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Characterization of Extracellular Protease from the Haloarcheon *Halococcus* sp. Strain GUGFAWS-3 (MF425611)

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Abstract

Halococcus agarilyticus GUGFAWS-3 (MF425611) was isolated from a marine white sponge of *Haliclona* sp., inhabiting the rocks in the intertidal region of Anjuna, Goa, India. Uniquely, the microbe simultaneously produces two halo-extremozymes in 25% NaCl, namely protease and lipase at 49.5 ± 0.4 and 3.67 ± 0.02 (U mL^{-1}), respectively. The protease is constitutively produced in starch mineral salts medium with consistent 4 ± 1.0 mm zone of enzyme production, regardless of the non-availability of protein as substrate. The ethanol precipitated enzyme on dialysis and Sephadex G-200 gel filtration chromatography was partially purified to 12.26-fold and was active between 20 and 80 °C, 0–5 M NaCl, and pH 3–13. Optimum activity, however, was at 70 °C, 3 M NaCl, and pH 7. The enzyme was thermo stable at 70 °C with 50.26 ± 2.40% of relative enzyme activity at 75 min. Furthermore, it was stable in the presence of polar and non-polar organic solvents, detergents, and hydrocarbons. Several metal cations enhanced its activity in the order of $Ca^{2+} > Ni^{2+} > Fe^{3+} > Co^{2+} > Mg^{2+} > Cu^{2+} > Mn^{2+}$. Dependence of enzyme on cysteine; serine, and metal ions was confirmed by β-mercaptoethanol; PMSF and EDTA, respectively which induced its partial inhibition. Additionally, protease inhibited in vitro biofilm formation in *Staphylococcus aureus.* Conclusively, the production of a neutral halo-thermophilic protease is reported for the first time in the genus *Halococcus*.

Introduction

Haloarchaea are true extremophiles of phylum Euryarcheota requiring high salt (2–4 M NaCl) and temperature (40–50 °C) for optimal growth [1, 2] and thrives in low salinity environments to hypersaline lakes, solar salterns, and other salt-saturated environments [3–6]. Haloarchaea are proteolytic in nature and protease produced by them are active in extreme conditions of pH, NaCl concentration, temperature, and in the presence of different additives due to the possession of unusual traits like salt-in strategy, accumulation of compatible solutes, and glycosylation of proteins [7–9].

pH-dependent protease activity is reported in several species of halophilic archaea from the order halobacteriales: (i) neutral proteases in *Halobacterium* sp. [10–14] and (ii) alkaline proteases in *Natronomonas pharaonis* [15], *Halobacterium* sp.[8, 16–18]. Globally, halophilic proteases are sought to be used in meat tenderization, in leather, pharmaceutical, and detergent industries, apart from various biotechnological and bioremediation applications [19–21]. Hence, an extensive screening effort on haloarchaeal diversity in hypersaline systems is still underway [22–28]

In our previous study [29], we reported the secretion of an extracellular protease and lipase in a single fermentation medium containing 25% NaCl by haloarchaeal sponge biont *Halococcus* sp. strain GUGFAWS-3.

To the best of our knowledge, there has been no report on the production and characterization of protease isolated from the members of genus *Halococcus* belonging to the family *Halococcaceae* of the order *Halobacteriales.* Hence, we directed this study to characterize the protease of *Halococcus* sp. strain GUGFAWS-3 and determine its ability to inhibit the formation of *S. aureus* biofilm.

Authors dedicate this article to their Dad's Krishnanath Gaonkar and Jeronimo Furtado.

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Materials and Methods

Extracellular Protease Production

Isolate *Halococcus* sp. strain GUGFAWS-3 from sponge *Haliclona* sp. was routinely cultured in NaCl Tryptone Yeast Extract medium (25% NTYE), as described previously by Gaonkar and Furtado [29]. The strain was checked for growth at room temperature (RT, 28 to 30 °C) at 150 rpm by monitoring optical density at 600 nm using the Shimadzu UV–Vis spectrophotometer-160, Japan. The production and activity of extracellular protease were also being checked over a period of 7 days.

Agar well diffusion assay (AWDA) was performed by taking 100 µL of the cell-free supernatant (CFS) of log phase culture broth of GUGFAWS-3 grown in (a) NTYE and (b) Mineral salt synthetic medium with 20% NaCl (NSM) containing 0.5% starch. The CFS obtained by centrifuging at 9217×*g* for 10 min (Eppendorf 5417 R centrifuge) was introduced into wells of 0.6 mm diameter, bored in NSM agar medium supplemented with 0.5% (w/v) gelatin. The CFS was allowed to diffuse completely into agar at RT for 24 h. The plates were then flooded with acidified $HgCl_2$ solution.

The clear CFS was also checked for the extremozyme protease activity according to our method [29]. One unit of protease activity is the amount of enzyme essential to release 1 µg of tyrosine per min, with absorbance recorded at 280 nm.

Protease Purification

CFS was used as the crude enzyme source. The CFS (500 mL) was concentrated to 250 mL by passing through dry Sephadex-10, Sigma. The enzyme was precipitated out from CFS by the dropwise addition of 80% chilled ethanol (125 mL) with constant stirring, with reference to minor modification by Vidhyasagar et al. [21]. After 2 h, the mixture was centrifuged at 10,000×*g* for 10 min and then the precipitate was dissolved in 5 mL of 0.1 M Tris–HCl buffer (pH 7) containing 2 M NaCl. The sample was then dialyzed overnight against the same buffer and then passed through a Sephadex G-200 (Sigma Aldrich, USA) gel permeation column (2 × 30 cm size) equilibrated with 0.1 M Tris–HCl buffer of pH 7 containing 2 M NaCl. The fractions were collected at a flow rate of 0.5 mL min^{-1}. Every 1 mL fraction was collected to determine the protein content and protease activity. The active protease fractions which showed positive clearance for gelatin degradation were pooled, concentrated to 2 mL, and re-analyzed for total protein and protease activity.

