



Characterization of multicomponent antioxidants from *Haloferax alexandrinus* GUSF-1 (KF796625)

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Abstract

The present study was aimed to exploit the haloarchaeon *Haloferax alexandrinus* GUSF-1 (KF796625) for the presence of biomolecules possessing antioxidant activity. The culture produced a bright orange pigment when grown aerobically in nutrient rich medium with 25% crude solar salt. Biomolecules from cell-free supernatant and from the cells of the culture were individually extracted through the assistance of solvents of different polarities, such as ethanol, methanol and hexane, and monitored for scavenging of stable free radicals. Each of the extracts showed varying capacities to scavenge DPPH• (20, 31, and 80% DPPH• RSA; 160.19, 248.29 and 640.76 AAE $\mu\text{g g}^{-1}$ of cells) at 1 mg mL⁻¹. The extracellular ethanolic extract was polysaccharide in nature, equivalent to 47 $\mu\text{g mL}^{-1}$ of glucose when assayed with the phenol-sulfuric acid method. The Fourier Transform-Infrared spectroscopy confirmed the characteristic glycosidic peaks between 2000 and 1000 cm⁻¹. Similarly, the glycerol diether moiety separated from hydroxylated methanolysates through thin-layer chromatography scavenged free radicals (10.47% DPPH• RSA; 80.03 AAE $\mu\text{g g}^{-1}$ of cells). Further, the hexanolic extract exhibited spectral characteristics of red carotenoids and resolved into distinct compounds when separated by thin-layer chromatography using different developing systems. All separated compounds were positive for the DPPH• reaction (13–30% DPPH• RSA; 100–240 AAE $\mu\text{g g}^{-1}$). Chemical profiling of the hexanolic extract using the high resolution-liquid chromatography–mass spectroscopy–diode array detector analysis confirmed the presence of different carbon length isoprenoids; C₃₀: tetrahydrosqualene, C₄₀: 3-hydroxyechinenone, astaxanthin, canthaxanthin, lycopene, phytofluene, phytoene and C₅₀: bisanhydrobacterioruberin, monoanhydrobacterioruberin, bacterioruberin and haloxanthin. Thus, we conclude that the synergistic actions of all these components contribute to the antioxidant activity of the culture and that the antioxidant activity of the exopolysaccharide, glycerol diether moiety, tetrahydrosqualene, haloxanthin and 3-hydroxyechinenone is recorded as the first report for *Haloferax alexandrinus* GUSF-1 (KF796625). Therefore, recommended for use in microbial industrial biotechnology.

Keywords Carotenoids · DPPH• · Exopolysaccharides · Glycerol diether moiety · Haloarchaea · HR-LC/MS–DAD

This article is dedicated to Corresponding Author's mother, Berta Faleiro Furtado and to Co-author's father, Philip John Alvares.

Haloferax alexandrinus GUSF-1 (GenBank accession number KF796625). Gen bank: <http://www.ncbi.nlm.nih.gov>.

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Introduction

Free radicals from atoms, molecules, or ions with highly unstable unpaired electrons are reported to be produced by oxidative reactions from biota and the environment (Lu et al. 2010). The balance between free radicals and oxidative stress caused by them in a living system is maintained by antioxidants (Wan et al. 2016; Alvares and Furtado 2018). Nutraceuticals, cosmetics, and food industries seek natural antioxidants, because they harbor phenolic components that scavenge free radicals (Utkina et al. 2004; Alvares and Furtado 2018). The antioxidant potential of marine halophilic eubacterial bionts isolated from several dominant marine invertebrates (sponges, corals, and bivalves) and haloarchaeal bionts are reported by (Velho Pereira and Furtado

2014; Velho Pereira et al. 2015), but their presence in Haloarchaeal cultures from solar salt is relatively less explored.

Reports of multiple biotechnological potentials in Haloarchaea, which belong to the third domain of life, are available (Singh and Singh 2017). Besides, they are known to degrade organic pollutants (Raghavan and Furtado 2000), mineralize manganese (Naik and Furtado 2019), produce extremozymes (Gaonkar and Furtado 2018; Malik and Furtado 2019), and synthesize nanoparticles (Patil et al. 2014).

Haloferax alexandrinus GUSF-1 (KF796625) retrieved from solar salt of Goa, India was selected because it biosynthesized silver nanoparticles (Patil et al. 2014), produced rhodochrosite (Naik and Furtado 2019) and its ability to scavenge free radicals (Alvares and Furtado 2018), all of significance, in the pharmaceutical and biotechnological industry.

This present study, therefore, is a record of the: (1) extraction of the biomolecules from the cells of *Haloferax alexandrinus* GUSF-1 (KF796625) assisted by the use of solvents such as ethanol, methanol and hexane; (2) the ability of the extracts to scavenge stable DPPH• free radicals and (3) the use of TLC, UV–visible, FTIR spectroscopy and HR-LC/MS–DAD for separation, identification and chemical profiling of the biomolecules responsible for the antioxidant activity of *Haloferax alexandrinus* GUSF-1 (KF796625).

Materials and methods

Chemicals and reagents

All chemicals and reagents used were of analytical grade. 1, 1-diphenyl-2-picrylhydrazyl (DPPH•) was acquired from Sigma-Aldrich and prepared in methanol (purity 97%)/hexane (purity > 95%) as required. Thin-layer chromatography (TLC) sheets (silica gel GF254 plates) used in this study were from Merck, Darmstadt, Germany.

Growth of *Haloferax alexandrinus* GUSF-1 (KF796625)

Haloferax alexandrinus GUSF-1 (GenBank accession number KF796625), a haloarchaeon, isolated from natural solar salt by Sequeira (1992), was used in this study. Cells were cultured in 5 L of Tryptone Yeast Extract containing 25% crude solar salt (NTYE) (Raghavan and Furtado 2000) at 42 °C and 150 rpm for 6 days (REMI CIS-24 PLUS, India), resulting in a wet cell mass of 107 g. Separation of cell mass from culture broth was achieved using a high-speed centrifuge at 12,857×g and 4 °C (Biofuge-Heraeus Stratos). The supernatant obtained was passed through a 0.2 µm membrane filter (MF-Millipore™) to give the cell-free supernatant (CFS).

