

Article

Marine Archaeal Extracellular Polymeric Substances from *Halococcus* AMS12, Their Characterization, and Biological Properties

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Abstract: In the present study, halophilic archaea were isolated from a marine sediment sample. Totally, 15 isolates (AMS 1–15) were identified by molecular identification as belonging to the ten genera. Further, their extracellular polymeric substances (EPS) were extracted (3.172 g/L), and their bioactivity was determined in terms of biosurfactant, emulsification, enzymatic and non-enzymatic antioxidants, and anticancer activity. The highest amount of EPS has been produced by *Halococcus* sp., AMS12. It is made up of 54.28% carbohydrates, 32.91% proteins, 2.41% lipids, and other compounds. Further, EPS has 43.69 ± 1.89 U/mg of gelatinase enzyme by degrading the substrate. The potential total antioxidant activity of 103.80 ± 0.02 (ascorbic acid equivalence (AAE)), total reducing power of 86.1 ± 0.25 AAE, 1,1-diphenyl-2-picrylhydrazyl (DPPH) activity of $97.23 \pm 0.21\%$, the hydrogen peroxide scavenging of $60.8 \pm 0.21\%$, and nitric oxide scavenging activity of $89.37 \pm 0.24\%$ were observed at 100 $\mu\text{g}/\text{mL}$ of EPS. Hence, we conclude that the archaeal EPS is multifunctional and useful for developing natural polymers for industrial, food, and pharmaceutical applications.

Keywords: marine sediment; archaea; EPS; antioxidant; cell viability

1. Introduction

Archaea have the ability to live in extreme environments with various adaptations. They have adapted to live in extreme environments, such as in soaring salt, pH, and pressure, to produce novel metabolites and are economically important [1]. These extremophilic archaea are identified from a moderate environment, which have the ability to fix nitrogen, reduce nitrate and nitrite, denitrify, and do all other activities [2]. The overview, general structure, and biological activities of EPS from archaea are given in Figure 1. Although, archaea have numerous prospective applications in different fields of biotechnology and it produces pigments, ammonia-oxidizing, hydrolytic enzymes such as lipase, DNase, amylase, gelatinase, chitinase, protease, cellulose, and polymers [3], only a few of them are involved in a major part in the environment through halophilic and halo-tolerant enzymes [4]. However, the archaeal role in the environment is not yet clear, where they have competed with bacteria at a high-salt concentration by their enzymes and EPS. Generally, these EPS are produced with high molecular weight during their growth

periods and thereby create a protective layer on the cell surfaces to form a biofilm [5]. The supple matrix of EPS from archaea has glycoproteins, enzymes, lipids, extracellular DNA, and other chelating substances. The heterogenic polymers have non-carbohydrates and monosaccharide units that differ from each species and each environment [6]. The chains of polysaccharides were built up by the repeating unit of oligosaccharides, and their sizes may vary according to the degree of polymerization [7]. These EPS are well known to be negatively charged, which has potential applications in pharmaceutical, food, and other industries. Additionally, the EPS from microbes remove organic contaminants and heavy metals from the environment [8]. In addition, they provide a defensive fence against UV radiation, desiccation, temperature fluctuation, pH changes, and other chemical substances. The EPS plays a key part in the biofilm formation on liquid and solid interfaces, and air–liquid interface [9] EPS producing thermophilic and halophilic archaea have been isolated from the hydrothermal vent and deep sea [2,10]. Extremophilic archaeal genera *Archaeoglobus*, *Sulfolobus* and *Thermococcus* have produced large amounts of EPS with antimicrobial activity [11]. The EPS from halophilic archaea have an odd range of sulfate and uronic acid content [12]. Although a large number of archaeal EPS were reported from various extreme environments, biosynthesis, composition, structure, and active properties [12], studies on archaea from marine sediment sample and their potential EPS production are very limited. Therefore, we focused on the archaeal domains of marine sediments and the bioactive potential of their EPS. In this study, we focused on the archaeal domains from marine sediments and the bioactive potential of EPS and their characterization.

