitutive production of amylase by moderate halophilic bacterium, *Halobacillus* sp. strain MA-2 in nutrient broth devoid of any carbohydrate.

3.4. Analysis of Starch Hydrolysis and End Product

Scanning Electron Microscopy (SEM) was used to visualize the enzymatic degradation of starch granules. Since the halophilic isolate GUVSC8 produced extracellular amylase, the cell-free supernatant from the culture was used as a source of enzyme. The SEM micrographs represents clear hydrolysis of the starch granules upon incubation with the CFS of isolate GUVSC8. The surface of the starch granules appeared smooth in the control sample, that is, the one incubated without enzyme. Interestingly, after 24 h of incubation pits on the surface of the granules were observed indicating partial degradation of the granules (Figure 2). Further increase in incubation time of 48 and 72 h, produced numerous pores in starch granules indicating vigorous degradation thereby confirming the amylolytic activity of the CFS. Degradation patterns of the crude enzyme on the starch granules was from surface toward to center (centripetal) which compared well with the report for α -amylases from *Bacillus amyloliquefaciens*.^[48]

The reaction products of soluble starch incubated with crude cell-free supernatants of *Halococcus* strain GUVSC8 for various durations were analyzed by TLC (Figure 3). At the early stage of hydrolysis (12 h), maltohexose and maltooligosaccharides larger than maltohexose was the end product, whereas after prolonged incubations (24 h), the products were maltotriose and maltotetrose. Similar pattern of hydrolysis has been previously reported in many other α -amylases from halophilic archaea. Kanai et al.^[17] isolated extracellular α -amylase-producing haloalkaliphilic archaeon *Natronococcus amylolyticus* strain Ah-36T from a Kenyan soda lake, Lake Magadi. The α -amylase was reported to produce maltotriose with small amounts of maltose and glucose on hydrolysis of soluble starch. Another study on α -amylase from *Haloferax mediterranei* demonstrated maltose and lower proportion maltohexose as the main products of starch hydrolysis.^[19]

LC-ESI-MS analysis was performed in order to determine the products of starch hydrolysis after treating it with the crude enzyme from *Halococcus* strain GUVSC8. The chromatogram exhibited only one elution peak, however ESI-MS analysis revealed the major molecular ion obtained at m/z 202.8, 364.9, 526.8, 688.8, and 851.0 corresponding to sodium adduct of glucose (m/z 180 + 23), maltose (m/z 342 + 23), maltotriose (m/z 504 + 23), maltotetrose (m/z 666 + 23), and maltopentose (m/z 828 + 23), respectively (Figure 4). The in-source ionization in the mass spectrometer could have resulted in the formation of sodium adduct.^[49]

The standard carbohydrates, glucose (m/z 202.9), maltose (m/z 364.8), maltotriose (m/z 526.9), maltotetrose (m/z 689), maltopentose (m/z 851.1), and maltohexose (m/z 1013.2) were recorded at respective retention times of 1.3 (glucose), 1.28 (maltose), and 1.26 min (maltooligosaccharides). The molecular mass of individual compounds obtained from the samples was compared well with the sodium adduct of standard carbohydrates (Figure S3, Supporting Information). The retention time was in the range of 1.2–1.3 indicating that the variation in the gradient method employed (Acetonitrile/ H_2O) and the column (C18) could have resulted in well separation of the hydrolytic products.

TLC and LC-ESI-MS analysis revealed that the major end products obtained after enzymatic hydrolysis of soluble starch