Preparation of ethanolic extract from CFS, hydroxylated methanolysate and hexanolic extract

Cell-free supernatant was subjected to extraction by the Del Gallo and Haegi (1990) method using 96% cold ethanol. After addition, the mixture was kept standing overnight at -20 °C and centrifuged. The pellet obtained was dispersed in sterile distilled water and checked for the presence of total carbohydrate using the phenol–sulfuric acid method, according to Dubois et al. (1956) with glucose as the standard.

Hydroxylated methanolysate of cells was prepared by the Ross et al. (1981) method. One gram of cells was mixed with methanol:toluene:concentrated H₂SO₄ (3:3:1 v/v), heated at 50 °C in a water bath for 18 h, and extraction of the hydroxylated methanolysate was accomplished by adding hexane.

To the remaining cells, methanol was added in minimum light exposure and kept overnight at 4 °C. The cell debris were then separated by centrifuging to obtain a clear cell-free methanolic extract of cells. Components from this were fractionated with hexane and distilled water according to the method of Asker et al. (2002) to obtain the hexanolic extract. The ethanolic extract, hydroxylated methanolysates and the phase fractionated hexanolic extract were concentrated under N₂ gas, wrapped in aluminium foil to avoid light exposure and stored at -20 °C.

Detection of free radical scavenging activity (RSA) of the ethanolic extract, hydroxylated methanolysate and hexanolic extract

Individual extracts at 1 mg mL⁻¹ were checked for RSA by the method of Alvares and Furtado (2018). Each was mixed with 1 mL of 0.2 mM DPPH• reagent (prepared in hexane or methanol as required; Supplementary Fig. 1) kept in the dark at room temperature (28 ± 2 °C), for 30 min and the absorbance checked at 517 nm, against hexane/methanol as the reference solution.

The % radical scavenging activity was calculated using Eq. 1;

$$\%DPPH \cdot RSA = \frac{AB - AS}{AB} \times 100, \quad (1)$$

where AB is the absorbance of 0.2 mM DPPH• in the respective solvent (hexane or methanol). AS is the absorbance of the reaction mixture (DPPH• + extract). Ascorbic acid was used as the standard ($R^2 = 0.9835$) and results were also expressed as Ascorbic Acid Equivalent (AAE) µg g⁻¹ of cells. The individual reaction mixtures were then scanned between 190 and 800 nm after 30 min of incubation.

Characterization of ethanolic extract, hydroxylated methanolysate and hexanolic extract

UV–Vis spectroscopy

The ethanolic and the fractionated hexanolic extracts were individually scanned between 190 and 800 nm using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) and against distilled water/hexane as the reference solution, as required.

TLC analysis

Components in the hydroxylated methanolysate were chromatographed on four different TLC sheets and resolved separately in the developing system consisting of petroleum

ether (60–80 °C):diethyl ether (85:15, v/v). The first chromatogram was placed in an iodine chamber to check for colorless compounds (Kushwaha et al. 1974). The second was sprayed with 10% dodecaphosphomolybdic acid and heated at 150 °C for 15 min (Ross et al. 1981). Detection of free radical scavenging activity was carried out by spraying the third chromatogram with 0.2 mM DPPH[•] and incubating in the dark. The fourth chromatogram was left untreated.

Similarly, components in the hexanolic extract were chromatographed and resolved using different developing systems: acetone:petroleum ether (60–80 °C) (20:80, v/v), methanol:chloroform (7:93, v/v) (Asker et al. 2002), acetone:hexane (50:50, v/v) (Ronnekleiv and Liaaen-Jensen 1995), and *n*-heptane:benzene (90:10, v/v) (Tornabene et al. 1969), respectively.