Molecular Mass Determination and Zymography

The 10% Native polyacrylamide gel electrophoresis (Native-PAGE) and Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were performed. The molecular mass of *Halococcus* protease was determined by running a mixture consisting of catalase (210 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), lactoglobulin (18.4 kDa), and trypsin soybean inhibitor (20.1 kDa) as Native-PAGE markers. Protease activity was examined by running electrophoresis of Native-PAGE incorporated with 1% gelatin, and staining overnight in coomassie brilliant blue followed by destaining, so as to visualize the zone of proteolysis as a colorless band against the blue background [30].

Evaluation of Physico-chemical Properties of Protease

Effect of pH

The effect of pH on protease stability was examined by incubating the crude enzyme with 100 mM buffer solutions of Citrate–Phosphate; Tris; Glycine–NaOH, and KCl–NaOH buffers corresponding to pH values of 3–6; 7–9; 10–11, and 12–13, respectively for 15 min at 37 °C. Aliquots were withdrawn at regular time intervals to test the relative activity.

Effect of Temperature

The influence of temperature on enzyme activity was determined by separately incubating the reaction mixture between 20 to 80 °C for 15 min. The stability of the enzyme was measured after every 15 min by incubating the enzyme mixture at 70 °C for a period of 75 min under standard assay conditions.

Effect of NaCl

Enzyme activity was evaluated in the presence of 0–5 M NaCl at 70 °C for 15 min and expressed as % relative activity under standard assay conditions.

Effect of Different Additives

The stability of protease was determined, at 70 °C for 2 h, in the presence of different additives such as organic solvents, detergents, surfactants, hydrocarbons [0.5% (v/v)], and [1%

(v/v)]; metal salts and inhibitors (1 mM, 5 mM) were separately prepared in 0.1 M Tris buffer (pH 7.0).

Determination of K_m and V_{max}

Protease activity was determined using 0.2–2 mg mL^{-1} concentration of casein in 0.1 M tris buffer (pH 7) and 3 M NaCl at 70 °C for 15 min. The values of K_m and V_{max} were calculated using the Michaelis– Menten kinetics equation and the Lineweaver–Burk plot.

Effect of Protease on Biofilm formation

The effect of protease and proteinase K was determined by both spectrophotometric (Shimadzu UV–Vis spectrophotometer-1601, Japan) assays and microscopic visualization. Each of the enzymes fractions of 25, 50, and 100 µg were separately added to a sterile nutrient broth (NB) of pH 7.0, prepared in 1 M NaCl containing 25 µL of the *Staphylococcus* suspension of 1, at 600 nm in ninety-six-well plates. A final volume was made up to 250 µL, with NB. Wells without the enzymes served as the positive control. The plates were incubated at 37 °C for 48 h. The crystal violet staining assay was performed in accordance with Thenmozhi et al. [31].

Halococcal protease action on biofilm formation was confirmed by pre-treating 100 µL of protease with a mixture of 5 mM of PMSF, EDTA, and β-mercaptoethanol at RT. After 2 h, the treated protease was checked for its enzymatic activity on gelatin agar plates, as well as its effect on biofilm formation in sterile wells as, above. Additionally, a sterile coverslip was placed in each well containing 100 µg of protease and 25 µL of the bacterial suspension. Positive controls without protease/proteinase K and treated as protease in NB with a bacterial suspension maintained in microtiter wells. Each coverslip was removed, treated, and examined under the scanning electron microscope (SEM) (ZEISS E VO 18 special edition).

Dispersion of Mature Biofilm by *Halococcus* protease

Pre-developed biofilms of 48 h on glass pieces (0.5 cm^2) were incubated at RT with 100 µL of *Halococcus* protease (100 µg) and Proteinase K (100 µg), respectively. After 1 h, each slide was observed under the light microscope (Nikon H600L) [32].

Results

The *Halococcus* sp. strain GUGFAWS-3 isolated from marine *Haliclona* sp. by Gaonkar and Furtado [29] produced extracellular protease at 49.5 ± 0.4 and lipase at 3.67 ± 0.02 (U mL^{-1}) under optimal growth conditions of 25% NaCl, pH 7, and at 37 °C. The protease from *Halococcus* sp. was isolated and partially purified to 12.26-fold by ethanol precipitation and gel filtration chromatography.

Determination of Mode of Protease Production: Constitutive or Induced

A 4 ± 1.0 mm zone was consistently produced by CFS of *Halococcus* grown in 20% NSM with starch as the carbon and energy source and thereafter repeated in every serial subculture (Fig. 1). The CFS of *Halococcus* sp. strain GUGFAWS-3 grown in NTYE, however, gave a zone diameter of 13.2 ± 1.5 mm.

