Research Article

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

Bhakti B. Salgaonkar[1,2]*, *Divya T. Sawant*[2], *Saranya Harinarayanan*[1] *and* *Judith M. Bragança*[1]

[1]Dept. of Biological Sciences, Birla Institute of Technology and Science Pilani, K K Birla, Goa Campus, NH-17B, Zuarinagar, Goa, India. 403 726

[2]Dept. of Microbiology, Goa University, Taleigao Plateau, Goa 403 206, India

email: salgaonkarbhakti@gmail.com,

Ph. No: +91-8322580352 / 08408064984; Fax No: +918322557033

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/star.201800018].

Received: January 18, 2018 / Revised: October 26, 2018 / Accepted: December 11, 2018

Abstract

Starch hydrolyzing isolate GUVSC8 was obtained from commercial crude salt crystals of Vedaranyam, Tamil Nadu, India. GUVSC8 was categorized as an extreme halophile based on its growth on complex medium containing 25% (w/v) NaCl. Morphological, chemotaxonomic and 16S rRNA gene sequences, revealed the cells to be coccus, producing bright orange C-50 bacterioruberin pigmentation with 95.95% similarity to *Halococcus hamelinensis* DQ017835. The strain GUVSC8 grown in complex medium devoid of starch did not exhibit amylolytic activity, thereby confirming the induction of amylase production in presence of starch. On incubation of the starch with crude enzyme, numerous pores were observed on the starch granules after 24 hrs of incubation, indicating vigorous degradation and confirming the amylolytic activity. Thin-layer chromatography (TLC) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis revealed the major end product obtained after amylolytic activity were 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, revealed best amfylase activity at 2M NaCl, pH6 and 45ºC. The amylase was stable and active in presence of divalent cations such as Mg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+} and Co^{2+}. Enzyme activity significantly reduced with Cu^{2+} and increased in presence of Mn^{2+}. The amylase was characterized as a α-amylase enzyme as it lost 87% of its activity in presence of EDTA. To the best of our knowledge, this is the first report on alpha-amylase production by salt crystal isolate belonging to the genus *Halococcus*.

Keywords

Halophiles ; Alpha-Amylase ; Archaea ; *Halococcus* ; Salt Crystal

Abbreviations

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

1 Introduction

Hypersaline regions such as the solar salterns, Great salt Lake, Dead Sea, etc are extreme environments with salinity approaching near saturation (≥ 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 (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 unfavourable 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, 9, 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 amylolytic enzymes for starch processing. The amylolytic 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 with 3-D structure determined. Subfamily GH13_36 represent α-amylases AmyA a polyextremophilic α-amylase from the thermophilic halophile *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 *Har. hispanica* from Spain [22], *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 biofulel/chemicals [25, 26, 27].

Among the 48 genera and 177 species, of halophilic archaea, of the family of *Halobacteriacea* 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 crude salt crystals of Vedaranyam, Tamil Nadu, India. The strain GUVSC8 showed 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 Materials and methods

2.1 Halophilic microorganism and culture conditions

Halophilic isolate GUVSC strain 8 (**G**oa **U**niversity **V**edaranyam **S**alt **C**rystals) was isolated from crude salt crystals of Vedaranyam, Tamil Nadu, India. The isolate was maintained on EHM (**E**xtremely **H**alophilic **M**edium) comprising of (g/L) NaCl 250, $MgSO_4.7H_2O$ 20, KCl 2, $CaCl_2.2H_2O$ 0.36, $NaHCO_3$ 0.06, NaBr 0.23, Peptone 5, Yeast Extract 10, $FeCl_3$. $6H_2O$ 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 (**N**orberg and **H**ofstein) medium comprising of (g/L) NaCl 200, $MgSO_4.6H_2O$ 10, KCl 5, $CaCl_2.2H_2O$ 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 on 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^{o}C for 15 days.

Screening of the extracellular hydrolytic enzyme production was determined using the methods described by Rohban, *et al.*, (2009) and the diameter of the zone of clearance/precipitation (devoid of the culture growth diameter) was directly proportional to amount of hydrolysis [31]. Briefly; (i) amylolytic 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, Mumbai, India)

and 0.2% carboxy methyl cellulose (CMC) Sodium salt (Thomas Baker, Mumbai, India) and detected with 0.1% congo red solution, destained 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]. (iv) phosphatase activity was detected using 0.5% Para Nitro phenol (pNP) phosphate (HiMedia Lab, Mumbai, India).

2.3 Parameters optimization for growth and amylase production

Halophilic isolate GUVSC8 showed excellent starch hydrolysis and was studied further. Various parameters were optimized and the amylase activity was qualitatively estimated using starch agar plate assay. Briefly, Ten 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^{o}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), i.e., 0%, 5%, 10%, 15%, 20%, 25% and 30%. (ii) Starch concentration: NH agar plates containing various concentration of starch (w/v), i.e., 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^{o}C, 27^{o}C, 37^{o}C and 45^{o}C. (iv) NH agar plates with a range of pH from acidic to alkaline, i.e., 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 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. One mililitre 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 mins. Hundred microlitres of the cell free supernatant (CFS) was introduced in wells (0.5mm 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 hours, 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

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) 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 10mins, 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, Netherlands). The magnification and other details are represented on the micrographs.