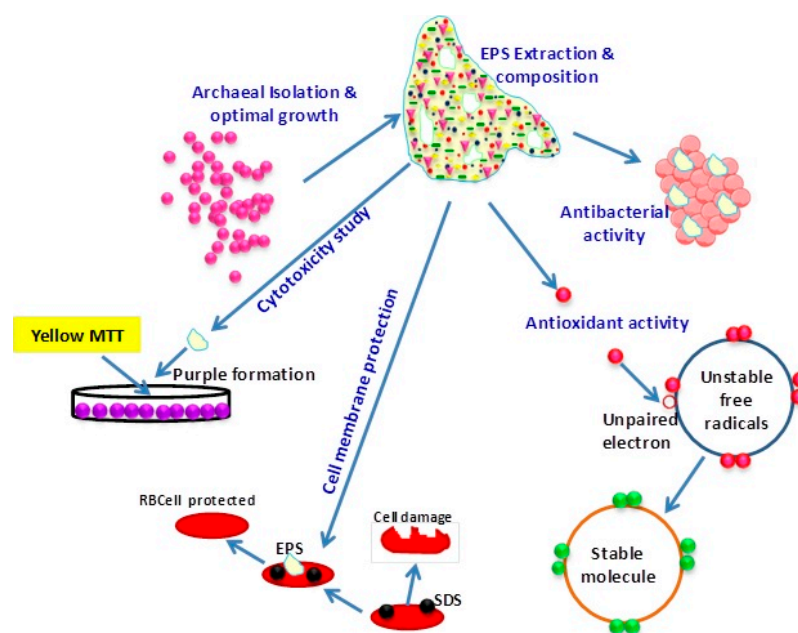


Figure 1. Archaeal EPS structure and their different biological activities.

2. Materials and Methods

2.1. Sample Collection

The sediment sample was collected from a depth of 25 m in the offshore region (13.003837° N, 80.329445° E) of Chennai using Van Veen grab of 0.04 m². The sediment sample was collected in a sterile plastic container (Himedia-HPW016T), and it was safely transferred to the laboratory.

2.2. Isolation and Identification of Marine Archaea

One gram of sediment sample was diluted with 10 mL of sterile distilled water (10^{-1}), and the same diluents were continued up to 10^{-6} . From those diluents, 1 mL of the sample was pipetted into Petri plates containing halo archaeal agar (HAA) (per litre: 250 g of NaCl,

2 g of KCl, 20 g of MgSO₄, 3 g of Na₃C₆H₅O₇, 10 g of yeast extract, 20 g of agar, and 7.2 pH). The plates were nurtured at a temperature of 35 ± 2 °C for 20 to 25 days. The archaeal population density was enumerated, and it has been stated as a CFU/mL (colony-forming unit per mL). Isolates were selected according to their distinct colony morphology and color of the colonies. Further, pure archaeal isolates were picked by recurrent modifications of different isolates on HAA plates [13].

The archaeal cell pellet was suspended in 1 mL of cell suspension solution (500 mM Tris HCl; 200 mM EDTA; 460 mM NaOH; pH 8) and 50 µL of RNase 100 µL of cell lysis solution (15% sodium dodecyl sulfate (SDS)) was incubated for 15 min at 55 °C and then added to the 25 µL of proteinase K incubated at 55 °C for 60 min, and 500 µL of 5 M NaCl was then added to incubate at 4 °C for 10 min. After incubation, 2 mL of TE buffer and 8 mL of ethanol were added to the aqueous layer of centrifuged samples and incubated for 2 min at room temperature and centrifuged for 15 min at 5000 rpm; subsequently, the dried condition pellet was dispersed in DNase free water [14]. The extracted DNA was visualized in agarose gel (0.7%) electrophoresis at 100 V. The archaeal 16SrDNA gene was amplified according to the Delong method [15] with 4 F and 1492 R archaeal primers. Amplified DNA contents were refined and sequenced by Bio-innovation Company (Mumbai). Further, the obtained sequence was analyzed using NCBI-BLAST search. Cell morphology and gram staining were measured by phase-contrast microscopy.

2.3. Optimal Growth Parameters of Archaea

Salt requirement (10–30%), temperature (35 °C–60 °C), and optimal growth pH (6 to 9) were determined in HAA medium, and the cultures were placed on a rotary shaker at 150 rpm at 30 °C without light sources for 120 h. Cell growth was noticed in halo-archaeal broth (per liter: 250 g of NaCl, 2 g of KCl, 20 g of MgSO₄, 3 g of Na₃C₆H₅O₇, 10 g of yeast extract) for 168 h on a rotary shaker at 200 rpm. Every 24 h, 2 mL of broth were centrifuged, and the cells were suspended with halo-archaeal broth to measure the absorbance at 600 nm. Further, 50 µL of cell suspension was observed in the microscopic observation on the Neubauer counting chamber [16].

2.4. Production and Purification of EPS

The EPS were isolated from *Halococcus* sp., AMS12 in halo-archaeal broth at 45 °C for 120 h. The EPS was extracted using chemical and physical methods followed by methods used by Forster and Clarke [17] and Li and Yang [18] with slight modification. The EPS from archaea were separated by centrifugation at 10,000 rpm for 30 min, the entire process was maintained at 4 °C, and the pellets were further suspended using ice-cold ethanol (double volume) and incubated at 4 °C for overnight.