Purification of Protease

An extracellular protease from *Halococcus* sp. was partially purified for the first time in four steps: (1) Concentration of protein on dry Sephadex G-10, (2) Ethanol precipitation, (3)

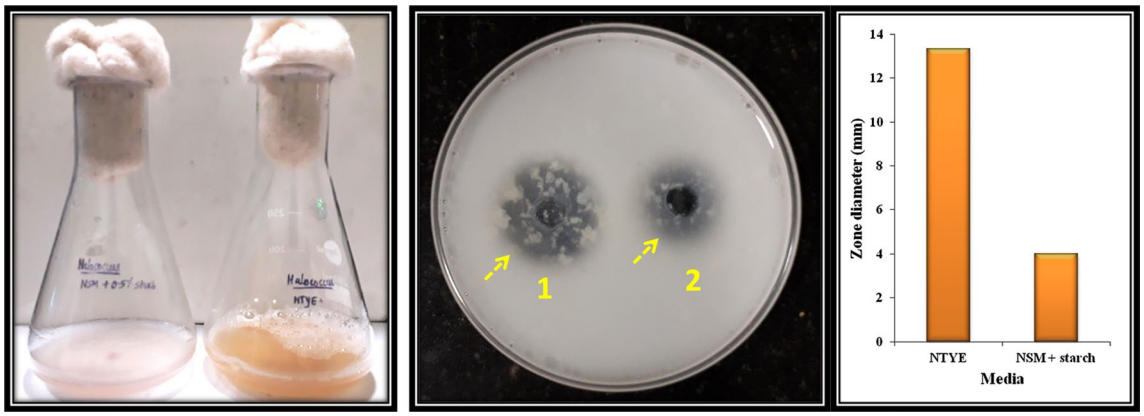

Fig. 1 Constitutive mode of protease production: **1** CFS from a culture grown in NTYE; **2** CFS from a culture grown in 20% NSM with starch

Dialysis, and (4) Gel permeation chromatography on Sephadex G-200. This process yielded a purity increase of 12.26-fold and 0.2% recovery yield (S1, Supplementary Table).

The chromatogram showed several elution peaks for protein, but only three peaks showed enzyme activity. The prominent peak was covered by elution fractions, numbered 45 to 56 of the gel filtration column (Fig. 2a), and gave intense clearance of gelatin digestion (Fig. 2b). Our results showed the presence of three protein bands with a molecular mass of approximately 220 kDa, 180 kDa, and 67 kDa on 10% Native polyacrylamide gel electrophoresis. In activity staining, protease activity was seen at 67 kDa with an intense zone of clearance (Fig. 2c). And, the molecular mass of the partially purified protease was confirmed to be ~ 67KD, a monomeric protein with the corresponding zone of clearance against SDS-PAGE gel with respective protein markers (Fig. 2d).

Response of Protease to Physico-chemical Parameters

The protease was very active in a wide pH range of 3–13 with optimal activity at pH 7.0, as depicted in Fig. 3a. There was 60% retention in activity across pH 4 to7, while 40% of its activity retained in alkaline pH range 8–11. The enzyme, however, lost 70% of enzyme activity at pH 3, 12, and 13.

Protease was active in 0–5 M NaCl concentration with optimum activity at a concentration of 3 M NaCl. At NaCl concentration of 1 M, the activity was 50% of its maximum (Fig. 3b). Protease in the absence of NaCl showed 80% loss