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*., (2006) [33]. An aliquot (3 µl) 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, maltopentaose, and maltohexaose, were used as molecular weight standards and were purchased from SISCO Research Laboratories and Sigma.

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 x 100 mm column) (Agilent, USA) at the temperature of 35°C. The mobile phase A consisted of Acetonitrile (ACN) /H_2O (80/20, v/v) and B ACN/H_2O (30/70, v/v) each with 0.1% (v/v) NH4OH solution pumped at gradient elution. The running a gradient of 40 to 60 % B for 5 min, followed by reset and equilibrating with 60% to 40% of B for 2 min. Flow rate was 170 µL/min with total run time was 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 10L/min and ionization source temperature was 350°C. Capillary voltage was set at 4000V. The

instrumentation parameters were as follows: 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^{o}C, 110 rpm and after every 24hrs interval the growth was measured by recording the absorbance of culture broth at 600 nm using UV-Visible spectrophotometer (SHIMADZU UV-1800). After 8 days, the culture was centrifuged at 8,000 rpm for 15 min at 4^{o}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^{o}C using cooling centrifuge (REMI C-24 PLUS). The aqueous layer was discarded and the precipitate was dissolved in sterile Tris–HCl buffer [19] 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 one mL of 3,5-dinitrosalicylic acid (DNSA) reagent to the enzyme substrate mixture. A control was maintained simultaneously with one mL of 1% soluble starch without enzyme. The mixture was placed in a boiling water bath (100^{o}C) for 10 min, cooled to room temperature (28^{o}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-1800) and

quantified using DNSA with maltose as standard. The amylase activity was determined in terms of U/mL; i.e., One unit of α-amylase activity is defined as the amount of enzyme that releases one μ mol 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, i.e., 0.5M, 1M, 2M, 3M, 4M and 5M. (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 pH 4, 5, 6, 7, 8, 9, 10. (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 ^{o}C, 4 ^{o}C,27 ^{o}C,37 ^{o}C, 45 ^{o}C, 55 ^{o}C, 70 ^{o}C, 100^{o}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 mM and 5mM concentration, of various metal ions and EDTA (ethylenediamine tetraacetic acid), respectively. The various divalent metals tested were $MgSO_4.7H_2O$, $NiCl_2.6H_2O$, $ZnCl_2.7H_2O$, $CuCl_2.2H_2O$, $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, and $CoCl_2.6H_2O$. 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 day old actively growing culture of halophilic isolate GUVSC8 was determined using Scanning Electron Microscopy (SEM) as described by Mani *et al*., (2012) [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*., (2012) [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 (**D**NA **D**ata **B**ank of **J**apan).

3 Results and discussion

3.1 Halophilic microorganism GUVSC8 and extracellular hydrolytic enzymes

A bright orange pigmented, halophilic isolate GUVSC8 was obtained from crude 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 EMH 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 hydrolyse skimmed milk, xylan, CMC and pNP phosphate indicting absence of protease, xylanase, cellulase, and phosphatase activity. Among the seven substrates screened, culture GUVSC8 showed 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* and *Halorubrum* [41]. Study by Kakhki *et al*., (2011) 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* [42]. Kharroub, *et al*., (2014) reported, amylase, gelatinase and lipase positive strains belonging to genera *Halorubrum*, *Haloferax*, *Haloterrigena*, *Halogeometricum*, *Halobacterium*, *Halomicrobium*, and *Haloarcula* from the Algerian hypersaline environments [43]. To the best of our knowledge, this is the first study on hydrolytic enzymes from extremely halophilic isolates obtained from commercial crude salt crystal of Vedaranyam, India.

3.2 Optimization of culture growth and amylase production

The culture halophilic isolate GUVSC8 could grow and exhibited amylase activity over a wide range of salt concentrations, i.e., 10–30 % NaCl, 0.5-4% starch concentration, 5 to 10 pH and temperatures of 15-45^{o}C (Figure S1), with maximum starch hydrolysis, indicating highest amylase activity at 20% NaCl (~3.4M), 0.5 % starch, pH 8 and temperature of 45^{o}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., (2015)

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 [20].

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). 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, and colleagues (2003) reported constitutive production of amylase by moderate halophilic bacterium, *Halobacillus* sp. strain MA-2 in nutrient broth devoid of any carbohydrate [47].

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 incubated with the CFS of isolate GUVSC8. The surface of the starch granules appeared smooth in the control sample, i.e., the one incubated without enzyme. Interestingly, after 24 hrs 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 hrs, 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 centre (centripental) 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 (12hrs), maltohexose and maltooligosaccharides larger than maltohexose was the end product, whereas after prolonged incubations (24hrs), the products were maltotriose and maltotetrose. Similar pattern of hydrolysis has been previously reported in many other α-amylases from halophilic archaea. Kanai, and colleagues (1995) isolated extracellular α-amylase-producing haloalkaliphilic archaeon *Natronococcus arnylolyticus* strain Ah-36T from a Kenyan soda lake, Lake Magadi [17]. 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). The retention time was in the range of 1.2-1.3 indicating 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 the major end products obtained after enzymatic hydrolysis of soluble starch 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 was 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].