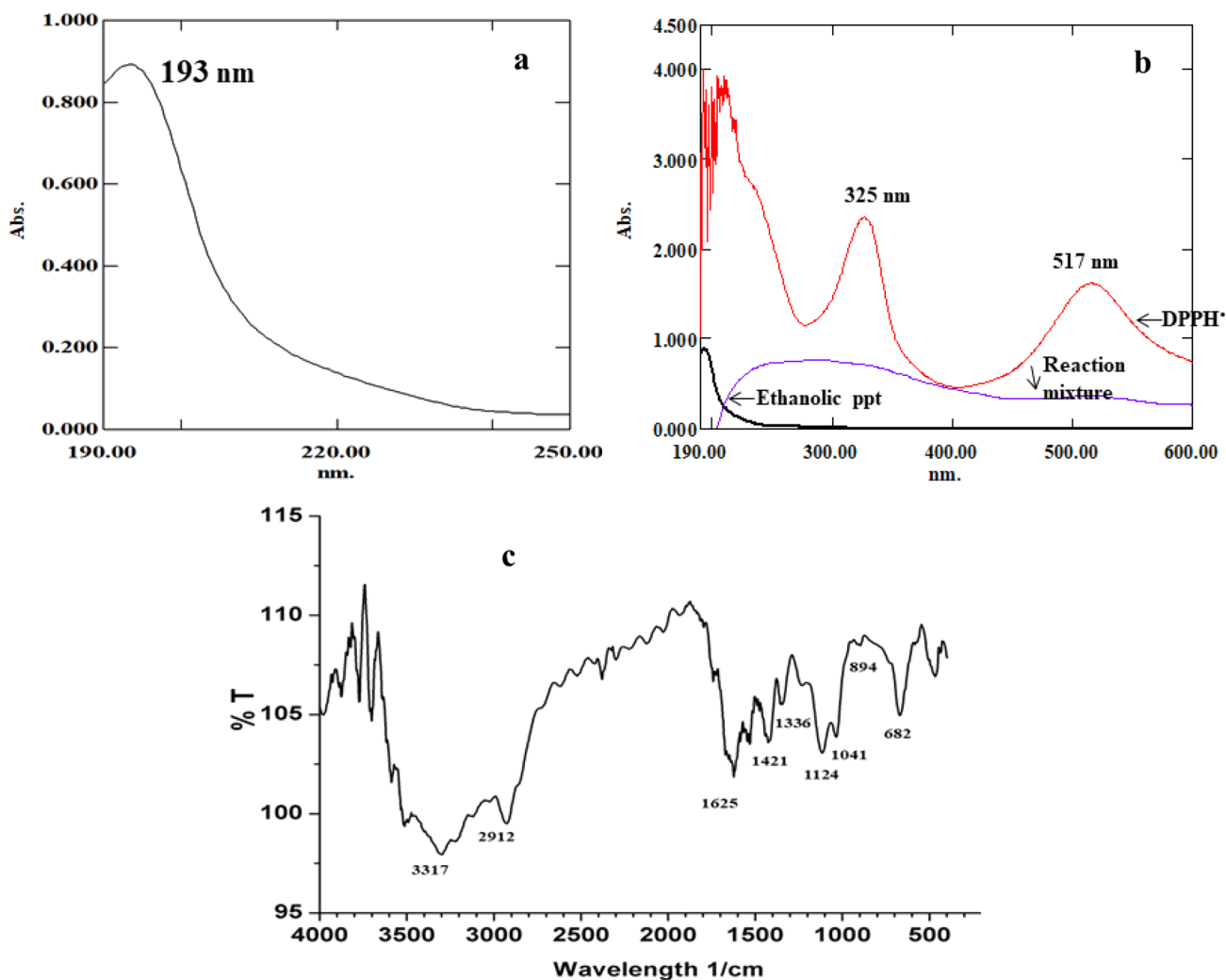


Fig. 1 Characterization of ethanolic extract from *Haloferax alexandrinus* GUSF-1 (KF796625). **a** UV spectrum showing a single peak at 193 nm, **b** DPPH[•] decolorization of ethanolic extract monitored by the spectrophotometer; which shows that both the peaks of DPPH[•]

(325 and 517 nm) are abolished by the ethanolic extract, indicating the free radical scavenging capacity, **c** FTIR spectrum revealing the characteristic glycosidic peaks between 2000 and 1000 cm^{-1}

In each case, sets of three different TLC sheets were used. In each set, spotting on a single chromatogram was done at a distance of 1.5 cm apart. After resolving in respective developing system, all the chromatograms were first air-dried at room temperature in the dark. Visible compounds, if any that were separated, were noted. The chromatogram developed in *n*-heptane:benzene (90:10, v/v) was visualized with iodine vapors. In each case, the detection of free radical scavenging activity was carried out by spraying the second set of chromatograms with 0.2 mM DPPH[•]. The third set was left untreated. The retention factor, R_f , of the resolved spots that decolorized the purple DPPH[•] was recorded and compared with that reported in literature using identical developing systems.

Select spots from individual chromatograms that were positive for the DPPH[•] activity, were scrapped off from the respective resolved untreated chromatogram. Under minimum light, each compound was individually eluted out in the respective solvent, concentrated, and weighed. Each elute was checked for their RSA and UV–Vis spectral characteristics.

Quantification of the purified colored compound was carried out using Eq. 2 (Kushwaha et al. 1974) and as reported by Raghavan and Furtado (2005):

$$\text{Conc } (\mu\text{g/g of cellular protein}) = \frac{AY}{E^{1\%}}, \quad (2)$$

where A is the absorbance at λ_{max} , Y = volume in mL (in this case, 1 mL cuvette was used). $E^{1\%}$ is the

extinction coefficient specific for each separated compounds (Britton 1995). C₅₀ carotenoids—2450 at λ_{490} (Kushwaha et al. 1974); Canthaxanthin—2092 at λ_{476} (Warren and Weedon 1958); 3-hydroxyechinenone—2100 at λ_{476} (An et al. 1989).

Fourier Transform Infrared (FTIR) spectroscopy

The ethanolic extract, the compound resolved on silica plate at R_f 0.2 from the hydroxylated methanolysate and hexanolic extract were individually mixed with KBr to form a pellet. Samples were then analyzed in the 4000 and 400 cm^{-1} spectral region at a resolution of 4.0 cm^{-1} using the FTIR spectrometer (Shimadzu IR Prestige-21).

High resolution-liquid chromatography–mass spectroscopy–diode array detector (HR-LC/MS–DAD) analysis

Chemical profiling of the constituents in hexanolic extract was achieved using a HR-LC/MS–DAD instrument; Agilent 1290 Infinity UHPLC system, 1260 Infinity Nano HPLC with Chipcube series (Agilent Technologies, USA) and a hypersil gold 3 microns 100×2.1 mm C-18 reverse-phase column from Agilent Technologies. The ESI interface consisted of a binary pump, an autosampler and 6550 i funnel QTOF-LC/MS. The two mobile phases used were: (A) 0.1% (v/v) formic acid in water and (B) 70% acetonitrile in 30% (v/v) methanol. The flow rate was adjusted for 0.3 mL/min at 25 °C. The UV–Vis

Fig. 2 Characterization of hydroxylated methanolysate from cells of *Haloferax alexandrinus* GUSF-1 (KF796625): The thin-layer chromatogram was resolved in petroleum ether (40–60 °C): diethyl ether (85:15, v/v) and developed with **a** iodine vapor, **b** 10% dodecaphosphomolybdic acid in absolute ethanol and heated at 150 °C, **c** decolorization of hexanolic DPPH[•] solution by compound at R_f 0.2 and **d** DPPH[•] spot assay