The archaeal EPS mixture in ethanol was obtained by the centrifugation method (13,000 rpm condition for 30 min at 4 °C). The pellet was washed with ethanol in absolute condition to further concentrate and purify. The excess salts and impurities were removed from the crude EPS using dialyzed membrane (HiMedia-Mumbai, LA401) for 48 h at 4 °C. The dialyzed EPS were freeze dried to make the powder [19], and total EPS was expressed in mg/L.

2.5. Characterization of Marine Archaeal EPS

The carbohydrate from the EPS was estimated by the phenol-sulphuric acid digestion method, glucose was maintained as standard [20], the protein level was determined by bicinchoninic assay (BCA), and BSA was used as standard. The nucleic acid was estimated by the method of [21] with a slight modification. In detail, the EPS solution was also diluted to the predefined volume after transferring through a 0.45 µm cellulose acetate membrane. A UV-Vis spectrophotometer was utilized to record the evolution of the dissolved solutions. Total uronic acid concentration levels were determined using a sulfamate/m-hydroxydiphenyl assay in a concentrated sulfuric acid [22], and total lipid contents were estimated [23] using ferric acetate-uranium acetate in a mixture of solvents,

such as acetic acid, sulfuric acid, and ferrous sulfate. The lyophilized EPS surface morphological characteristics and shape were examined by FE-SEM [24].

2.6. Biosurfactant and Cell Membrane Protection Assay

Emulsification activity of EPS was measured by mixing 1 mL of EPS with an equal volume of aromatic hydrocarbons, such as benzene, and was vigorously vortexed for 3 min. Then, the mixture was incubated at 4 °C for 24 h. After incubation, the height of the emulsification was measured. Triton X-100 was used as a positive control, and cell-free PBS was used as a blank.

The emulsification index = height of emulsion (mm)/total height of mixture (mm) × 100

The cellular membrane defense activity of EPS from archaea was analyzed by the method of Sajjad et al. [25] with a slight modification. The red blood cells (RBC) were centrifuged at 5000 rpm for 5 min and then diluted with saline phosphate buffer (1:10). A 5 mL of EPS (0.1 to 0.5 mg/mL concentrations) was inoculated to diluted cells and kept for 10 min. Further, SDS was added to the reaction mixture made up to 10 mL and incubated for 30 min. The supernatant of the reaction mixture was collected by centrifugation at 5000 rpm for 5 min. The absorbance of the supernatant was measured at 600 nm. The percentage of cell-lysis was calculated by the given formula: % of cell lyses = (control-test/control) × 100. The diluted cell with SDS was used as the negative control and the cell with glycerol was a positive control.

2.7. Enzyme Activity of Marine Archaeal EPS

To determine the enzyme activity, the archaeal isolates were centrifuged at 10,000 rpm for 10 min at 4 °C, and the cell-free supernatant was used as a source of gelatinase enzyme. The assay was then carried out with a 1mg/mL concentration of gelatin as a substratum at 35 °C [25].

2.8. Antioxidant Activity of EPS

The antioxidant activity of the EPS from marine archaea was determined by the methods of Kamala et al. [26] at various concentrations in terms of DPPH scavenging activity, total antioxidant, hydrogen peroxide scavenging activity, total reducing power, and nitrous oxide inhibition activity. The enzymatic in-vitro antioxidant activity was performed by Sivaperumal et al. [27] at different concentrations in terms of superoxide dismutase (SOD), catalase (CAT), glutathione activity (GSH), lipid peroxidation, and malondialdehyde (MDA).

2.9. Cell Viability (MTT) and DCFH-DA Assay

Mouse fibroblast cell line (3T3) was purchased from National Centre for Cell Science (NCCS), Pune, and the obtained cells were incubated in T25 culture flasks of DMEM (Dulbecco's Modified Eagle Medium) supplemented with 1% antibiotics and 10% FBS. Further, cells were incubated with 5% of CO₂ (humidified atmosphere) and maintained at 37 °C. After accomplishment of confluence, the cells were used for further experiment. Cell viability was determined through MTT assay by the method of Bhavna et al. [28]. The viable cells in the control were 100%, and the viability of treated cells was calculated by the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells] × 100.