3.3 Effect of NaCl, pH and temperature on amylase

As represented in Figure 5(A), ≥ 80% of amylase activity was observed from 0.5 to 2 M NaCl concentration, with the maximum activity at 2M NaCl. Reduction in the amylase activity was observed from 3M to 5M NaCl, however, the activity was more than 50% of the maximum activity. Santorelli *et al*., (2016) 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 at 2 to 4.3 M [19, 20, 22].

The α-amylase of halophilic isolate GUVSC8 was found to be active in broad pH range (Figure 5B), i.e., pH 6 to 9, with an optimum pH of 6.0. The relative enzyme activity started decreasing from pH 7 to 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-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., (2015) and Fukushima *et al*., (2005) reported α-amylase from *Haloferax* sp. HA10 and *Haloarcula* stain 1 with best activity at neutral pH 7 [20, 23].

Enzyme activity was found to be optimum at 45°C and was ≥ 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 [21, 19, 20, 22, 18, 17]. 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.

3.4 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). Bajpai and coworkers, reported that Co^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+} drastically inhibited the activity of α-amylase from halophilic 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 & Chandra, (2008) and Prakash *et al* (2009) 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 [50, 51].

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 increases nor decreases the amylase activity of GUVSC8 suggesting that the enzyme activity could be calcium-independent. Santorelli, *et al*., (2016) reported the activity of

AmyA, an α-amylase from halophilic archaeon *Haloterrigena turkmenica* to be independent of of calcium ions or EDTA [24].

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 and colleagues, (2011) 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 [52]. Similarly, Marco, *et al* (1996), reported Mn^{2+}/Co^{2+} enhanced the activity of truncated *Bacillus subtilis* α-amylase, produced by *Escherichia coli* cells [53]. Alariya, *et al*., (2013) reported that media supplemented with $MnSO_4$ enhanced the production and activity of α-amylase by four soil isolates, *Pseudomonas fluorescens*, *Bacillus subtilus*, *Escherichia coli* and *Serratia marscens* [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.

3.5 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). SEM analysis revealed the cell morphology to be cocci mostly appearing as tetrads of ~ 0.8–1.0 µm in diameter (Figure S5B) which compared well with the cell size of halophilic archaeon *Halococcus hamelinensis* (0.8-1.2 µm) [55]. Spectrophotometric analysis of the Chloroform:Methanol (2:1 v/v) extract of the pigments from halophilic isolate GUVSC8 showed a characteristic carotenoid pigment spectrum with absorption maxima at 499 nm with

two shoulder peaks at 472 nm 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 (**N**ational **C**enter for **B**iotechnology **I**nformation), USA, the isolates GUVSC8 was identified as member of the genus *Halococcus* and closely related to *Halococcus hamelinensis* DQ017835 (95.95%). The tree showing the phylogenetic position of the isolates is represented in Figure S5(C) and the 16S rRNA sequence has been deposited in NCBI GenBank with accession number MG008998 (https://www.ncbi.nlm.nih.gov/nuccore/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, 23, 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 unfavourable 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, amylase, glycogen, and pullulan.

Acknowledgements

BBS and DS are grateful to Head of Dept. of Microbiology, Goa University, for the facilities and infrastructure. BBS thanks Council of Scientific and Industrial Research (CSIR) India for Research Assciateship (RA) (Ref No: 09/919(0030)/2016-EMR-I). Authors are grateful to Dr. Kabilan Mani, Dept. 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.

References

[1] G. Z. L. Dalmaso, D. Ferreira, A. B. Vermelho, Marine extremophiles: a source of hydrolases for biotechnological applications. Marine drugs. **2015**, *13*, 1925-1965.

[2] L. Montoya, C. Vizioli, N. Rodríguez, M. J. Rastoll, R. Amils, I. Marin, Microbial community composition of Tirez lagoon (Spain), a highly sulfated athalassohaline environment. Aquat. Biosyst. **2013**, *9*, 19.

[3] H. Stan-Lotter, S. Fendrihan, Halophilic archaea: life with desiccation, radiation and oligotrophy over geological times, Life. **2015**, *5*, 1487-1496.

[4] B. Van Den Burg, Extremophiles as a source for novel enzymes. Curr. Opin. Microbiol. **2003**, *6*, 213-218.

[5] F. Sarmiento, R. Peralta, J. M. Blamey, Cold and hot extremozymes: industrial relevance and current trends. Front. Bioeng. Biotechnol. **2015**, 3.

[6] Š. Janeček, B. Svensson, E. A. MacGregor, α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. Cell. Mol. Life Sci. **2014**, *71*, 1149-117.

[7] B. J. Green, D. H. Beezhold, Industrial fungal enzymes: an occupational allergen perspective. J. Allergy. **2011**.

[8] J. A. Littlechild, Archaeal enzymes and applications in industrial biocatalysts. Archaea. **2015**.

[9] D. Mehta, T. Satyanarayana, Bacterial and archaeal α-amylases: diversity and amelioration of the desirable characteristics for industrial applications. Front. Microbiol. **2016**, *7*.

[10] S. Kumar, J. Grewal, A. Sadaf, R. Hemamalini, S. K. Khare, Halophiles as a source of polyextremophilic α-amylase for industrial applications. Aims. Microbiol. **2016**, *2*, 1-26.