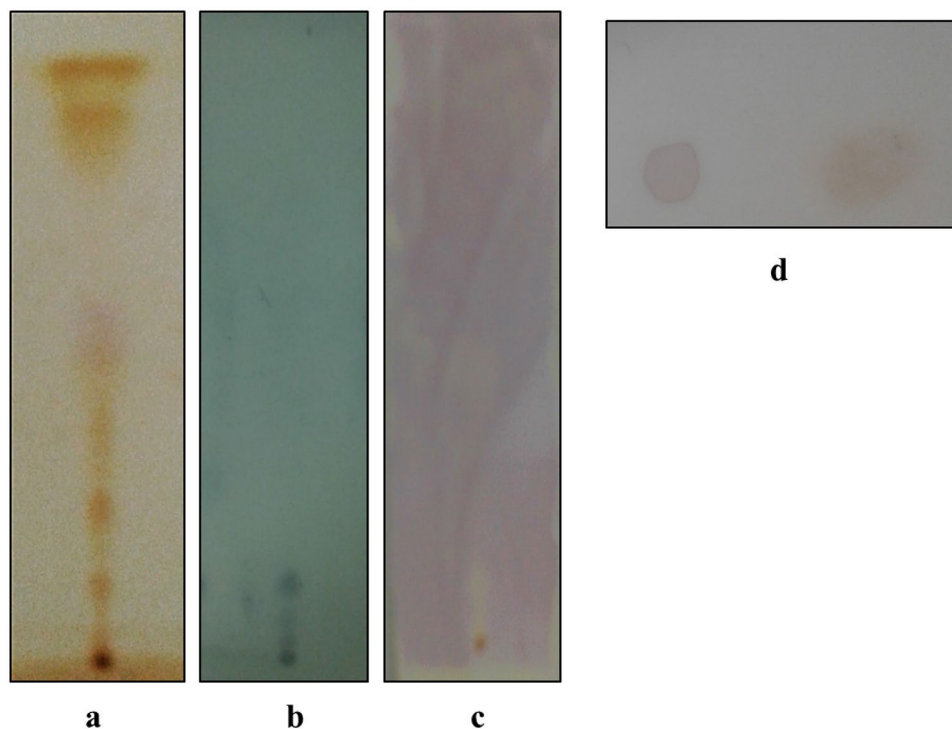
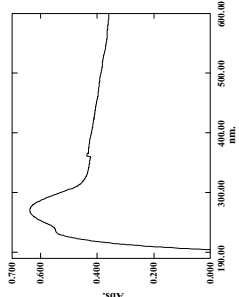
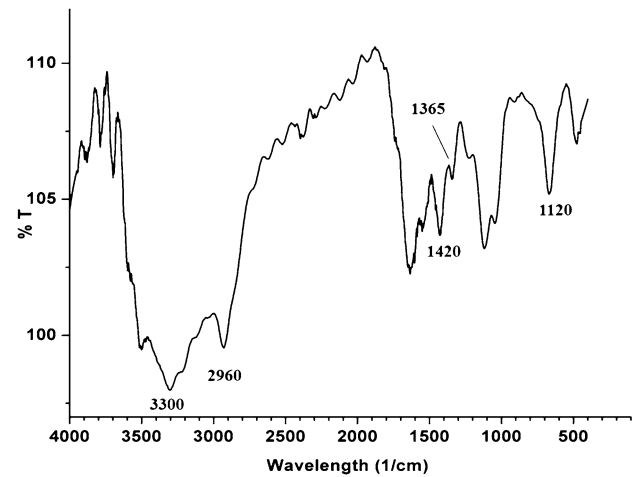
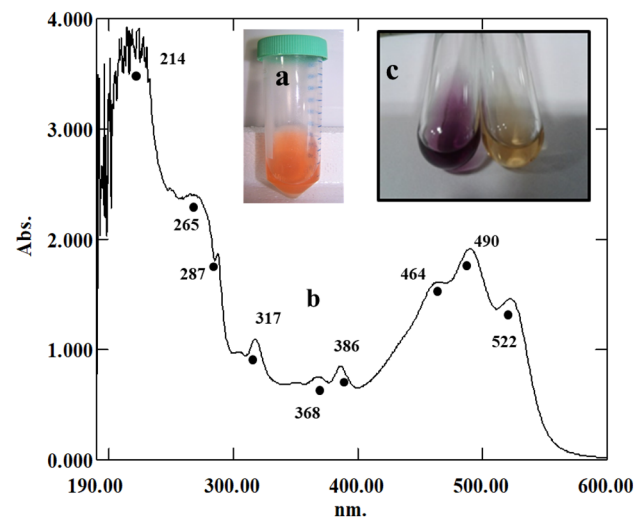


Table 1 Characterization and quantification of glycerol diether moiety extracted from cells of *Haloferax alexandrinus* GUSF-1 (KF796625) and separated using thin-layer chromatography

Solvent system	R_f of compound with antioxidant activity	DPPH [•] reaction	Quantity obtained after TLC separation (mg 100 g ⁻¹ cells)	% DPPH [•] RSA; AAE $\mu\text{g g}^{-1}$ of cells (mean \pm SD)	UV-visible absorption spectrum	UV-visible absorption spectral peaks (nm)	Identification
Petroleum ether(60–80 °C); Diethyl ether (85:15, v/v)	0.2	+	6.66	10.47 \pm 0.21; 80.03 \pm 0.32		256, 263, 270	Glycerol diether moiety (GDEM)

**Fig. 3** FTIR spectrum of glycerol diether moiety extracted from hydroxylated methanolsate from cells of *Haloferax alexandrinus* GUSF-1 (KF796625)**Fig. 4** Decolorization of DPPH[•] by hexanolic extract from cells of *Haloferax alexandrinus* GUSF-1 (KF796625). **a** Bright orange hexanolic extract, **b** UV-Vis absorption spectrum of hexanolic extract with peaks in the UV and visible region and **c** the deep purple color of DPPH[•] (left) is decolorized by the hexanolic extract rendering it straw yellow (right), an indication of its potent free radical scavenging capacity

spectrum was obtained between 190 and 600 nm and the chromatograms were processed at 320, 447 and 490 nm, respectively. Separated compounds were identified by comparing the obtained m/z values with the Agilent Mass Hunter Qualitative Analysis Software version (B.05.00), Japanese carotenoid database, Pubchem and earlier reported literature.

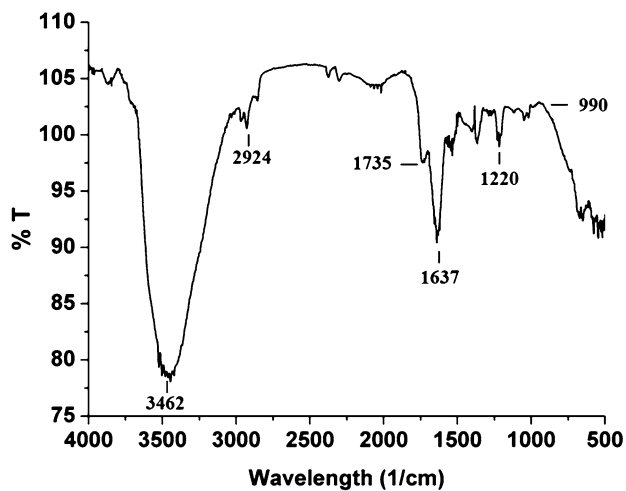


Fig. 5 FTIR spectrum of hexanolic extract from cells of *Haloferax alexandrinus* GUSF-1 (KF796625)

Results and discussion

Cells of *Haloferax alexandrinus* GUSF-1 (KF796625)

Haloferax alexandrinus GUSF-1 (KF796625) produced a bright orange pigment when grown aerobically in 25% NTYE broth and a cell mass of 107 g was recovered from the fully grown culture (Supplementary Fig. 2). The colonies of the culture have been reported to scavenge free radicals by the agar growth method in a previous study (Alvares and Furtado 2018).