2.10. Dichloro-Dihydro-Fluorescein Diacetate (DCFH-DA) Assay

The DCFH-DA assay was conducted to evaluate the reactive oxygen species (ROS) generation. The production of intracellular ROS in untreated and EPS-treated 3T3 cells was measured. Hydrogen peroxide treated cells were used as a positive control for ROS generation. A total volume of 2 mL was obtained by diluting an aliquot of 8 × 10⁶ cells/mL in PBS with 7.4 pH condition. The mixture of one ml cell suspension and 100 µL of DCFH-

DA (5 $\mu\text{M}/\text{mL}$) were incubated for 30 min at 37 °C. An inverted fluorescence microscope was used to observe and photograph the stained cells from each group.

3. Results

3.1. Isolation and Identification of Marine Archaea

A total of 34 archaeal isolates were obtained from marine sediment samples using HAA medium (Figure 2a). Out of them, 15 isolate had distinct colony morphology which had been taken for further studies. The potential study strain of *Halococcus* sp. AMS12 showed the cocci-shaped morphology (Figure 2b) in the microscopic view. Genomic DNA was isolated and amplified with archaeal primers (4 F with 1492 R) at 1488 bp. Archaeal 16S rDNA analysis revealed the presence of members from *Euryarchaeota* phyla, which were identified as *Halococcus* sp. AMS1, *Halorubrum* sp. AMS2, *Natronorubrum* sp. AMS3, *Halobacterium* sp. AMS4, *Halomicrobium* sp. AMS5, *Halorhabdus* sp. AMS6, *Natrinema* sp. AMS7, *Haloferax* sp. AMS8, *Haloplanus* sp. AMS9, *Natrinema* sp. AMS10, *Haloferax* sp. AMS11, *Halococcus* sp. AMS12, *Halomicrobium* sp. AMS13, *Natrinema* sp. AMS14, and *Haloarcula* sp. AMS15. Phylogenetic analysis of the 16S rDNA of these isolates had II clusters; cluster I had eight genera (*Halococcus*, *Halorubrum*, *Halobacterium*, *Halomicrobium*, *Haloferax*, *Haloplanus*, *Halorhabdus*, and *Haloarcula*). Cluster II had two genera (*Natrinema* and *Natronorubrum*), and *Escherichia coli* (AB269763) was used for our group. All the isolates were submitted to the gene bank to obtain an accession number (KU995301-KU995315) with 90–99.9% similarity with the query sequences.

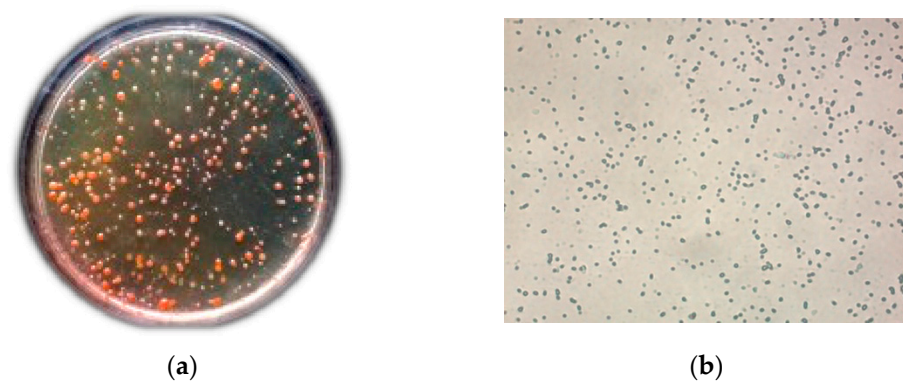


Figure 2. Archaeal colonies on halo-archaeal agar (HAA) and micro morphology of *Halococcus* sp. AMS12: (a) isolated colonies; (b) microscopic view.

Totally, 26 nucleotide sequences were used for the phylogenetic study which has 1400 base pairs in the ending dataset [29]. The optimal branch length of the phylogenetic tree was 1.0678 [30]. The percentage of repeat trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The phylogenetic tree was constructed by ten clusters; each genus separated into a single cluster; cluster I was constructed with seven isolated from five genera with five reference sequences (*Halomicrobium* (HM063952), *Haloarcula* (EF645680), *Halorhabdus* (NR113464), *Halobacterium* (AB663362), and *Halococcus* (AB935407)) at 100% bootstrap level with respective isolates. Cluster II has four isolates belonging to three genera with reference sequences (*Haloferax* (D14128), *Halorubrum* (AY510708), and *Haloplanus* (NR113462)); cluster III has four isolates belonging to *Natrinema* with the reference sequence (AB663465) and *Escherichia coli* (AB269763) was used as the out group (Figure 3).