[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, Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and

biochemical characterization of the recombinant enzyme. Appl. Environ. Microbiol. **1997**, *63*, 3569–3576.

[13] S. Jorgensen, C. E. Vorgias, G. Antranikian, Cloning, sequencing, characterization, and expression of an extracellular α-amylase from the hyperthermophilic archaeon *Pyrococcus furiosus* in *Escherichia coli* and *Bacillus subtilis*. J. Biol. Chem. **1997**, *272*, 16335–16342.

[14] E. Leveque, B. Haye, A. Belarbi, Cloning and expression of an α-amylase encoding gene from the hyperthermophilic archaebacterium *Thermococcus hydrothermalis* and biochemical characterisation of the recombinant enzyme. FEMS Microbiol. Lett. **2000**, *186*, 67–71.

[15] J. K. Lim, H. S. Lee, Y. J. Kim, S. S. Bae, J. H. Jeon, S. G. Kang, J. H. Lee, Critical factors to high thermostability of an α-amylase from hyperthermophilic archaeon *Thermococcus onnurineus* NA1. J. Microbiol. Biotechnol. **2007**, *17*, 1242–1248.

[16] N. Li, B. K. Patel, B. N. Mijts, K. Swaminathan, Crystallization of an α-amylase, AmyA, from the thermophilic halophile *Halothermothrix orenii*. Acta Crystallogr. D*:* Biol. Crystallogr. **2002**, *58*, 2125-2126.

[17] H. Kanai, T. Kobayashi, R. Aono, T. Kudo, *Natronococcus amylolyticus* sp. nov., a haloalkaliphilic archaeon. Int. J. Syst. Evol. Microbiol. **1995**, *45*, 762-766.

[18] W. A. Good, P. A. Hartman, Properties of the amylase from *Halobacterium halobium*. J. Bacteriol. **1970**, *104*, 601-603.

[19] F. Pérez-Pomares, V. Bautista, J. Ferrer, C. Pire, F. C. Marhuenda-Egea, M. J. Bonete, α-Amylase activity from the halophilic archaeon *Haloferax mediterranei*. Extremophiles. **2003**, *7*, 299-306.

[20] B. Bajpai, M. Chaudhary, J. Saxena, Production and Characterization of α-Amylase from an Extremely Halophilic Archaeon, *Haloferax* sp. HA10. Food. Technol. Biotech. **2015**, *53*, 11.

[21] T. Fukushima, T. Mizuki, A. Echigo, A. Inoue, R. Usami, Organic solvent tolerance of halophilic α-amylase from a Haloarchaeon, *Haloarcula* sp. strain S-1. Extremophiles. **2005**, *9*, 85-89.

[22] G. W. Hutcheon, N. Vasisht, A. Bolhuis, Characterisation of a highly stable α-amylase from the halophilic archaeon *Haloarcula hispanica*. Extremophiles. **2005**, *9*, 487-495.

[23] M. Moshfegh, A. R. Shahverdi, G. Zarrini, M. A. Faramarzi, Biochemical characterization of an extracellular polyextremophilic α-amylase from the halophilic archaeon *Halorubrum xinjiangense*. Extremophiles. **2013**, *17*, 677-687.

[24] M. Santorelli, L. Maurelli, G. Pocsfalvi, I. Fiume, G. Squillaci, F. La Cara, G. del Monaco, A. Morana, Isolation and characterisation of a novel alpha-amylase from the extreme haloarchaeon *Haloterrigena turkmenica*. Int. J. Biol. Macromol. **2016**, *92*, 174-184.

[25] K. Majzlova, Z. Pukajova, Š. Janeček, Tracing the evolution of the α-amylase subfamily GH13_36 covering the amylolytic enzymes intermediate between oligo-1, 6-glucosidases and neopullulanases. Carbohydr. Res. **2013**, *367*, 48-57.

[26] R. Karan, M. D. Capes, S. DasSarma, Function and biotechnology of extremophilic enzymes in low water activity. Aquat. Biosyst. **2012**, *8*, 4.

[27] M. Delgado-García, B. Valdivia-Urdiales, C. N. Aguilar-González, J. C. Contreras-Esquivel, R. Rodríguez-Herrera, Halophilic hydrolases as a new tool for the biotechnological industries. J. Sci. Food Agric, **2012**, *92*, 2575-2580.

[28] A. Oren, Taxonomy of halophilic Archaea: current status and future challenges. Extremophiles. **2014**, *18*, 825–834.

[29] S. Chen, Y. Xu, H. C. Liu, A. N. Yang, L. X. Ke, *Halobaculum roseum* sp. nov., isolated from underground salt deposits. Int. J. Syst. Evol. Microbiol. **2017**, *67*, 818-823.

[30] P. Norberg, B. V. Hofstein, Proteolytic enzymes from extremely halophilic bacteria. J. Gen. Microbiol. **1969**, *55*, 251-256.

[31] R. Rohban, M. A. Amoozegar, A. Ventosa, Screening and isolation of halophilic bacteria producing extracellular hydrolyses from Howz Soltan Lake, Iran. J. Ind. Microbiol. Biotechnol. **2009**, *36*, 333-340.