Characterization of ethanolic extract

Ethanolic extracts from the cell-free supernatant resulted in a white precipitate soluble in distilled water and exhibited a value of $20 \pm 0.4\%$ DPPH[•] RSA ($160.19 \text{ AAE } \mu\text{g g}^{-1}$ of cells). The UV spectrum of this precipitate showed a single peak at 193 nm (Fig. 1a). The spectrum of the reaction mixture revealed that both peaks of the pure DPPH[•] radical seen at 517 and 325 nm were abolished along with the single peak at 193 nm during the decolorization process suggesting its involvement (Fig. 1b). In the FTIR spectrum, absorption peaks were seen between 3900 and 500 cm^{-1} (Fig. 1c). Characteristic peaks were also seen at 3317 cm^{-1} for the OH group, at 2912 cm^{-1} indicating the presence of the C-H group and at 1625 cm^{-1} for the carbonyl group. A broad stretch at 1041 cm^{-1} followed by an intense peak at 1124 cm^{-1} is indicative of the C–OH side groups and the C–O–C glycosidic band vibrations (Zhang et al. 2011). The ethanolic extract tested positive for carbohydrates with 47 mg mL^{-1} equivalent of glucose, hence, polysaccharide in nature (Supplementary Fig. 3).

Little is reported about exopolysaccharide (EPS) production from haloarchaea with Antón et al. (1988), first reporting the production of EPS by the *Haloferax mediterranei* (ATCC 33500). Antioxidant activity of EPS has been reported in the *Haloterrigena turkmenica* by Squillaci et al. (2016), at a value three times lower than our activity, although assayed at the same concentration.

Fig. 6 Thin-layer chromatograms of hexanolic extract from cells of *Haloferax alexandrinus* GUSF-1 (KF796625) developed in **a** acetone:petroleum ether (20:80; v/v), **b** methanol:chloroform (7:93; v/v), and **c** acetone:hexane (50:50; v/v) and viewed as (1) visible spots and (2) after spraying with DPPH[•]. The hexanolic extract was also resolved in **d** *n*-heptane: benzene (90:10, v/v) and viewed as (1) visible spots, (2) after spraying with DPPH[•] and (3) exposed to iodine vapor

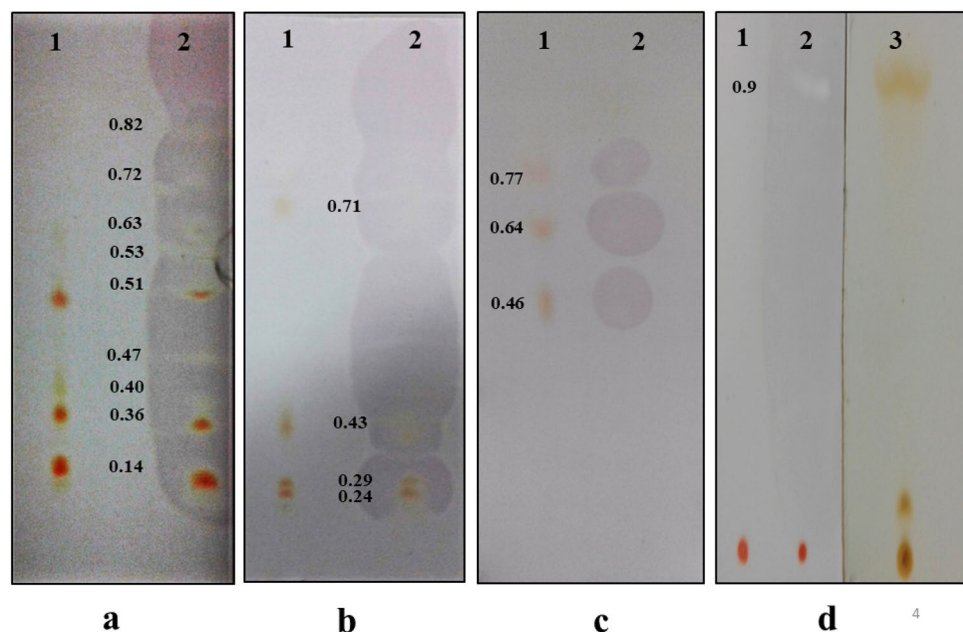


Table 2 Separation of biomolecules from hexanolic extract of *Haloferax alexandrinus* GUSF-1 (KF796625) achieved using thin-layer chromatography and different developing systems

Solvent system	R_f of separated carotenoids	DPPH [•] reaction
Acetone–petroleum ether (20: 80, v/v)	0.82	+
	0.72	+
	0.63	+
	0.53	+
	0.51	+
	0.47	+
	0.4	+
	0.36	+
	0.14	+
Methanol–chloroform (7:93, v/v)	0.71	+
	0.43	+
	0.29	+
	0.24	+
Acetone: hexane (50:50, v/v)	0.71	+
	0.58	+
	0.46	+
<i>n</i> -heptane: benzene (90:10, v/v)	0.9	+

Characterization of hydroxylated methanolysate

The hydroxylated methanolysate extract was pale yellow and of oily consistency and gave a value of $31 \pm 0.24\%$ DPPH[•] RSA ($248.29 \text{ AAE } \mu\text{g g}^{-1}$ of cells). The chromatogram revealed the presence of 6 spots in iodine chamber at R_f 0.2, 0.3, 0.48, 0.59, 0.78 and 0.89, respectively (Fig. 2a). On spraying with 10% dodecaphosphomolybdic acid, a blue spot was visualized at R_f 0.2 (Fig. 2b). Interestingly, the compound at R_f 0.2 decolorized DPPH[•] on the chromatogram and on the spot test (Fig. 2c, d) and the RSA of the compound after elution in hexane, gave a value of $10.47 \pm 0.21\%$ DPPH[•] RSA ($80.03 \pm 0.32 \text{ AAE } \mu\text{g g}^{-1}$ of cells) (Table 1). These results also confirmed our earlier report (Alvares and Furtado 2018). The UV spectrum exhibited absorption maxima at 256, 263, and 270 nm (Table 1). Notable in the FTIR spectra was the complete absence of ester absorption peaks between 1730 and 1750 cm^{-1} . However, the spectra did show strong absorption bands indicative of long-chain groups (2960 cm^{-1}), OH groups (3300 cm^{-1}), ether C–O–C groups (1120 cm^{-1}) and the isopropyl units between 1365 and 1420 (Fig. 3). This spectrum matched with that reported for the purified glycerol diether moiety from *Halobacterium cutirubrum* NCMB 763 confirming the identity as glycerol diether moiety (GDEM), a compound that is unique to haloarchaea and which distinguishes it from the eubacterial domain (Ross et al. 1981, 1985). Furthermore, the result was also consistent with reports by Braganca and Furtado (2009)