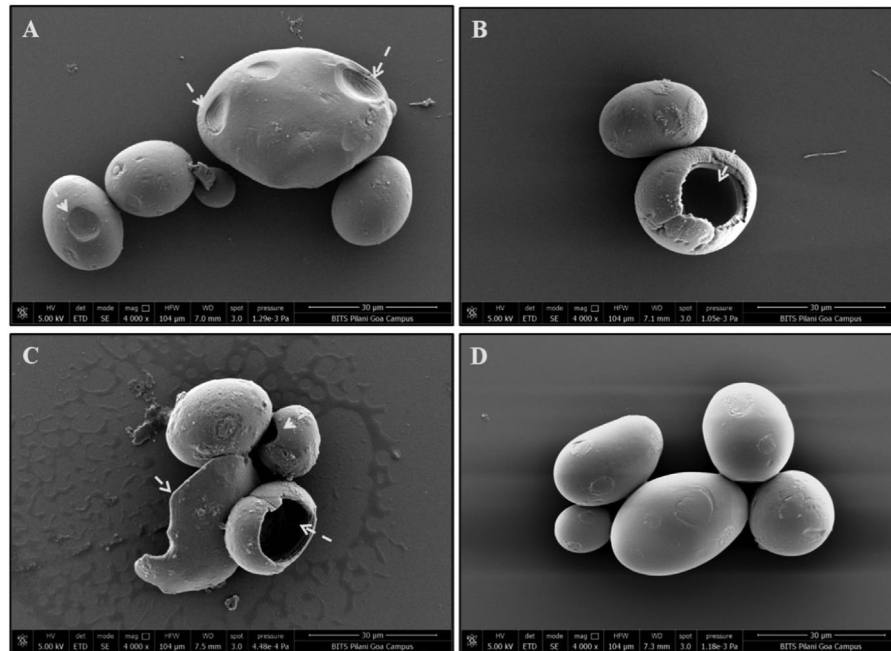


Figure 2. Scanning electron microscopy images of starch granules incubated with cell-free supernatants of *Halococcus* strain GUVSC8 for a) 24 h, b) 48 h, c) 72 h, and d) Starch granules without CFS (control).

were glucose, maltose, maltotriose, maltotetrose, maltopentose, and other maltooligosaccharides. This indicated that the amylase produced by *Halococcus* strain GUVSC8 possessed endoamylase activity and was conclude as α -amylase. The α -amylase (EC 3.2.1.1) (α -1,4-glucan-4-glucanohydrolase) belong to the family of endoamylases which are able to cleave α ,1–4 glycosidic bonds present in the inner part (endo-) of the glucose polymer of starch (both of the amylose or amylopectin chain) into varying lengths of shorter oligosaccharides such as a mixture of maltose, maltotriose, and branched oligosaccharides. They are produced by organisms belonging to all three domains of life.^[11]

3.5. Effect of NaCl, pH, and Temperature on Amylase

As represented in **Figure 5A**, $\geq 80\%$ of amylase activity was observed from 0.5 to 2M NaCl concentration, with the maximum activity at 2M NaCl. Reduction in the amylase activity was observed from 3 to 5M NaCl, however, the activity was more than 50% of the maximum activity. Santorelli et al.^[24] recently reported α -amylases from *Haloterrigena turkmenica* to exhibit optimum activity at 2M NaCl. Alpha-amylases from halophilic archaea, are reported to exhibit activities at NaCl concentration as low as 0.5M (*Haloferax* sp. HA10) to as high as 5M (*Haloarcula* stain 1) with optimum activity in the range of 2–4.3M.^[19,20,22]

The α -amylase of halophilic isolate GUVSC8 was found to be active in broad pH range (Figure 5B), that is, pH 6–9, with an optimum pH of 6.0. The relative enzyme activity started decreasing from pH 7–9, with 64% at pH 8 and 58% at pH 9, of that at pH 6.0. Among the limited studies on α -amylases from halophilic archaea, to the best of our knowledge, to date, *Haloarcula hispanica* was the only isolate reported to exhibit optimum activities at acidic pH (pH 5–6).^[20] Recent study on α -