[32] S. S. Balan, R. Nethaji, S. Sankar, S. Jayalakshmi, Production of gelatinase enzyme from *Bacillus* spp isolated from the sediment sample of Porto Novo Coastal sites. Asian. Pac. J. Trop. Biomed. **2012**, *2*, S1811-S1816.

[33] S. M. Ryan, G. F. Fitzgerald, D. van Sinderen, Screening for and identification of starch-, amylopectin-, and pullulan-degrading activities in *Bifidobacterial* strains. Appl. Environ. Microbiol. **2006**, *72*, 5289-5296.

[34] P. Bernfeld, Amylases, alpha and beta. Methods Enzymol. **1955**, *I*, 149-158.

[35] K. Mani, B. B. Salgaonkar, J. M. Braganca, Culturable halophilic archaea at the initial and crystallization stages of salt production in a natural solar saltern of Goa, India. Aquat. Biosyst. **2012**, *8*, 15.

[36] M. Dyall-Smith, The halohandbook: protocols for halobacterial genetics. http://www.haloarchaea.com/resources/halohandbook/index.html **2008**.

[37] M. Birbir, B. Calli, B. Mertoglu, R. E. Bardavid, A. Oren, M. N. Ogmen, A. Ogen, Extremely halophilic Archaea from Tuz Lake, Turkey and the adjacent Kaldirim and Kayacik salterns. World. J. Microbiol. Biotechnol. **2007**, *23*, 309-316.

[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, Introducing EzTaxon-e: A prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. Int. J. Syst. Evol. Microbiol. **2012**, *62*, 716-721.

[39] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5:Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. **2011**, *28*, 2731–2739.

[40] M. de Lourdes Moreno, D. Pérez, M. T. García, E. Mellado, Halophilic bacteria as a source of novel hydrolytic enzymes. Life. **2013**, *3*, 38-51.

[41] M. Birbir, A. Ogan, B. Calli, B. Mertoglu, Enzyme characteristics of extremely halophilic archaeal community in Tuzkoy Salt Mine, Turkey. World J. Microbiol. Biotechnol. **2004**, *20*, 613-621.

[42] A. M. Kakhki, M. A. Amoozegar, E. M. Khaledi, Diversity of hydrolytic enzymes in haloarchaeal strains isolated from salt lake. Int. J. Environ. Sci. Technol. **2011**, *8*, 705-714.

[43] K. Kharroub, M. A. Gomri, M. Aguilera, M. Monteoliva-Sanchez, Diversity of hydrolytic enzymes in haloarchaea isolated from Algerian sabkhas. Afr. J. Microbiol. Res. **2014**, *8*, 3992-4001.

[44] S. Patel, N. Jain, D. Madamwar, Production of α-amylase from *Halobacterium halobium*. World J. Microbiol. Biotechnol. **1993**, *9*, 25-28.

[45] M. J. Coronado, C. Vargas, J. Hofemeister, A. Ventosa, J. J. Nieto, Production and biochemical characterization of an α-amylase from the moderate halophile *Halomonas meridiana*. FEMS Microbiol. Lett. **2000**, *183*, 67-71.

[46] H. Onishi, Halophilic amylase from a moderately halophilic *Micrococcus*. J. Bacterial. **1972**, *109*, 570-574.

[47] M. A. Amoozegar, F. Malekzadeh, K. A. Malik, Production of amylase by newly isolated moderate halophile, *Halobacillus* sp. strain MA-2. J. Microbiol. Methods. **2003**, 52, 353–359.

[48] E. S. Demirkan, B. Mikami, M. Adachi, T. Higasa, S. Utsumi, α-Amylase from *B. amyloliquefaciens*: purification, characterization, raw starch degradation and expression in *E. coli*. Process Biochem. **2005**, *40*, 2629-2636.

[49] H. Tong, D. Bell, K. Tabei, M. M. Siegel, Automated data massaging, interpretation, and e-mailing modules for high throughput open access mass spectrometry. J. Am. Soc. Mass Spectrom. **1999**, *10*, 1174-1187.

[50] K. K. Kiran, T. S. Chandra, Production of surfactant and detergent-stable, halophilic, and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. Strain TSCVKK. Appl. Microbiol. Biotechnol. **2008**, *77*, 1023–1031.

[51] B. Prakash, M. Vidyasagar, M. Madhukumar, G. Muralikrishna, K. Sreeramulu, Production, purification, and characterization of two extremely halotolerant, thermostable, and alkali-stable α-amylases from *Chromohalobacter* sp. TVSP 101. Process Biochem. **2009**, *44*, 210–215.

[52] S. Chakraborty, A. Khopade, R. Biao, W. Jian, X. Y. Liu, K. Mahadik, B. Chopade, L. Zhang, C. Kokare, Characterization and stability studies on surfactant, detergent and oxidant stable α-amylase from marine haloalkaliphilic *Saccharopolyspora* sp. A9. J. Mol. Catal. B-Enzym. **2011**, 68, 52–58.

[53] J. L. Marco, L. A. Bataus, F. F. Valencia, C. J. Ulhoa, S. Astolfi-Filho, C. R. Felix, Purification and characterization of a truncated *Bacillus subtilis* α-amylase produced by *Escherichia coli*. Appl. Microbiol. Biotechnol. **1996**, *44*, 746-752.