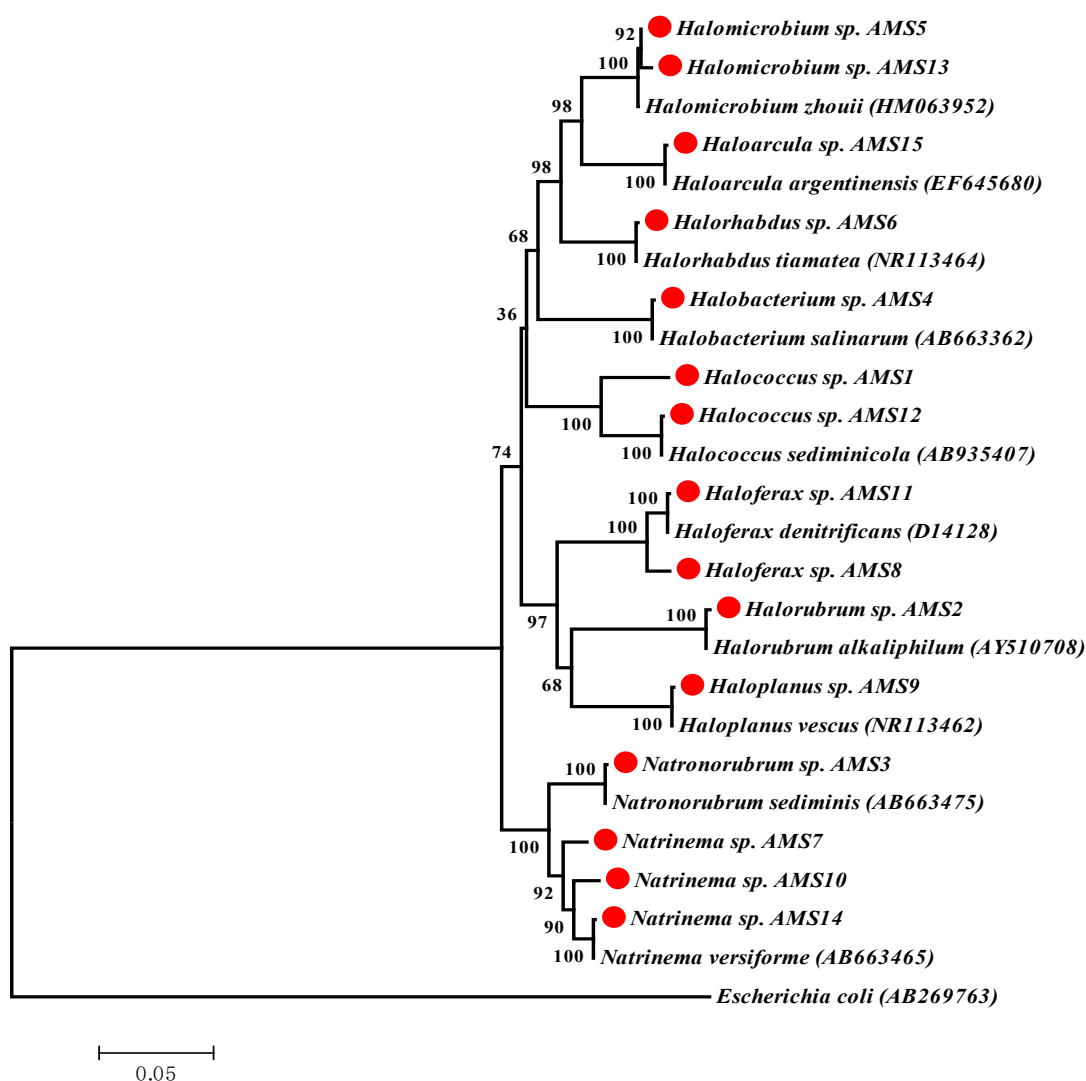


Figure 3. Phylogenetic analysis of EPS-producing marine archaeal from sediment sample.

3.2. Optimal Growth Condition of *Halococcus* sp. AMS12

A total of 15 archaeal isolates (AMS1 to AMS15) were used to screen the maximum EPS production. Compared to other isolates, *Halococcus* sp. AMS12 produced a maximum quantity of EPS (3.172 g/L). For further study, we used the potential isolates of *Halococcus* sp. AMS12 (Figure 4). The *Halococcus* sp. AMS12 optimal growth condition was noted in the presence of various salt concentrations ranging from 10% to 30% of NaCl, but the optimal growth was observed at 25% NaCl concentration. The result confirmed the halophilic nature of the isolate *Halococcus* sp. AMS12. The growth level of AMS12 at various pH has been given in Figure 5 which showed the optimal growth at pH 7.5. Similarly, the growth in different temperatures was also investigated, and the optimal growth was observed at 45 °C. The optimal growth was observed after 48 to 76 h at 45 °C with a 25% salt concentration at pH 7.5.