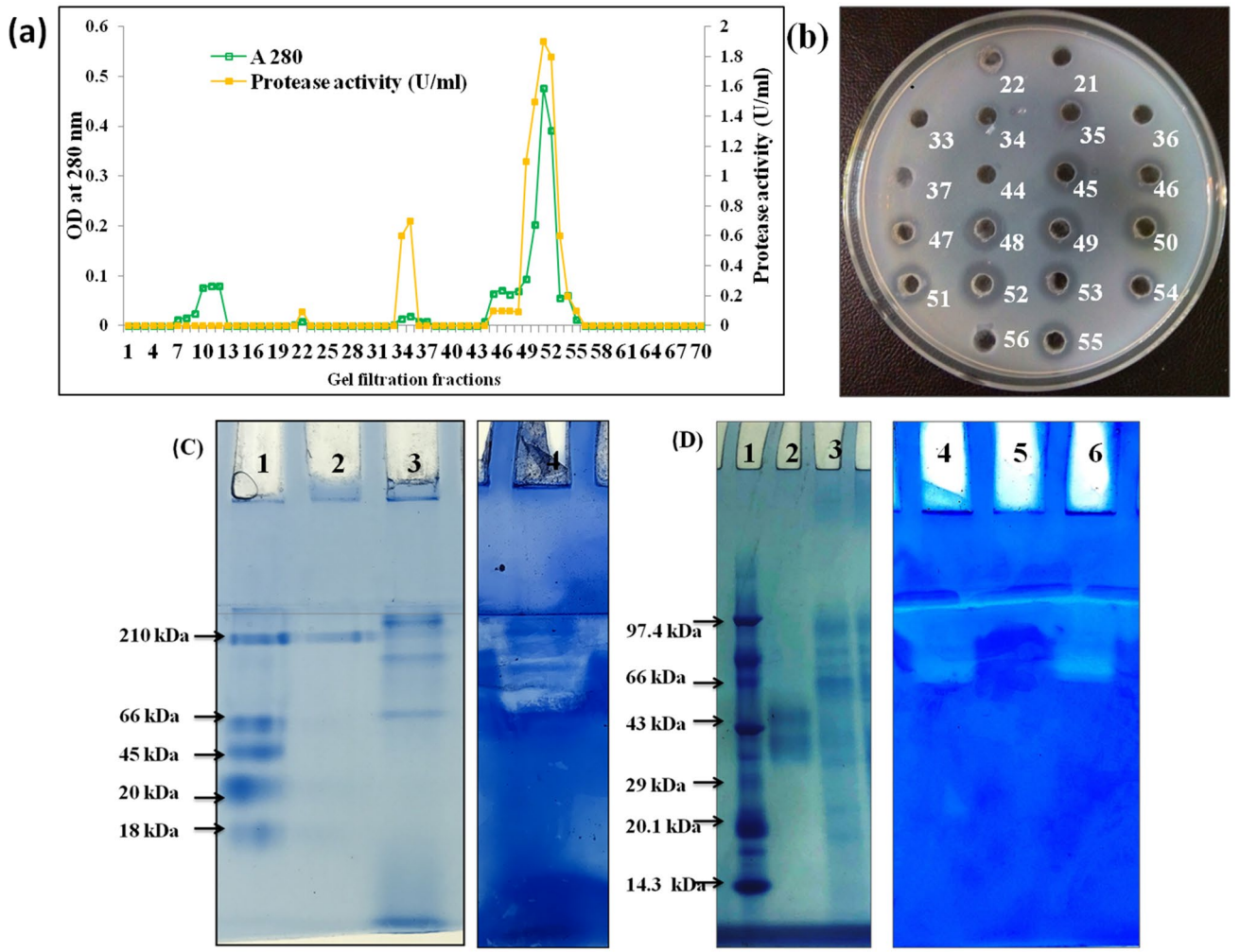

Fig. 2 Purification of extracellular protease from *Halococcus* sp. strain GUGFAWS-3 **a** elution profile of Sephadex G-200 gel filtration chromatography; **b** Protease-active fractions seen as a zone of clearance on 20% NSM with gelatin agar; **c** Native-PAGE 10%. Lane 1 Molecular weight marker, Lane 2 blank, Lane 3 concentrated samples containing protease activity from gel filtration on Sephadex G-200, and Lane 4 protease activity staining; **d** SDS-PAGE profile. Lane 1 Molecular weight marker, Lane 2 blank, Lane 3 concentrated samples containing protease activity from gel filtration on Sephadex G-200, Lane 4 and Lane 6 protease activity staining, and Lane 5 blank

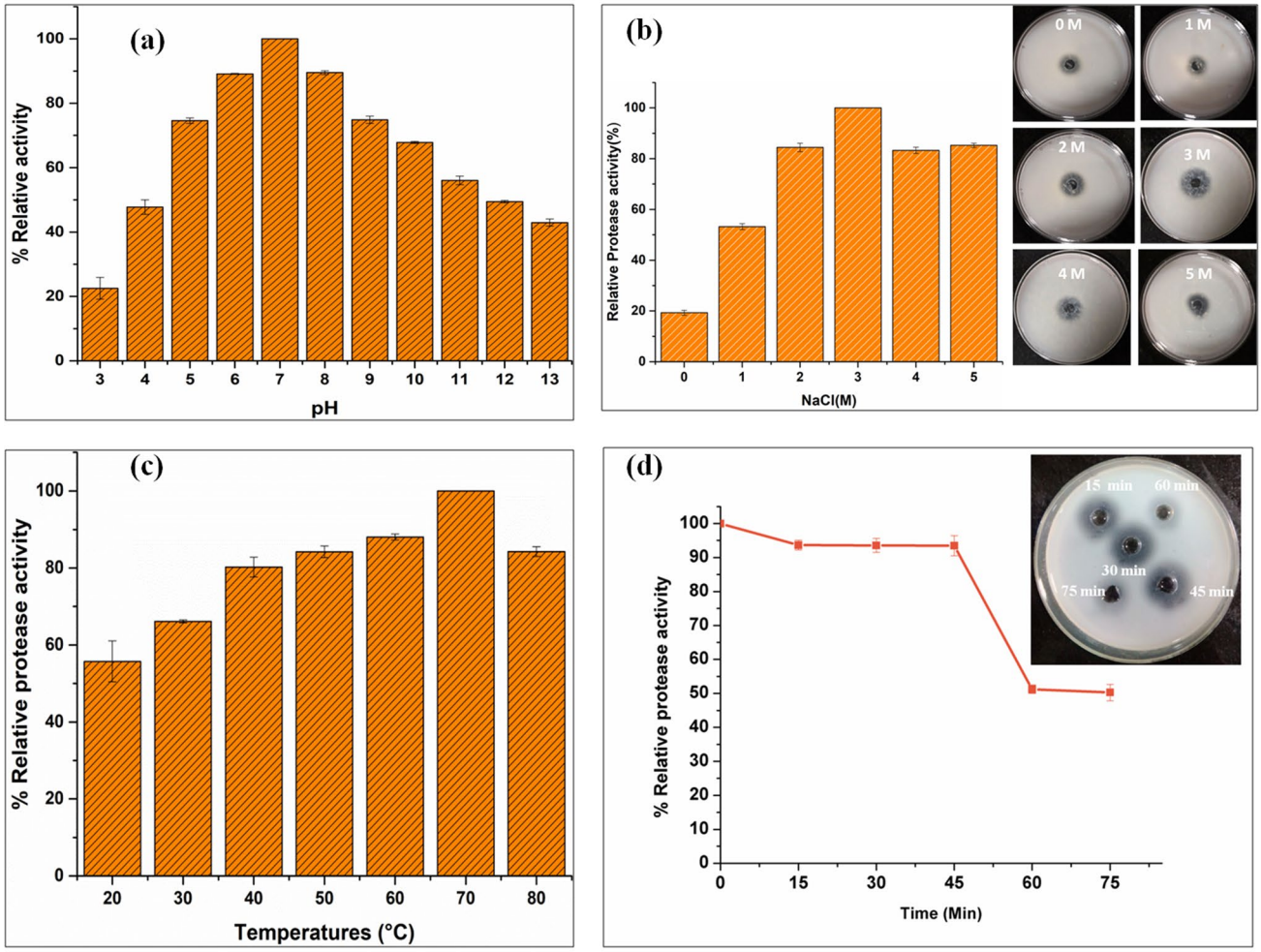

Fig. 3 The effect of pH (**a**), NaCl (**b**), temperature (**c**), and (**d**) on the activity and stability of protease. The results are the means of 2 independent experiments, and the bars correspond to the standard deviation

of activity. However, protease retained more than 85% of relative enzyme activity at 4 M and 5 M NaCl concentration.