[54] S. S. Alariya, S. Sethi, S. Gupta, G. B. Lal, Amylase activity of a starch degrading bacteria isolated from soil. Arch. Appl. Sci. Res. **2013**, *5*, 15-24.

[55] F. Goh, S. Leuko, M. A. Allen, J. P. Bowman, M. Kamekura, B. A. Neilan, B. P. Burns, *Halococcus hamelinensis* sp. nov., a novel halophilic archaeon isolated from stromatolites in Shark Bay, Australia. Int. J. Syst. Evol. Microbiol. **2006**, *56*, 1323-1329.

[56] A. Oren, Molecular ecology of extremely halophilic Archaea and Bacteria. FEMS Microbiol. Ecol. **2002**, *39*, 1–7.

Figure captions

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.

Figure 2. Scanning electron microscopy images of starch granules incubated with cell-free supernatants of *Halococcus* strain GUVSC8 for A) 24 hrs, B) 48 hrs, C) 72 hrs and D) Starch granules without CFS (control).

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 12hrs and 24 hrs respectively. Lane 3-8 contained standards, including glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentose (G5) and maltohexaose (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) maltotetrose and (e) maltopentose.

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

Figure S1. Zone of clearance on starch agar produced by extracellular amylase of halophilic isolate GUVSC8 when grown in minimal medium at various temperatures.

Figure S2. Screening for amylolytic activity of the cell free supernatant (CFS) of the halophilic archaeon *Halococcus* strain GUVSC8 grown in (A) complex medium and (B) minimal medium (NH) supplemented with 0.5% (w/v) starch.

Figure S3. ESI-MS spectra of sodium adducts of the standard carbohydrates, A) glucose, B) maltose, C) maltotriose, D) maltotetrose, E) maltopentose and F) maltohexose.

Figure S4. Effect of metal ions on activity of α-amylase produced by halophilic isolate GUVSC8 as compared to standard condition.

Figure S5. (A) Pure culture of bright orange pigmented, halophilic isolate GUVSC8 obtained from salt crystal of Vedaranyam, Tamil Nadu. (B) The scanning electron micrograph of the halophilic isolate GUVSC8. (C) Phylogenetic tree based on the partial 16S rRNA gene sequence, showing the position of the halophilic isolates GUVSC8, obtained by the neighbor-joining method and constructed with MEGA 5.0. The accession numbers for the reference strains are included in brackets. Percentage bootstrap values based on 1,000 replications and displaying for 100 are indicated at nodes. *Methanosarcina barkeri* AJ012094T is represented as an out-group of the tree.