3.3. Production and Characterization of Marine Archaeal EPS

During the log phase, the isolate of AMS12 produced 3.172 g/L of EPS (Figure 5) which contained 54.28% of carbohydrate, 32.91% of proteins, 2.41% of lipids, 1.3% nucleic acid, 0.76% of uronic acid, and 8.34% of unidentified compounds (Figure 6). In the archaeal EPS, contributions of carbohydrates are a higher percentage followed by proteins and other substances. The unidentified compound may be phosphorous and other minor contents [31]. Further, the surface morphology of EPS was analyzed through FE-SEM and mentioned in Figure 7. The FESEM image indicates that the surface morphology is

rough, and hence, it will have strong binding capacity. Therefore, the EPS showed good biological activity.

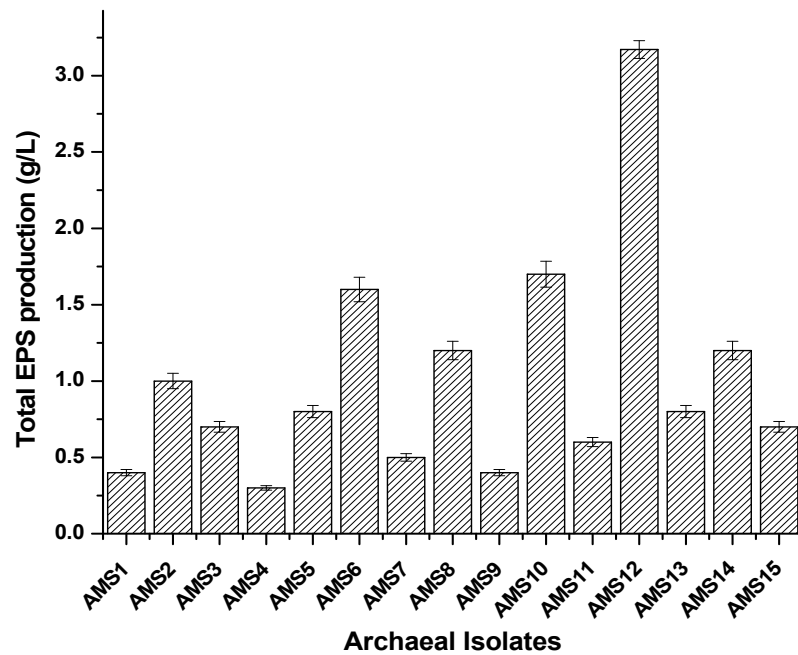


Figure 4. Total EPS production of archaeal isolates AMS12 on HAA.