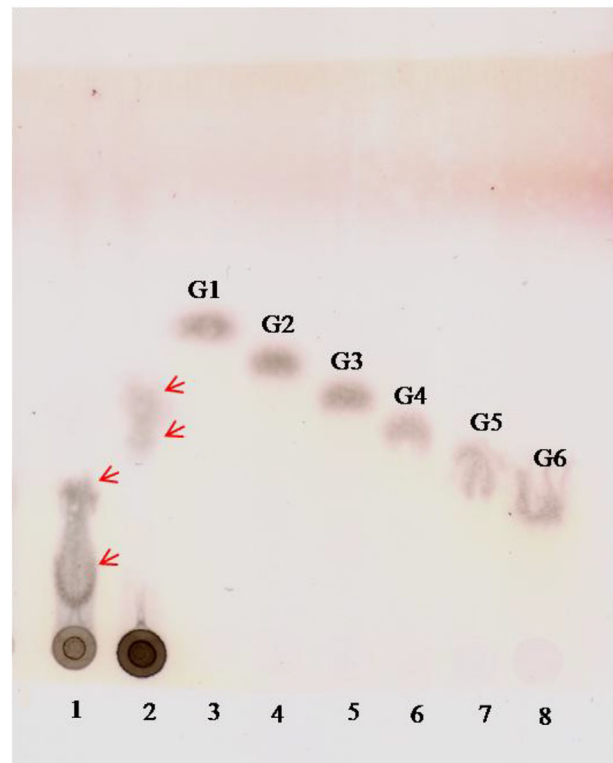


Figure 3. TLC analysis of the products obtained from starch hydrolysis following incubation with the cell-free supernatant of *Halococcus* strain GUVSC8. Lanes 1 and 2 contained sample after incubation for 12 and 24 h, respectively. Lane 3–8 contained standards, including glucose (G1), maltose (G2), maltotriose (G3), maltotetrose (G4), maltopentose (G5), and maltohexose (G6).