Figure 1

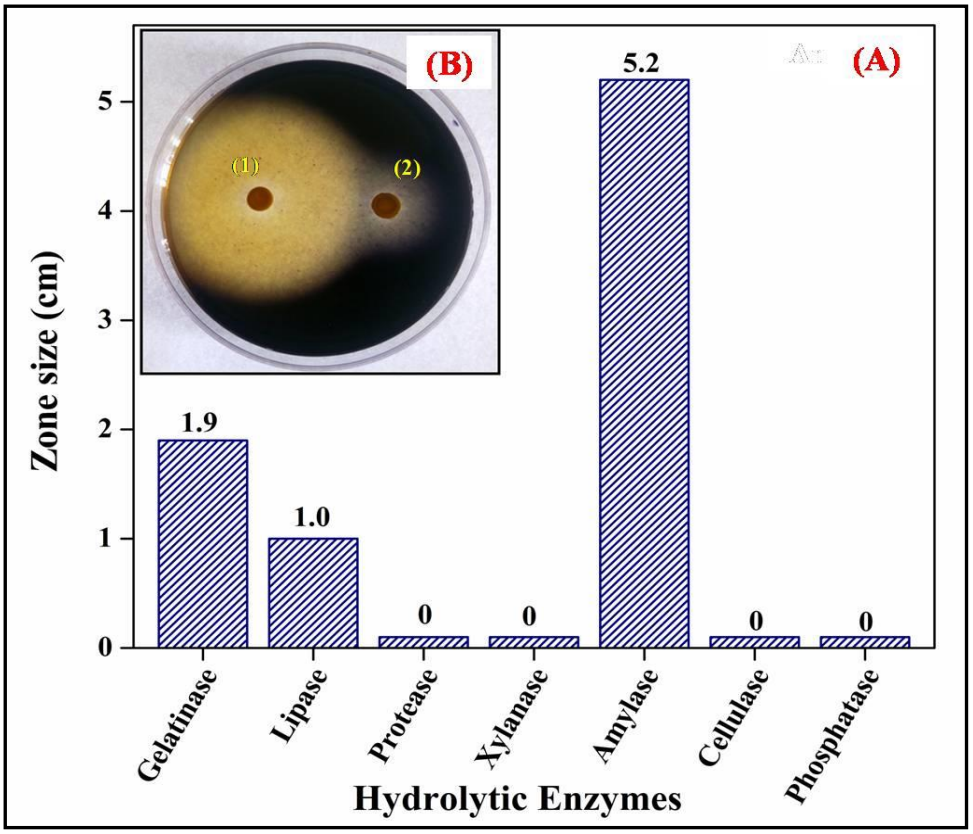

Figure 2

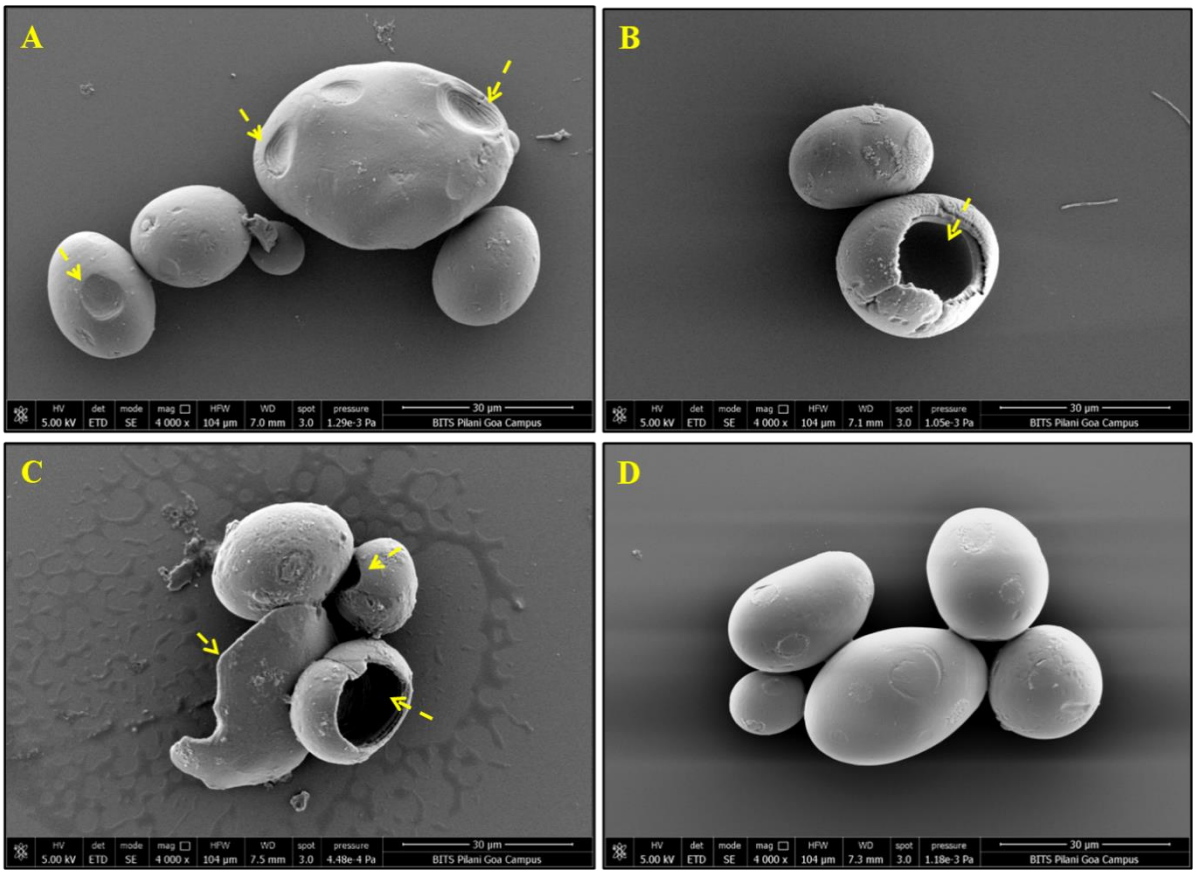