and Gaonkar and Furtado (2018). Though GDEM is reported to protect Archaea and haloarchaea against hydrolysis of their membrane phospholipids, their role as scavengers of DPPH[•] observed by us in this study is the first record.

Characterization of hexanolic extract

The hexanolic extract was bright orange, with absorption peaks both in the UV (190–300 nm) and the visible (300–700 nm) range (Fig. 4a, b). These peaks corroborated with red carotenoids and their derivatives, as reported earlier for the culture (Alvares and Furtado 2018) and also reported for Haloarchaea by Britton et al. (1995). The hexanolic extract decolorized the deep purple-colored stable DPPH[•] to straw yellow and showed a highly potent value of $80 \pm 0.32\%$ DPPH[•] RSA ($640.76 \text{ AAE } \mu\text{g g}^{-1}$) (Fig. 4c). The FTIR spectral analysis revealed intense absorption peaks at 3462 cm^{-1} and stretching vibrations were seen at 2924 cm^{-1} , while absorption bands were observed at 1735, 1637, 1220, and 990 cm^{-1} (Fig. 5). The constituents of the hexanolic extract resolved by TLC were found to be all positive for the DPPH[•] reaction with values in the range of 13–30% DPPH[•] RSA; $100\text{--}240 \text{ AAE } \mu\text{g g}^{-1}$. The TLC profiles, spectroscopic characteristics and quantification data obtained using the individual elutes' absorption coefficients are recorded in Fig. 6 and Tables 2 and 3.

This separation attained by TLC supported and confirmed the presence of canthaxanthin, 3-hydroxyechinenone, bacterioruberin, bisanhydrobacterioruberin, monoanhydrobacterioruberin, haloxanthin and tetrahydrosqualene. These results are in good agreement and consistent with that reported for *Haloferax alexandrinus* Strain TMT (Asker et al. 2002), *Haloferax volcanii* culture (Ronnekleiv et al. 1995) and in *Halobacterium cutirubrum* as described by Tornabene et al (1969).

Chemical profiling of hexanolic extract

The chemical profile of hexanolic extract by HR-LC/MS–DAD showed 12 peaks, which, in the increasing order of their retention time, eluted a total of 17 different compounds, with different m/z values (Fig. 7a). The diode array detector (DAD) revealed that peaks between R_t 29.943 and 31.68 absorbed at 320, 447, and 490 nm (Fig. 7b–d). These compounds were therefore, confirmed as red carotenoids. A combination of the DAD analyses (UV–Vis), the Agilent Mass Hunter software, the Japanese carotenoid database, and PubChem was used to identify the different compounds according to their m/z values. Compounds thus identified were canthaxanthin, tetrahydrosqualene, 3-hydroxyechinenone, phytoene, astaxanthin, bisanhydrobacterioruberin, monoanhydrobacterioruberin, bacterioruberin, lycopene,

Table 3 Purification and quantification of the antioxidant compounds separated from the hexanolic extract of *Haloflex alexandrinus* GUSF-1 (KF796625)

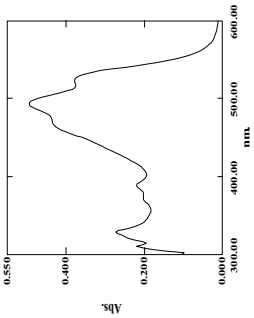
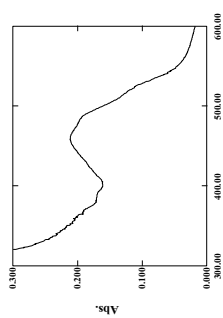
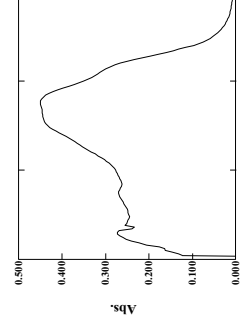
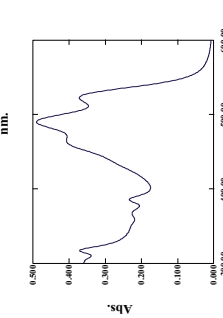
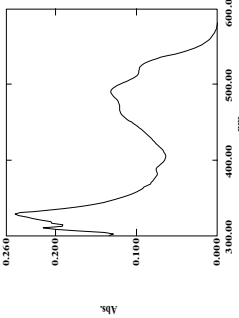
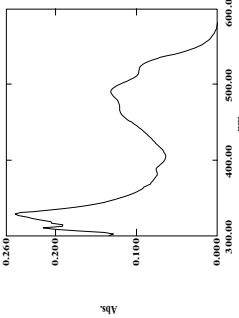
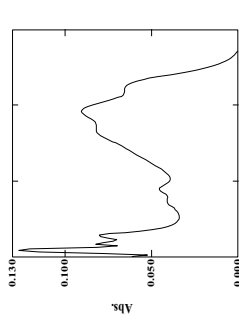
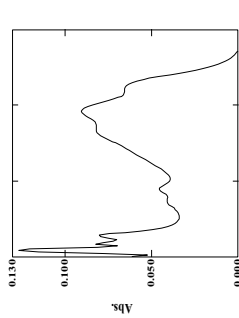
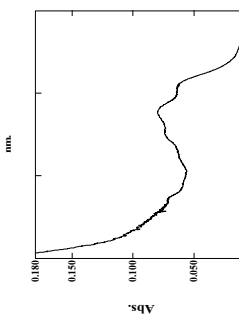
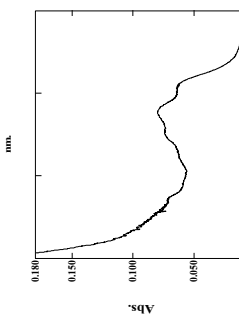
Solvent system	R_f of separated compounds with antioxidant activity	Quantity obtained after TLC separation ($\text{mg } 100 \text{ g}^{-1}$ cells)	% DPPH [•] RSA; AAE $\mu\text{g g}^{-1}$ of cells (mean \pm SD)	UV-visible absorption spectrum of pure compound	UV-visible absorption spectral peaks (nm)	Quantification of the purified compound using molar extinction coefficient ($\mu\text{g/g}$ of cellular protein) (Kushwaha et al. 1974)	Identification
Acetone-petroleum ether (20:80, v/v)	0.14	8	30 ± 0.04 ; 240.02 ± 0.5		368, 387, 463, 492, 525	2	Bacterioruberin
	0.36	5.6	17.51 ± 0.1 ; 140.0 ± 0.21		369, 388, 458	1.009	3-hydroxyechinenone
	0.53	2.93	14.56 ± 0.18 ; 110.6 ± 0.36		374, 476	2.151	Canthaxanthin
Methanol-chloroform (7:93, v/v)	0.24	8.53	28 ± 0.12 ; 220.4 ± 0.06		368, 386, 463, 492, 525	2	Bacterioruberin