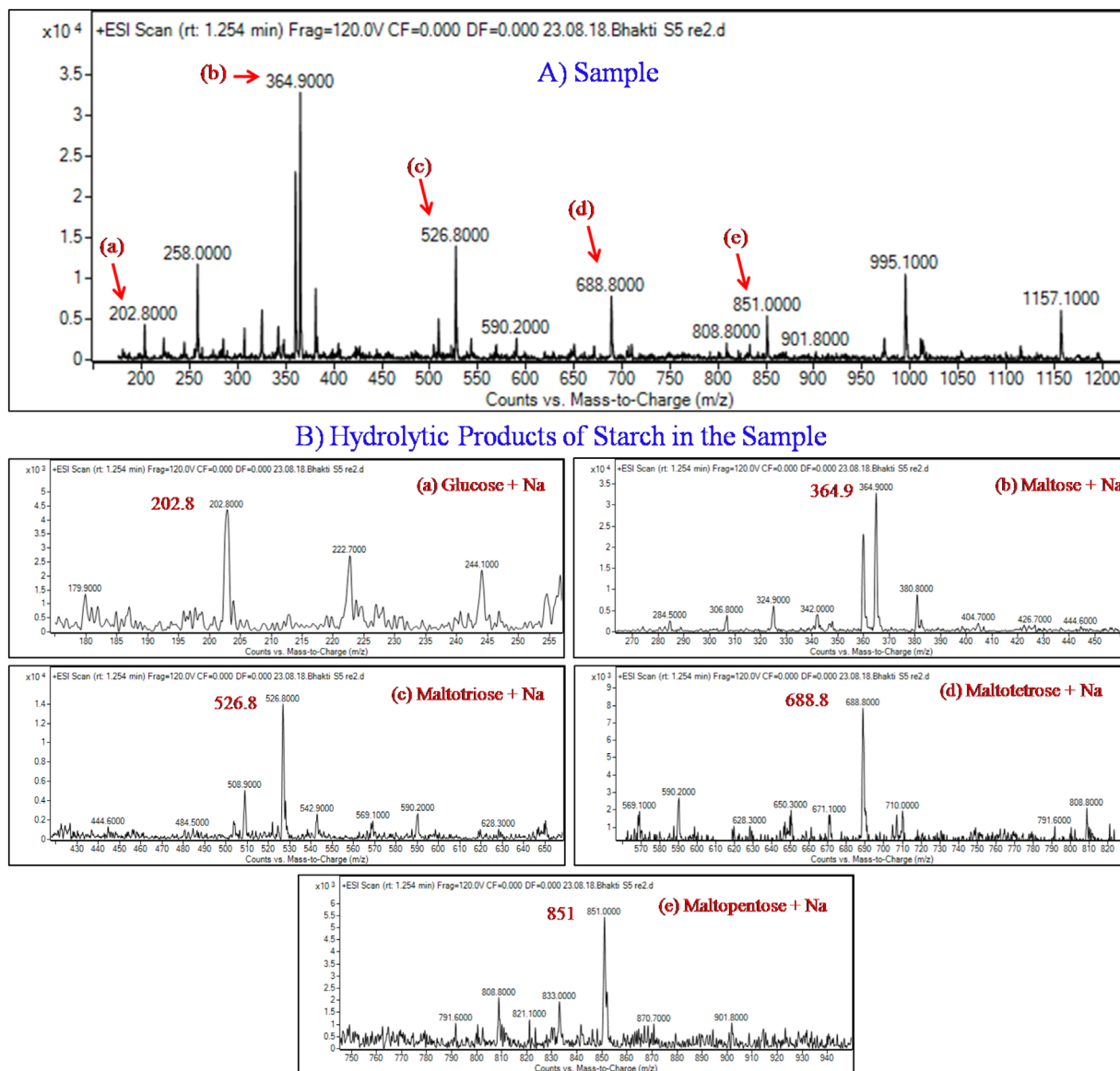


Figure 4. ESI-MS spectra of A) starch after treating with Cell free supernatant (CFS) of the *Halococcus* strain GUVSC8 and B) hydrolytic products of starch obtained, (a) glucose, (b) Maltose, (c) maltotriose, (d) maltotetraose and (e) maltopentose.

amylases from *Haloterrigena turkmenica* was reported to exhibit optimum activity at alkaline pH of 8.5.^[22] *Halorubrum xinjiangense* and *Haloferax mediterranei* are reported to produce α -amylase with best activity around neutral to alkaline pH (7–8).^[21,17] Bajpai et al.^[20] and Fukushima et al.^[21] reported α -amylase from *Haloferax* sp. HA10 and *Haloarcula* stain one with best activity at neutral pH 7.^[23]

Enzyme activity was found to be optimum at 45 °C and was $\geq 80\%$ in the range of 50–70 °C (Figure 5C). Steady reduction in the enzyme activity was observed at temperatures above 70 °C. However, 69% enzyme activity was retained even at 90 °C. Optimum temperature of α -amylase obtained from haloarchaeal isolates has been reported to be in the range of 40 °C (*Halorubrum xinjiangense*), 50 °C (*Haloarcula hispanica*; *Haloarcula* stain 1), 55 °C (*Haloterrigena turkmenica*; *Haloferax*

sp. HA10) to 60 °C.^[17–22] Alpha-amylases from halophilic archaea are heat stable and this thermostability is a desirable and attractive property for industrial starch liquefaction which could be achieved at elevated temperatures.

3.6. Effect of Metal Ions and EDTA on Amylase

Various metals exhibited different effects on the enzyme activity. The relative activity of α -amylase was not much affected in presence of Mg^{2+} , Ni^{2+} , Zn^{2+} , Ca^{2+} , and Co^{2+} when compared with control. In presence of Cu^{2+} the enzyme activity significantly reduced to 57% (Figure S4, Supporting Information). Bajpai et al.^[20] reported that Co^{2+} , Zn^{2+} , Cu^{2+} , and Mn^{2+} drastically inhibited the activity of α -amylase from halophilic