Figure 3

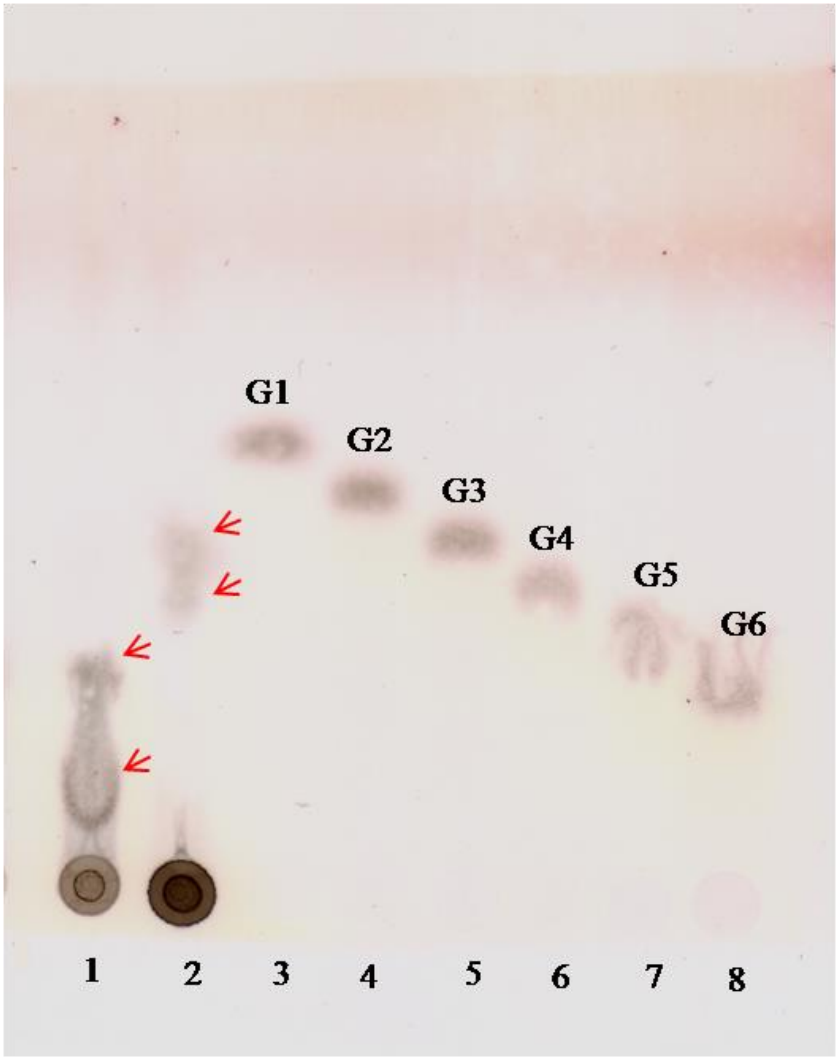

Figure 4

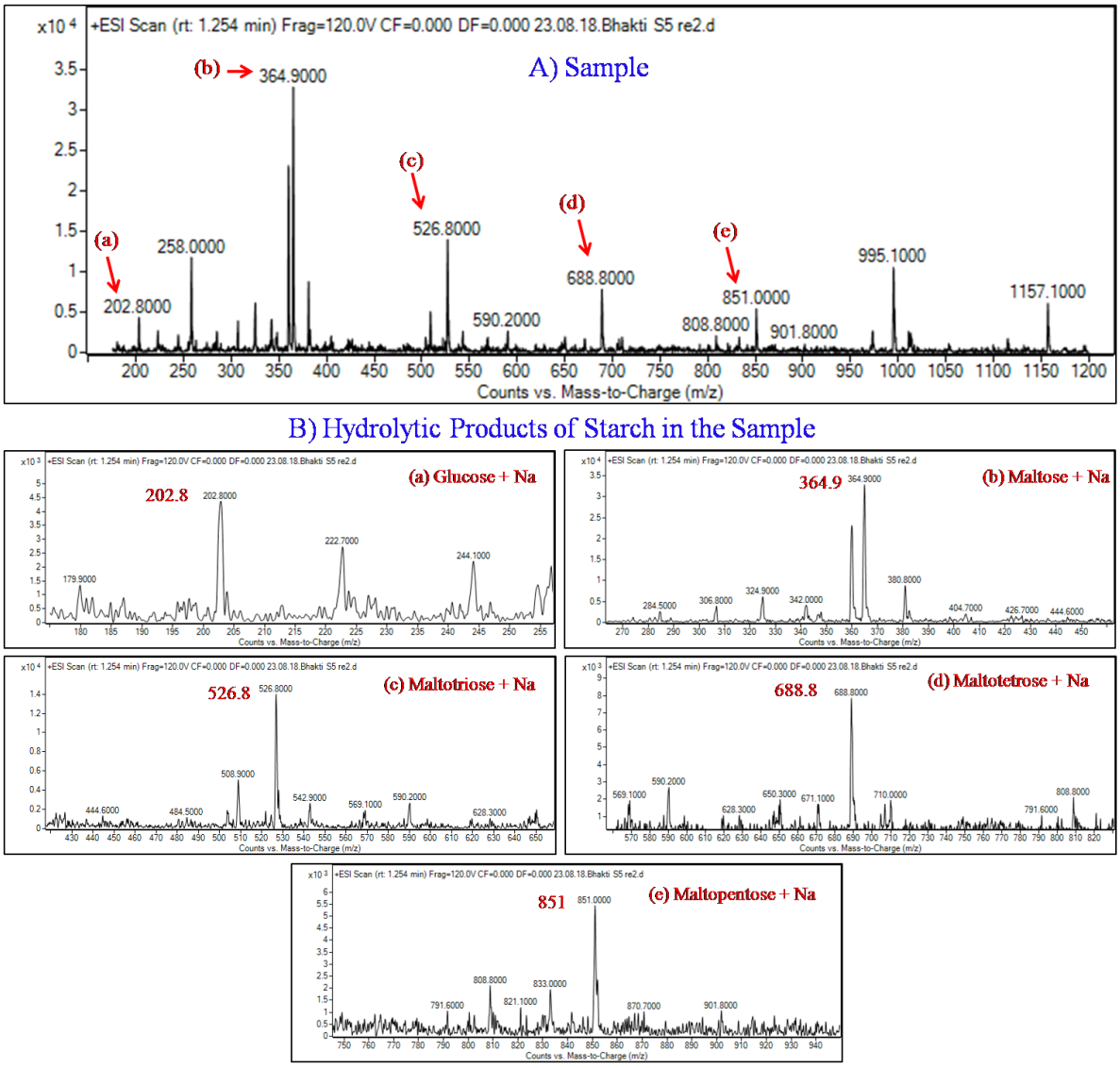

Figure 5