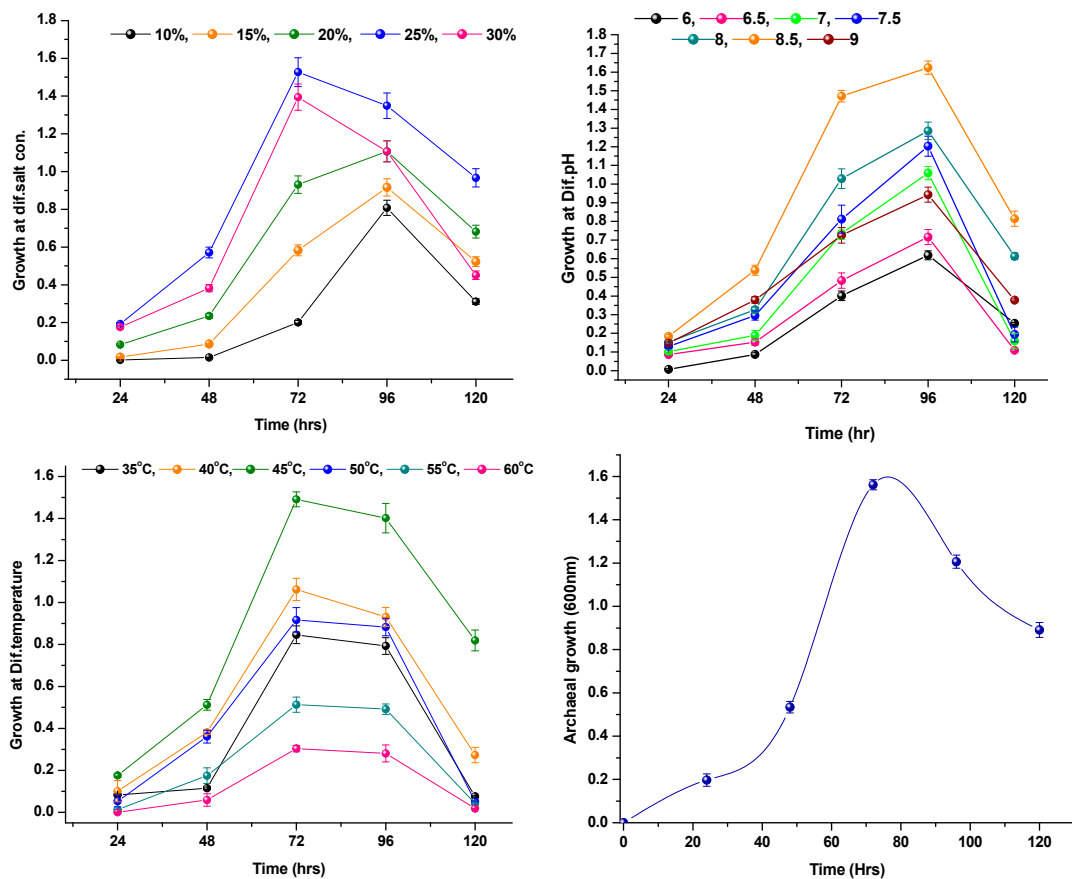


Figure 5. Optimal growth condition of marine archaea *Halococcus* AMS12.

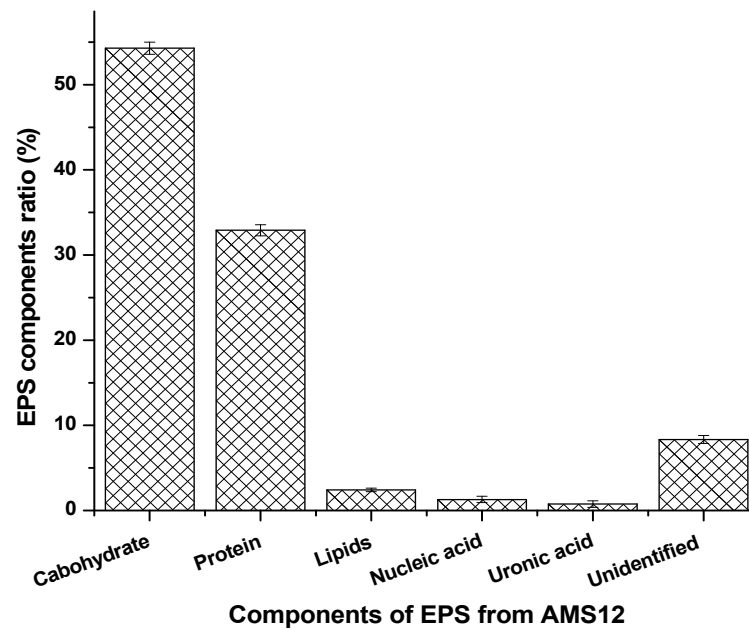


Figure 6. Components of EPS from marine archaea *Halococcus* sp. AMS12.

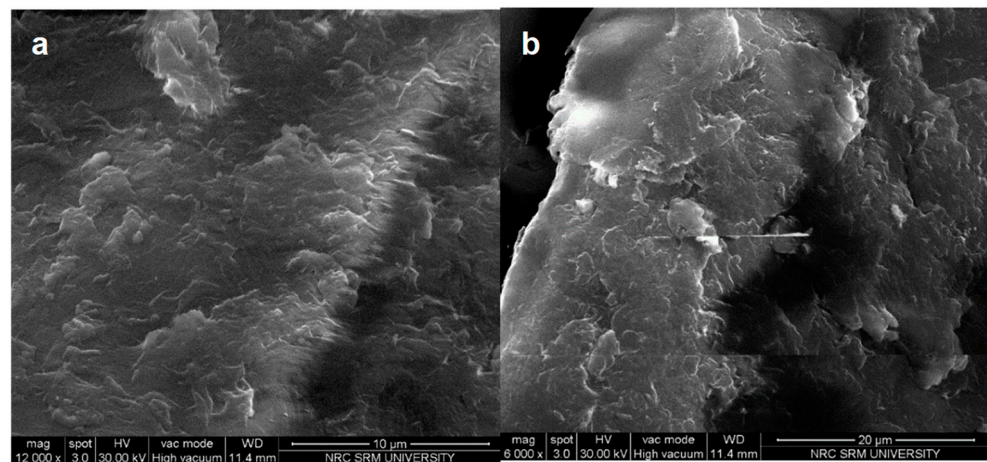


Figure 7. The surface morphology of purified EPS obtained from marine archaea *Halococcus* sp. AMS12 by SEM analysis. (a) EPS observation at 10-micron magnification and (b) at 20-micron magnification.