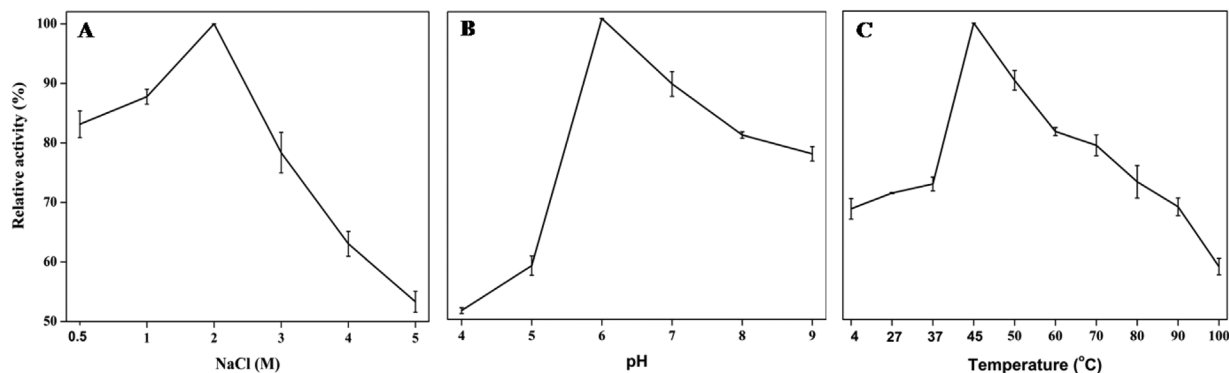


Figure 5. Effect of (A) NaCl, (B) pH, and (C) temperature on the activity of partially purified amylase from halophilic isolate GUVSC8.

archaeon, *Haloferax* sp. HA10.^[20] The decrease in activity of amylase in presence of CuSO_4 was also reported in amylase produced by *Halobacillus* sp. strain MA-2.^[47]

Stimulatory effects on amylase production in presence of divalent metal ions especially calcium ions is reported in halophilic microorganisms. In case of halophilic archaea, calcium ions dependent amylase catalysis has been reported in *Haloferax mediterranei* and *Haloarcula hispanica*.^[19,22] Kiran and Chandra^[50] and Prakash et al.^[51] reported CaCl_2 enhanced the activity of halo-philic/-tolerant, and alkali-tolerant/-stable α -amylase produced by *Bacillus* sp. strain TSCVKK and *Chromohalobacter* sp. TVSP 101.

Most of the microbial α -amylases are calcium ion-dependent enzymes which indicate that Ca^{2+} may play a role in stabilizing the architecture of the catalytic cleft. However, the presence of CaCl_2 , neither increased nor decreased the amylase activity of GUVSC8 suggesting that the enzyme activity could be calcium-independent. Santorelli et al.^[24] reported the activity of AmyA, an α -amylase from halophilic archaeon *Haloterrigena turkmenica* to be independent of calcium ions or EDTA.

In presence of Mn^{2+} the enzyme activity drastically increased to 64% as compared to control. Our study was comparable with the one by Chakraborty et al.^[52] which reported increase in amylase activity in presence of Mg^{2+} , Mn^{2+} , Co^{2+} , and Cu^{2+} by α -amylase from haloalkaliphilic marine actinomycetes *Saccharopolyspora* sp. A9. Similarly, Marco et al.^[53] reported $\text{Mn}^{2+}/\text{Co}^{2+}$ enhanced the activity of truncated *Bacillus subtilis* α -amylase, produced by *Escherichia coli* cells.^[53] Alariya et al.^[54] reported that media supplemented with MnSO_4 enhanced the production and activity of α -amylase by four soil isolates, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Escherichia coli*, and *Serratia marcescens*.^[54]

In presence of the EDTA (chelating agent) the enzyme activity drastically reduced to 13% and could not be restored by dialysis against CaCl_2 . Such irreversible loss in amylase activity in presence of EDTA was also reported in amylase from *Haloferax mediterranei*.^[19] The α -amylase from isolate GUVSC8 required metal ion for its activity.

3.7. Identification of Isolate GUVSC8

The halophilic isolate GUVSC8 appeared bright-orange pigmented with colonies of approximately 1 mm in diameter and

was categorized as extreme halophile based on its growth on complex media containing 25% NaCl (w/v) along with other salts^[40] (Figure S5a, Supporting Information). SEM analysis revealed the cell morphology to be cocci mostly appearing as tetrads of $\approx 0.8\text{--}1.0\ \mu\text{m}$ in diameter (Figure S5B, Supporting Information) which compared well with the cell size of halophilic archaeon *Halococcus hamelinensis* ($0.8\text{--}1.2\ \mu\text{m}$).^[55] Spectrophotometric analysis of the Chloroform:Methanol (2:1 v/v) extract of the pigments from halophilic isolate GUVSC8 exhibited a characteristic carotenoid pigment spectrum with absorption maxima at 499 nm with two shoulder peaks at 472 and 534 nm, and was well comparable with the archaeal C-50 bacterioruberin.^[56] On comparison of partial 16S rRNA gene sequences with GenBank, NCBI (National Center for Biotechnology Information), USA, the isolates GUVSC8 was identified as member of the genus *Halococcus* and closely related to *Halococcus hamelinensis* DQ017835 (95.95%). The tree exhibiting the phylogenetic position of the isolates is represented in Figure S5c, Supporting Information, and the 16S rRNA sequence has been deposited in NCBI GenBank with accession number MG008998 (<https://www.ncbi.nlm.nih.gov/nucleotide/1247128159>).