The maximum enzyme activity was observed at 70 °C and was able to retain above 50% activity in the range of 20–80°C (Fig. 3c). The relative protease activity of 84.26% was retained when the reaction mixture was incubated at 80 °C. This enzyme was highly stable at 70 °C and its incubation at this temperature did not have any effect on it for an hour. The relative enzyme activity at 70 °C was 93.66 ± 1.38, 93.57 ± 2.05, 93.48 ± 2.9, 51.22 ± 1.20 ,and 50.26 ± 2.40 (%) for 15, 30, 45, 60, and 75 min, respectively. It may be noted that the relative enzyme activity drops by ~ 50% after 45 min at 70 °C (Fig. 3d). Comparison between proteases from *Halococcus* sp. (our study) and other members of order Halobacteriales, with respect to enzyme optimal physicochemical parameters, are detailed in Table 1.

The enzyme was found to be active and stable in the presence of polar and non-polar organic solvents. It retains more than 60% of its activity in the presence of isopropanol, butanol, and ether. The enzyme activity increased and the enzyme was stable in the presence of acetone, ethanol, methanol, DMSO, and acetonitrile, with acetone the activity was enhanced to 156% (Fig. 4a).

The enzyme was stable and its activity increased in the presence of most of the hydrocarbons tested at 0.5 and 1% concentration. A maximum increase in the activity was observed for sodium benzoate. In contrast, the presence of 1% aliphatics like hexane and cyclohexane resulted in a 50% loss of enzyme activity (Fig. 4b).

Protease was highly stable in the presence of surfactants like Tween 20, Tween 40, Tween 80, and triton X-100. About 80% of enzyme activity was retained when protease was pre-incubated at 70 °C for 2 h in the presence of 1% lab detergent and CTAB in the assay reaction mixture (Fig. 4c). Anionic detergent SDS at 0.5% retained 40% of enzyme activity.

Table 1 Comparison between proteases from *Halococcus* sp. (our study) and other members of order Halobacteriales with respect to enzyme optimal physico-chemical parameters

Strain Name	NaCl Range (M)	Substrate	$NaCl_{optimal}$ (M)	$T_{optimal}$ (°C)	pH	References
Halococcus sp. strain GUGFAWS-3	0–5	Casein	3	70	7	Our study
Halobacterium sp. strain HP25	1–4.5	Casein	2.9	60	8	Elbanna et al. [17]
Halobacterium sp.	0.017–0.08	Casein	0.08	60	10	Vijayaraghavan et al. [18]
Halobacterium sp. sp1(1)	0.85–5.13	Casein	1.7	30–50	7–9	Akolkar and Desai [7]
Natronomonas pharaonis	0.5–4.5	Casein	0.5–4.5	61	10	Stan-Lotter et al. [15]
Halobacterium salinarum ATCC43214	0–4	Glycin P'1	4	ND	ND	Ryu et al. [13]
Halobacterium TuA4	0.3–4.3	Azocasein	3	ND	7	Schmitt et al. [14]
Hbt. halobium	2–4	Azocasein	4	37	7.2	Izotova et al. [10]
Halobacterium salinarium I & IM	2–4	Casein, gelatin	4	ND	7	Norberg and Hofsten [12]