Table 3 (continued)

Solvent system	R_f of separated compounds with antioxidant activity	Quantity obtained after TLC separation ($\text{mg } 100 \text{ g}^{-1}$ cells)	% DPPH* RSA; AAE $\mu\text{g g}^{-1}$ of cells (mean \pm SD)	UV-visible absorption spectrum of pure compound	UV-visible absorption spectrum of purified compound	UV-visible absorption spectral peaks (nm)	Quantification of the purified compound using molar extinction coefficient ($\mu\text{g/g}$ of cellular protein) (Kushwaha et al. 1974)	Identification
	0.29	5.06	23.24 \pm 0.20; 180.6 \pm 0.19			369, 389, 464, 490, 522	0.36	Bisanhydrobacterioruberin
	0.43	4.53	18.49 \pm 0.09; 140.8 \pm 0.01			368, 387, 464, 489, 522	0.534	Monoanhydrobacterioruberin
Acetone:hexane (50:50, v/v)	0.46	3.73	13.26 \pm 0.07; 100.6 \pm 0.14			377, 451, 478, 512, 522	0.3151	Haloxanthin
<i>n</i> -Heptane:benzene (90:10, v/v)	0.9	8	26.04 \pm 0.12; 200.85 \pm 0.1	—	—	—	—	Tetrahydro-squalene

Sample Name	GU-JA-1	Position	P1-D1	Instrument Name	Instrument 1	User Name	
Inj Vol	3	Inj Position		Sample Type	Sample	IRM Calibration Status	Success
Data Filename	GU-JA-1.d	ACQ Method	30mins_+ESI_10032014	Comment		Acquired Time	5/9/2018 11:39:03 AM

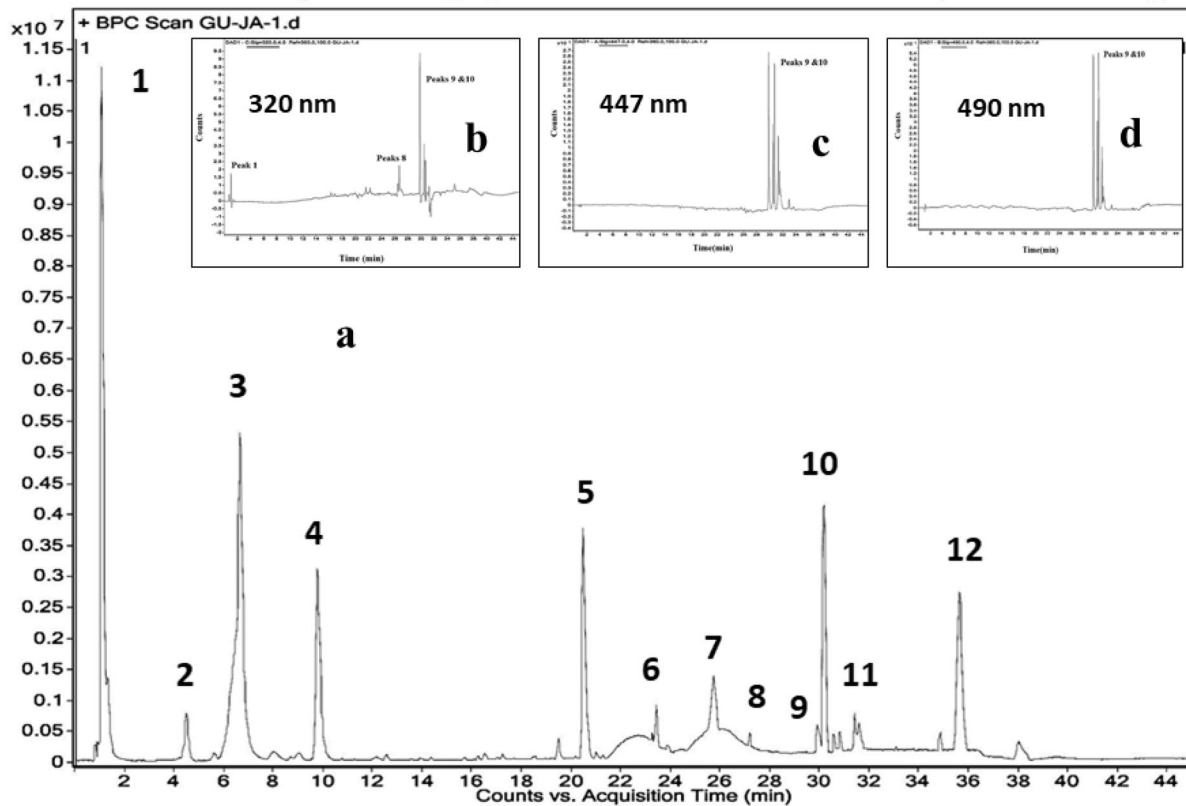


Fig. 7 Chemical profile of hexanolic extract from cells of *Haloferax alexandrinus* GUSF-1 (KF796625). **a** HR-LC/MS–DAD chromatogram of hexanolic extract revealing a total of 12 peaks labeled according to the increasing retention times (R_t). Identification of the separated compounds was achieved by comparing the m/z values

obtained with the Agilent Mass Hunter Qualitative Analysis Software version (B.05.00), Japanese carotenoid database, Pubchem, and reported literature. Diode Array Detector response at **b** 320, **c** 447 and **d** 490 nm

haloxanthin, and phytofluene, whose mass spectral data are presented in Table 4.