Few studies on the saccharification of starch by halophilic archaeal α -amylases have been reported by members of the genus *Haloferax*, *Haloarcula*, *Halorubrum*, and *Haloterrigena*.^[19,22–24] To the best of our knowledge, to date, α -amylases from halophilic archaeon belonging to the genus *Halococcus* has not yet been reported.

4. Conclusions

Hydrolases from halophilic archaea have been explored owing to their uniqueness and novelty with their optimum activities lying in polyextremophilic conditions. In this study, a new potent amylase producer halophilic archaeon, *Halococcus* strain GUVSC8 was isolated from commercial salt crystals of Vedaranyam salt pans, Tamil Nadu, India. The amylase exhibited optimum activities and stability at polyextremophilic conditions which are usually unfavorable to mesophilic organisms. The enzyme was characterized as α -amylase with enhanced activity in presence of divalent cation, manganese. Stability and activity of the enzyme at extreme salt concentration, acidic to alkaline pH,

thermophilic temperature, makes it a strong candidate as an industrial biocatalyst and could be used for the treatment of agricultural waste in saline media. Purification and scale up of amylase production using fermentor and by optimizing medium components by statistical tools for enhanced amylase yield could be studied. The substrate spectrum of amylase could be determined by incubating the enzyme with starch obtained from various other sources and also using amylose, amylopectin, glycogen, and pullulan.

Abbreviations

CFS, cell free supernatant; CMC, carboxy methyl cellulose; DDBJ, DNA Data Bank of Japan; DNSA, Dinitro salicylic acid; EDTA, ethylene diamine tetra acetic acid; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; MEGA, Molecular Evolutionary Genetics Analysis; MUSCLE, Multiple Sequence Comparison by Log Expectation; NCBI, National Center for Biotechnology Information; SEM, Scanning Electron Microscopy; TLC, Thin-layer chromatography.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgments

BBS and DS are grateful to Head of Department of Microbiology, Goa University, for the facilities and infrastructure. BBS thanks Council of Scientific and Industrial Research (CSIR) India for Research Associateship (RA) (Ref No: 09/919(0030)/2016-EMR-I). Authors are grateful to Dr. Kabilan Mani, Department of Biotechnology, P.S.G. college of Technology, Coimbatore, Tamil Nadu, for helping with phylogenetic analysis. The authors thank Dr. Devendra Kumar, (LC-MS In-charge) and Mr. Shivprakash Solanki, (FESEM In-charge) at Central Sophisticated Instrumentation Facility (CSIF) at BITS Pilani K K Birla Goa Campus, for helping with the analysis.

Conflict of Interest

The authors have declared no conflict of interest.

Keywords

alpha-amylase, archaea, *halococcus*, halophiles, salt crystal

Received: January 18, 2018

Revised: October 26, 2018

Published online:

- [1] G. Z. L. Dalmaso, D. Ferreira, A. B. Vermelho, *Marine drugs*. **2015**, *13*, 1925.
- [2] L. Montoya, C. Vizioli, N. Rodríguez, M. J. Rastoll, R. Amils, I. Marin, *Aquat. Biosyst.* **2013**, *9*, 19.
- [3] H. Stan-Lotter, S. Fendrihan, *Life*. **2015**, *5*, 1487.
- [4] B. Van Den Burg, *Curr. Opin. Microbiol.* **2003**, *6*, 213.
- [5] F. Sarmiento, R. Peralta, J. M. Blamey, *Front. Bioeng. Biotechnol.* **2015**, *3*, 148.
- [6] Š. Janeček, B. Svensson, E. A. MacGregor, *Cell. Mol. Life Sci.* **2014**, *71*, 1149.
- [7] B. J. Green, D. H. Beezhold, *J. Allergy*. **2011**, *2011*, Article ID 682574, 11.
- [8] J. A. Littlechild, Archaeal enzymes and applications in industrial biocatalysts. *Archaea*. **2015**.
- [9] D. Mehta, T. Satyanarayana, *Front. Microbiol.* **2016**, *7*, 1129.
- [10] S. Kumar, J. Grewal, A. Sadaf, R. Hemamalini, S. K. Khare, *Aims. Microbiol.* **2016**, *2*, 1.
- [11] Š. Janeček, α -Amylases from Archaea: sequences, structures and evolution. *Biotechnology of Extremophiles*, Springer, Cham. **2016**, 505–524.
- [12] G. Dong, C. Vieille, A. Savchenko, J. G. Zeikus, *Appl. Environ. Microbiol.* **1997**, *63*, 3569.
- [13] S. Jorgensen, C. E. Vorgias, G. Antranikian, *J. Biol. Chem.* **1997**, *272*, 16335.
- [14] E. Leveque, B. Haye, A. Belarbi, *FEMS Microbiol. Lett.* **2000**, *186*, 67.
- [15] J. K. Lim, H. S. Lee, Y. J. Kim, S. S. Bae, J. H. Jeon, S. G. Kang, J. H. Lee, *J. Microbiol. Biotechnol.* **2007**, *17*, 1242.
- [16] N. Li, B. K. Patel, B. N. Mijts, K. Swaminathan, *Acta Crystallogr. D: Biol. Crystallogr.* **2002**, *58*, 2125.
- [17] H. Kanai, T. Kobayashi, R. Aono, T. Kudo, *Int. J. Syst. Evol. Microbiol.* **1995**, *45*, 762.
- [18] W. A. Good, P. A. Hartman, *J. Bacteriol.* **1970**, *104*, 601.
- [19] F. Pérez-Pomares, V. Bautista, J. Ferrer, C. Pire, F. C. Marhuenda-Egea, M. J. Bonete, *Extremophiles*. **2003**, *7*, 299.
- [20] B. Bajpai, M. Chaudhary, J. Saxena, *Food. Technol. Biotech.* **2015**, *53*, 11.
- [21] T. Fukushima, T. Mizuki, A. Echigo, A. Inoue, R. Usami, *Extremophiles*. **2005**, *9*, 85.
- [22] G. W. Hutcheon, N. Vasisht, A. Bolhuis, *Extremophiles*. **2005**, *9*, 487.
- [23] M. Moshfegh, A. R. Shahverdi, G. Zarrini, M. A. Faramarzi, *Extremophiles*. **2013**, *17*, 677.
- [24] M. Santorelli, L. Maurelli, G. Pocsfalvi, I. Fiume, G. Squillaci, F. La Cara, G. del Monaco, A. Morana, *Int. J. Biol. Macromol.* **2016**, *92*, 174.
- [25] K. Majzlova, Z. Pukajova, Š. Janeček, *Carbohydr. Res.* **2013**, *367*, 48.
- [26] R. Karan, M. D. Capes, S. DasSarma, *Aquat. Biosyst.* **2012**, *8*, 4.
- [27] M. Delgado-García, B. Valdivia-Urdiales, C. N. Aguilar-González, J. C. Contreras-Esquivel, R. Rodríguez-Herrera, *J. Sci. Food Agric.* **2012**, *92*, 2575.
- [28] A. Oren, *Extremophiles*. **2014**, *18*, 825.
- [29] S. Chen, Y. Xu, H. C. Liu, A. N. Yang, L. X. Ke, *Int. J. Syst. Evol. Microbiol.* **2017**, *67*, 818.
- [30] P. Norberg, B. V. Hofstein, *J. Gen. Microbiol.* **1969**, *55*, 251.
- [31] R. Rohban, M. A. Amoozegar, A. Ventosa, *Iran. J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 333.
- [32] S. S. Balan, R. Nethaji, S. Sankar, S. Jayalakshmi, *Asian. Pac. J. Trop. Biomed.* **2012**, *2*, S1811.
- [33] S. M. Ryan, G. F. Fitzgerald, D. van Sinderen, *Appl. Environ. Microbiol.* **2006**, *72*, 5289.
- [34] P. Bernfeld, *Methods Enzymol.* **1955**, *1*, 149.
- [35] K. Mani, B. B. Salgaonkar, J. M. Braganca, *Aquat. Biosyst.* **2012**, *8*, 15.
- [36] M. Dyal-Smith, *The halo handbook: protocols for halobacterial genetics*. **2008**, <http://www.haloarchaea.com/resources/halo handbook/index.html> (accessed, November 2018).
- [37] M. Birbir, B. Calli, B. Mertoglu, R. E. Bardavid, A. Oren, M. N. Ogmen, A. Ogen, *World. J. Microbiol. Biotechnol.* **2007**, *23*, 309.
- [38] O. Kim, Y. J. Cho, K. Lee, S. H. Yoon, M. Kim, H. Na, S. C. Park, Y. S. Jeon, J. H. Lee, H. Yi, S. Won, J. Chun, *Int. J. Syst. Evol. Microbiol.* **2012**, *62*, 716.
- [39] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, *Mol. Biol. Evol.* **2011**, *28*, 2731.
- [40] M. de Lourdes Moreno, D. Pérez, M. T. García, E. Mellado, *Life*. **2013**, *3*, 38.
- [41] M. Birbir, A. Ogan, B. Calli, B. Mertoglu, *World J. Microbiol. Biotechnol.* **2004**, *20*, 613.