ND not determined

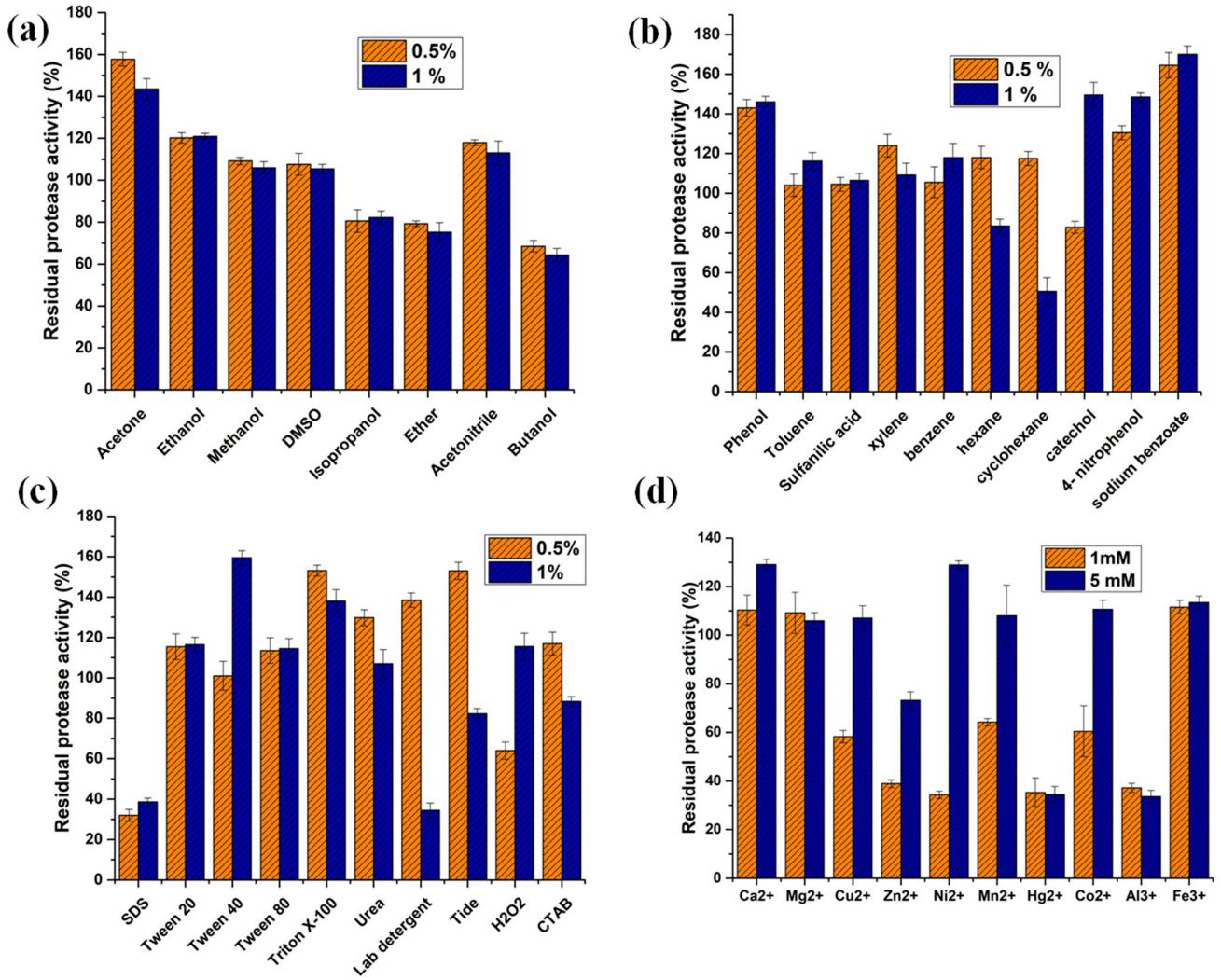

Fig. 4 Effect of organic solvents (**a**), hydrocarbons (**b**), surfactants (**c**), and metal ions (**d**) on protease activity. The results are the means of 2 independent experiments, and the bars correspond to the standard deviation

The enzyme was activated by 1 mM concentration of Ca^{2+}, Mg^{2+}, and Fe^{3+} ions. The residual activity after pre-incubation of the enzyme with these metal ions increased above 100% than the control (Fig. 4d). Metal ions like Cu^{2+}, Ni^{2+}, Mn^{2+}, and Co^{2+} at 5 mM concentration enhanced the protease activity while Hg^{+2}, Zn^{2+},and Al^{3+} ions inhibited the enzyme activity.

There was 58% and 78% loss of enzyme activity observed in the presence of 1 mM and 5 mM β-mercaptoethanol, respectively. Furthermore, the loss of more than 35% of relative protease activity was seen in the presence of EDTA and PMSF (S2, Supplementary figure).

K_m and V_{max}

K_m and V_{max} values determined through the Lineweaver–Burk plot for casein hydrolysis at 70 °C at pH 7 with NaCl were 3.99 mg mL^{-1} and 41.49 U mL^{-1}, respectively (S3, Supplementary Figure).

Effect of *Haloccocus* sp. Protease on Biofilm Formation

The spectrophotometric and microscopic studies revealed the potential of protease to inhibit biofilm formation. At 25, 50, and 100 µg, protease caused 31.33–70.07% biofilm inhibition (Fig. 5a). As observed under SEM, 100 µg protease and proteinase K distorted the protein matrix holding the cells and eventually disrupted the cells of *S. aureus* affecting its ability to form biofilms (Fig. 5b).

Upon treatment with PMSF, β-mercaptoethanol, and EDTA known to be protein denaturants or inhibitors; the *Haloccocus* protease completely lost its activity as seen on the gelatin agar plate. Moreover, the treated protease did not affect the biofilm-forming ability of *S. aureus* as seen on the SEM micrograph (S4, supplementary figure). These findings suggested that anti-biofilm activity was due to the protease degrading the protein matrix holding the bacterial cells.

The protease from *Halococcus* sp. and the standard proteinase K revealed their remarkable ability to disrupt already formed mature biofilms (S5, supplementary figure). Analysis of the glass slides using a light microscope (Nikon H600L)