Tetrahydro-squalene (C_{30} isoprenoid) attributes to stabilize the membrane structure in *Halobacterium cutirubrum* as reported by Tornabene et al. (1969) and as observed in this study, is recorded for its first-time role as a free radical scavenger (antioxidant). The C_{40} isoprenoids detected were 3-hydroxyechinenone, astaxanthin, canthaxanthin, lycopene, phytofluene and phytoene. Although all of these are reported in *Haloferax alexandrinus* strain TMT (Asker et al. 2002), but their biological property and role is not characterized. The antioxidant activity for astaxanthin, canthaxanthin, and lycopene is a known feature, but we demonstrated and recorded, for the first time, the free radical scavenging activity of 3-hydroxyechinenone. Yang et al. (2015) delineated phytofluene and phytoene as precursors in the biosynthetic pathway of C_{50} carotenoids in Haloarchaea. Phytoene detected in very low concentrations than the other

chemical constituents in this study is consistent with that reported by Rodrigo-Baños et al. (2015).

The C_{50} isoprenoids identified were bacterioruberin along with its precursors; monoanhydrobacterioruberin and bisanhydrobacterioruberin. All of these decolorized DPPH[•], confirming their antioxidant activity. Involvements of these carotenoids in free radical scavenging activity in a whole-cell extract of *Haloterrigena turkmenica* are known and reports are available (Squillaci et al. 2017). A dodecane C_{50} carotenoid, haloxanthin with a peroxide group detected in the hexanolic extract, has been identified in *Haloferax volcanii* (Ronnekleiv et al. 1995), but not in *Haloferax alexandrinus* GUSF-1 (KF796625) and its role in scavenging free radicals as observed in this study, is a novel report.

Conclusively, the present study is the first report of the antioxidant activity of exopolysaccharide, glycerol diether moiety, tetrahydro-squalene, haloxanthin, and 3-hydroxyechinenone of *Haloferax alexandrinus* GUSF-1 (KF796625). The synergistic actions of all of these

Table 4 Mass spectral analysis of the hexanolic extract of *Haloferox alexandrinus* GUSF-1 (KF796625)

Peak no	Retention time ^a	(m/z) ^a	z ^a	Ion ^a	Abundance ^a (conc)	Mass ^a	Molecular formula ^b	Identification ^b
1	1.121	202.17	1	(M+H) ⁺	1.12 × 10 ⁷	201.17	C ₁₁ H ₂₃ NO ₂	Unidentified
2	4.528	302.19	1	(M+H) ⁺	0.07 × 10 ⁷	301.18	C ₁₆ H ₂₃ N ₅ O	Unidentified
3	6.823	565.3	1	(M+H) ⁺	0.51 × 10 ⁷	564.3	C ₄₀ H ₅₂ O ₂	Canthaxanthin
4	9.801	430.24	1	(M+H) ⁺	0.31 × 10 ⁷	429.23	C ₂₂ H ₃₁ N ₅ O ₄	Unidentified
5	20.523	415.20	1	(M+H) ⁺	0.37 × 10 ⁷	414.20	C ₃₀ H ₅₄	Tetrahydroqualene
6	23.457	301.13	1	(M+Na) ⁺	0.02 × 10 ⁷	278.15	C ₁₆ H ₂₂ O ₄	Unidentified
7	25.471	568.45	1	(M+H) ⁺	0.03 × 10 ⁷	567.45	C ₄₀ H ₅₆ O ₂	3-hydroxyechinenone
		595.38	1	(M+Na) ⁺	0.08 × 10 ⁷	572.39	C ₄₀ H ₆₀ O ₂	Unidentified
		546.40	1	(M+H) ⁺	0.1 × 10 ⁴	545.39	C ₄₀ H ₆₄	Phytoene
		597.4	1	(M+H) ⁺	2 × 10 ⁴	596.6	C ₄₀ H ₅₂ O ₄	Astaxanthin
8	27.226	337.23	1	(M+Na) ⁺	0.04 × 10 ⁷	314.24	C ₁₈ H ₃₄ O ₄	Unidentified
9	29.943	705.55	1	(M+H) ⁺	0.3 × 10 ⁴	704.55	C ₅₀ H ₇₂ O ₂	Bisanhydrobacterioruberin
		723.57	1	(M+H) ⁺	0.9 × 10 ⁴	722.55	C ₅₀ H ₇₄ O ₃	Monoanhydrobacterioruberin
		741.58	1	(M+H) ⁺	0.03 × 10 ⁷	740.57	C ₅₀ H ₇₆ O ₄	Bacterioruberin
10	30.209	537.4	1	(M+H) ⁺	1 × 10 ³	536	C ₄₀ H ₅₆	Lycopene
11	31.68	739.56	1	(M+H) ⁺	0.07 × 10 ⁷	738.55	C ₅₀ H ₇₄ O ₄	Haloxanthin
12	35.658	543.2	1	(M+H) ⁺	2.1 × 10 ³	542.2	C ₄₀ H ₆₂	Phytofluene

^aThe hexanolic extract was chromatographed on a C-18 reverse-phase column; Hypersil gold 3 micron 100 × 2.1 mm column. Peaks are labeled based on increasing retention times (R_t) that they are eluted out

^bIdentified using the support system; Agilent Mass Hunter software, the Japanese carotenoid database, and Pubchem

components contribute to the antioxidant activity of the culture. The minimum aseptic conditions required to obtain cell mass possessing such high antioxidant ability gives it an economic advantage over other eubacterial counterparts for large-scale production. Therefore, the haloarchaeon, *Haloferox alexandrinus* GUSF-1(KF796625) is recommended for use in microbial industrial biotechnology.

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Author contributions Author IJ Furtado designed the experiments, analyzed the observations, and formulated the MS along with JJ Alvares who alone carried out all the experimental benchwork.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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