- [42] A. M. Kakhki, M. A. Amoozegar, E. M. Khaledi, *Int. J. Environ. Sci. Technol.* **2011**, *8*, 705.
- [43] K. Kharroub, M. A. Gomri, M. Aguilera, M. Monteoliva-Sanchez, *Afr. J. Microbiol. Res.* **2014**, *8*, 3992.
- [44] S. Patel, N. Jain, D. Madamwar, *World J. Microbiol. Biotechnol.* **1993**, *9*, 25.
- [45] M. J. Coronado, C. Vargas, J. Hofemeister, A. Ventosa, J. J. Nieto, *FEMS Microbiol. Lett.* **2000**, *183*, 67.
- [46] H. Onishi, *J. Bacteriol.* **1972**, *109*, 570.
- [47] M. A. Amoozegar, F. Malekzadeh, K. A. Malik, *J. Microbiol. Methods.* **2003**, *52*, 353.
- [48] E. S. Demirkan, B. Mikami, M. Adachi, T. Higasa, S. Utsumi, *Process Biochem.* **2005**, *40*, 2629.
- [49] H. Tong, D. Bell, K. Tabei, M. M. Siegel, *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 1174.
- [50] K. K. Kiran, T. S. Chandra, *Appl. Microbiol. Biotechnol.* **2008**, *77*, 1023.
- [51] B. Prakash, M. Vidyasagar, M. Madhukumar, G. Muralikrishna, K. Sreeramulu, *Process Biochem.* **2009**, *44*, 210.
- [52] S. Chakraborty, A. Khopade, R. Biao, W. Jian, X. Y. Liu, K. Mahadik, B. Chopade, L. Zhang, C. Kokare, *J. Mol. Catal. B-Enzym.* **2011**, *68*, 52.
- [53] J. L. Marco, L. A. Bataus, F. F. Valencia, C. J. Ulhoa, S. Astolfi-Filho, C. R. Felix, *Appl. Microbiol. Biotechnol.* **1996**, *44*, 746.
- [54] S. S. Alariya, S. Sethi, S. Gupta, G. B. Lal, *Arch. Appl. Sci. Res.* **2013**, *5*, 15.
- [55] F. Goh, S. Leuko, M. A. Allen, J. P. Bowman, M. Kamekura, B. A. Neilan, B. P. Burns, *Int. J. Syst. Evol. Microbiol.* **2006**, *56*, 1323.
- [56] A. Oren, *FEMS Microbiol. Ecol.* **2002**, *39*, 1.