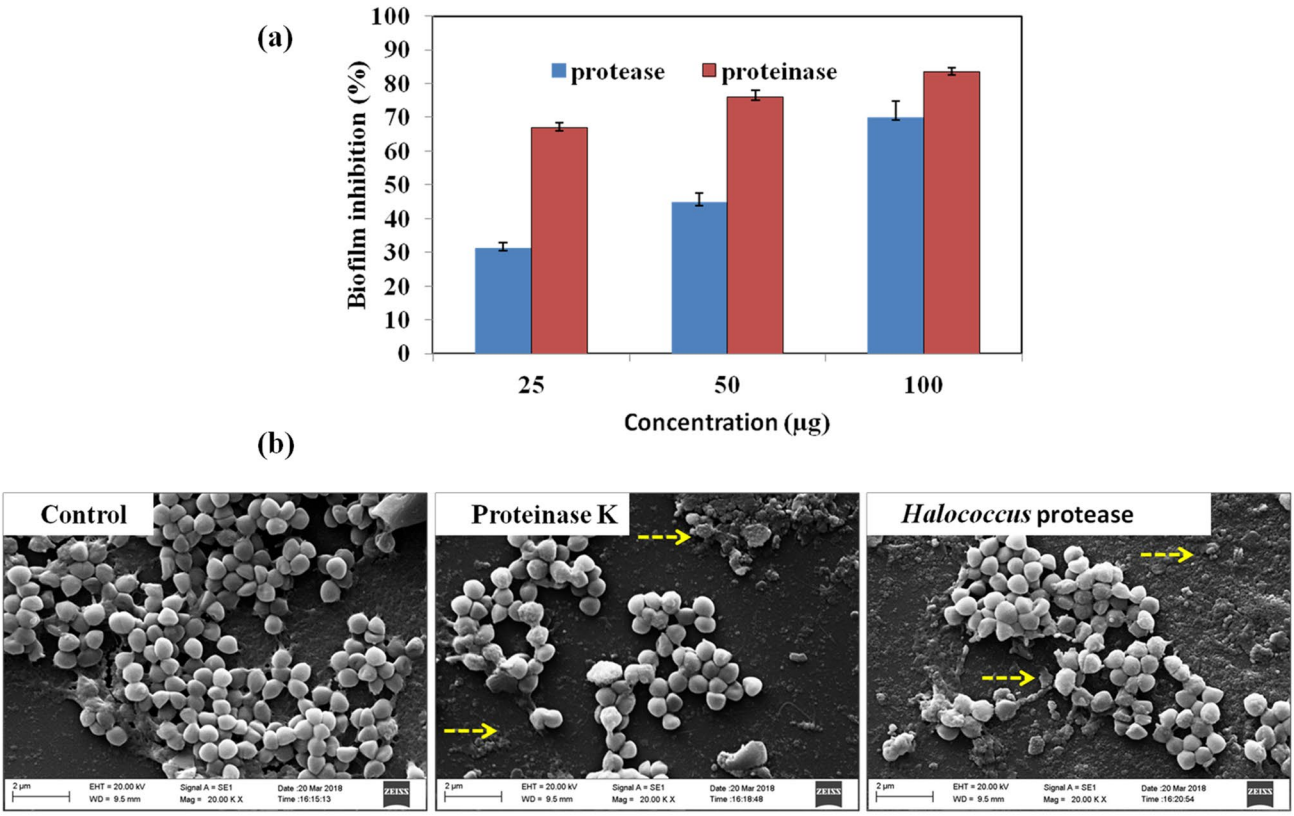

Fig. 5 Effect of *Halococcus* sp. strain GUGFAWS-3 protease and proteinase K on biofilm-forming ability of *S. aureus*. **a** % biofilm inhibition of *S. aureus* at 25, 50, and 100 µg of protease/proteinase K. **b** SEM micrograph showing biofilm of *S. aureus* (control) and disruption of cells by the action of protease and proteinase K on the extracellular protein matrix

indicated disruption of biofilm, proving the real potential of the protease at 100 µg in disturbing the biofilm of *S. aureus*.

Discussion

Members belonging to order Halobacteriales of class Halobacteria except genus *Halococcus* were studied for protease biosynthesis, optimization, and characterization properties. Genus *Halococcus* and other members of phylum Euryarcheota have been reported in low-salinity marine sediments [5, 33–35] and in association with marine invertebrates [4, 29, 36–39]. Further, biotechnological potentials such as the production of silver nanoparticles, PHA, EPS, agarase, and restriction enzymes from *Halococcus* sp. are reviewed by Ammozegar et al. [9].

Halococcus sp. strain GUGFAWS-3 produced an extracellular protease in the medium containing starch. However, the addition of proteins enhances its synthesis and production. Protease secretion by the microorganisms is generally constitutive and in a few cases, it may be inducible [40]. A study by Geisseler and Horwath [41] showed that the regulation of protease activity by microorganisms depends upon different sources of nitrogen and carbon availability in the soil. In haloarchaea, the constitutive property of protease synthesis by *Haloferax mediterranei* in glucose-ammonium chloride-based medium, with 20% sea water salt solution was reported [42]. In another study by Akolkar and Desai [8], protein-rich soybean flour was the best nitrogen source for protease production in *Halobacterium* sp. SP1 (1).

Partially purified protease of *Halococcus* sp. strain GUGFAWS-3 under study showed three bands on 10% Native gel with one band corresponding to the molecular mass of ~ 67 kDa with the intense zone of protease activity. Proteases reported from the order Halobacterials are of molecular mass between 21 to 60 kDa i.e., 41 kDa for *Halobacterium halobium* [10], 60 kDa for *Halobacterium halobium* protease TuA4 [14], 30 kDa for *Natronomonas pharaonis* [15], and 43 kDa for *Halobacterium* sp. [18]. However, *Halococcus* sp. strain GUGFAWS-3 weighs ~ 67 kDa. The report being first on genus *Halococcus* is of great significance.

Our reported protease was similar to neutral proteases reported from those of *Halobacterium* sp. optimally active at pH 7 [10, 12, 14]. However, *Nartronomonas pharaonis, Halobacterium* sp. SP1(1), and *Halobacterium* sp. produced proteases that are active under alkaline conditions [7, 15, 18]. The protease showed an optimum temperature for activity at 70 °C, with more than 50% activity retained between 20 and 80°C. This property of protease has potential applications in the baking industry, wherein some processes require complete inactivation of the enzyme with increasing temperature, which is seen when the protease is exposed to longer durations at 80 °C. Most of the proteases from haloarchaea showed good activity in the temperature range from 30 °C to 60 °C [7, 10, 15, 18]. For instance, protease from *Halobacterium halobium* had the maximum activity at 37 °C [10]. A report by Capiralla et al. [43] showed that the extracellular serine protease from *Halobacterium halobium* S9 was maximally active at 40 °C. Our protease activity was relatively stable at 70 °C for 75 min, thus proving the enzyme to be thermo stable with a possible application in industrial processes operating at high-temperature conditions. The relative protease activity of 23% at 70 °C for 30 min from *Halobacterium* sp. was reported by Vijayaraghavan et al. [18]. There is 50% residual activity retention by the protease from *Halobacterium* sp. SP1(1) in the presence of 4 M NaCl for 60 min [7].

The protease produced by *Halococcus* sp. showed activity in the absence of NaCl and at salinity as high as 5 M, with an optimum at 3 M. This enzyme property was similar to chymotrypsin-like serine protease from *Natronomonas pharaonis* which showed activity in the absence of NaCl. The activity of this salt-tolerant protease decreases with an increase in salt concentration [15]. In contrast to the present study, the enzyme retains above 85% of relative enzyme activity at 4 M and 5 M NaCl concentrations. In general, proteases from other extremely halophilic archaea of order halobacteriales are active at high salinity conditions and are irreversibly inactivated under low salt concentrations [10, 12]. The stability of protease in the absence of NaCl is useful in a wider range of industrial processes including the low-salt and high-salt fermentation industry. Our protease is stable in salt solutions over the range of 0–5 M and therefore serves the advantage of being capable of low-salt fermentation compared to the enzymes from other halophilic archaea. On the other hand, high activity and stability at high salt concentration have an added advantage over normal proteases that are unstable in the presence of salt. Organic solvents in the present study showed that acetone stimulated protease activity. Similar results were shown by protease from *Halobacterium* sp. AF1 [44] where acetone stimulated the activity of the enzyme.

Protease form *Halococcus* sp. was highly stable in the presence of aliphatic and aromatic hydrocarbons/solvents retaining more than 50% of its initial activity. A similar report on extracellular proteases from *Halobacterium* sp. [18]. and *Halobacterium* sp. SP1(1) [45] where solvents like hexane, toluene, and benzene stimulated the proteolytic activity of the enzyme.

In the present study, we found that residual enzyme activity was increased to 116%, 159%, 114%, and 138% at 1% concentration of tween 20, tween 40, tween 80, and triton X-100, respectively. However, the serine protease from *Halobacterium* sp. SP(1) retained 62.15% of its initial activity in presence of 0.5% anionic SDS, whereas neutral

detergents like tween 80 and triton X-100 did not have any effect on the enzyme activity [45].

The effect of metal ions on protease activities was studied by several authors and it differed for each protease, with Ca^{2+} ions showing more relative enzyme activity (129%) among other divalent ions tested. Similar results on increasing relative protease activity by Ca^{2+} were reported in the protease isolated from *Halobacterium* sp. [18] which showed 137% relative enzyme activity. In our study, $Hg^{2+}$and Al^{3+} at 1 mM and 5 mM concentration strongly inhibited protease activity, whereas Zn^{2+}, Mn^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+},and Fe^{3+} at 5 mM concentration enhanced the activity. Although inhibition of AF1 protease from *Halobacterium* [15] by Fe^{3+} has been reported, our protease showed the requirement of Fe^{3+} ions for its activity.

The increased activity by Ca^{2+} and Mg^{2+} and loss in enzyme activity with the addition of EDTA indicates the possibility of the metal-dependent enzyme. The inhibition by β-mercaptoethanol as well as SDS affirms the protease to be possibly cysteine protease consisting of cysteine in the active domain at the catalytic site of the enzyme. Most proteases from halophilic archaea are serine protease primarily inhibited by serine protease inhibitors like PMSF and DFP [8, 15, 18].

Higher V_{max} and lower K_m values suggested more active hydrolysis by enzyme using a low concentration of substrate. In accordance with this, our results suggested relatively more active hydrolysis of casein in the presence of NaCl (high V_{max}), though requiring more substrate for saturation (high K_m). Similar behavior was observed by serine protease from *Halobacterium* sp. (SP1) in the presence of 30% Na-glutamate for hydrolysis of casein [7].

It should be noted that these results could not be compared with proteases from non-halophilic microorganisms since their behavior at physico-chemical parameters such as higher salinity and temperature are not a routine parameter in biochemical characterization studies. Therefore, these proteases, including that of *Halococcus* sp., which are active at high salt concentrations and temperature may have interesting applications in the treatment of protein-rich saline waters.

Finally the extracellular protease from *Halococcus* sp. strain GUGFAWS-3 of marine *Haliclona* sp. was proved as an anti-biofilm agent against *S. aureus*. Exopolysaccharide produced by most of the bacteria acts as a barrier protecting the cells within the biofilm. The physical integrity of the EPS is due to proteins, lipids, and carbohydrate cross-links which are resistant to most of the antimicrobial agents. Enzymes from microbial origin have proven to be effective for the degradation of EPS holding the biofilm [46, 47]. However, the present study deals with assessing the anti-biofilm efficacy of protease from extremely halophilic archaea *Halococcus* sp. alone.

The morphological changes of the cells of *S. aureus* after treatment with *Halococcus* protease and Proteinase K were clearly visible under the SEM micrograph. The lysed cells of this pathogen clearly indicated the anti-biofilm activity of this protease by distorting the protein matrix between the cells. The lysis of the *B. proteolyticus* cells by protease is ascribed to be the cause of morphological changes seen in the SEM micrographs [47]. EPS degradation in mature biofilm was also found to be effective in the present study. Some studies reported that carbohydrates constitute a major portion of the EPS, while some studies found proteins to be dominating [47, 48].

Moreover, in the present study, the effect of inhibitors on protease and subsequently on biofilm was also investigated. Upon the treatment of inhibitors such as PMSF, β-mercaptoethanol, and EDTA capable of denaturing proteins, the protease tend to lose their activity. Hence, their influence on biofilm of *S. aureus* was not significant as observed with untreated protease thus confirming the proteinaceous nature of the protease enzyme.

In conclusion, there are few reports published on the protease production in the high salt medium in halophilic archaea. *Halococcus* sp. strain GUGFAWS-3 produces an extracellular protease which is salt, pH stable, and a solvent-tolerant enzyme. It functions optimally at 70 °C, thermostable with optimum activity temperature for over an hour, hence of biotechnological and industrial significance. Moreover, the newly characterized protease potentially inhibited the formation of biofilm and even disrupted already formed mature biofilms of *S. aureus*, thus implying new possibilities for such proteases in extracellular proteases which are salt stable, pH stable, and solvent-tolerant enzymes.

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Author Contributions SKG performed the experiments, analyzed the data, and drafted the manuscript. IJF helped in the analysis of the data and corrected the manuscript.

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Compliance with Ethical Standards

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

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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