3.4. Biosurfactant Activity and Cell Membrane Protection

The bio-surfactant activity was determined in terms of emulsification at various concentrations of archaeal EPS. The significant activity was 52% from archaeal EPS, and the titronX-100 shows 67% of emulsifying activity. The lowest emulsifying activities of 18% and 24% were observed at 25 $\mu\text{g}/\text{mL}$ concentration of EPS and standard of TitronX-100, respectively (Figure 8). Cell membrane stability of archaeal EPS was estimated with human RBC, which showed 12% cell lysis in the presence of detergent at 100 $\mu\text{g}/\text{mL}$ of EPS concentration, followed by 28% cell lysis in the presence of glycerol and 94% cell lysis with the addition of detergent (SDS). The minimal concentration (25 $\mu\text{g}/\text{mL}$) of detergent lysed 52% of cells, whereas 42% of cells were lysed with the addition of archaeal EPS.

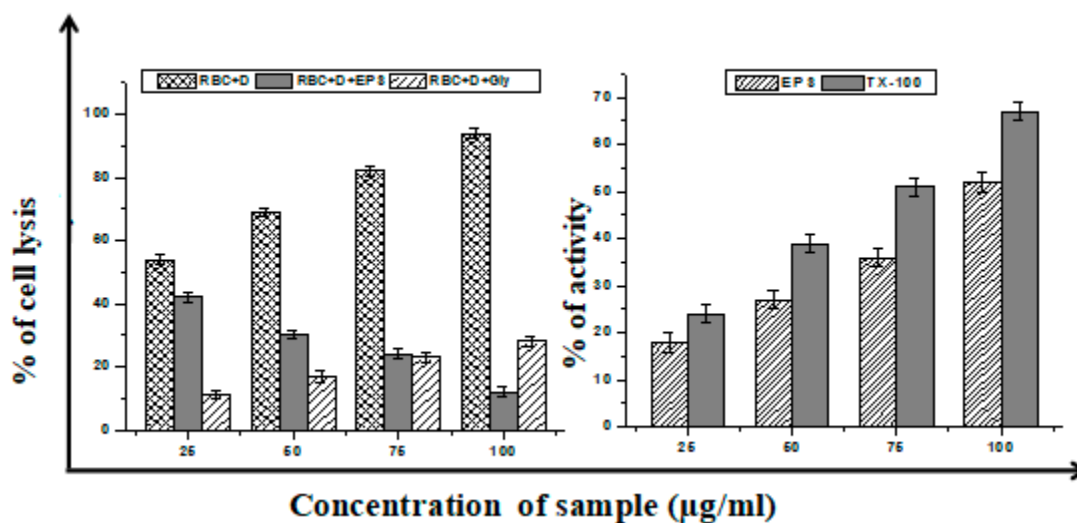


Figure 8. Cell-membrane protection and bio-surfactant activity of *Halococcus* sp. AMS12.

3.5. Enzymatic Activity of Marine *Halococcus* sp. AMS12

The extracellular enzyme from halophilic archaea received special attention due to the high stability in pH and temperature resistance, low water activity, and solvent resistance, which also facilitate survival in unfavorable conditions [32]. In the present study, the hydrolyzing activity of EPS from *Halococcus* sp. AMS12 exhibited 43.69 ± 1.89 U/mg gelatinase enzyme by degrading the substrates.

3.6. Antioxidant Activity of EPS Obtained from *Halococcus* sp. AMS12

In the present study, in vitro antioxidant activity was observed by the various antioxidant assays. The potential total antioxidant activity of 103.80 ± 0.02 (AAE), total reducing power of 86.1 ± 0.25 (AAE), DPPH of $97.23 \pm 0.21\%$, hydrogen peroxide scavenging of $60.8 \pm 0.21\%$, and nitric oxide scavenging activity of $89.37 \pm 0.24\%$ were observed at 100 µg/mL of archaeal EPS (Figure 9). The stable free-radical DPPH started to react with archaeal EPS and donated a hydrogen atom which led to reduction form (diphenyl picrylhydrazine), which had a deep yellow color. Through the inhalation of vapor, hydrogen peroxide will enter the human body, which is the source of lipid peroxidation, and it may lead to DNA damage in the body. The enzymatic antioxidant activity of EPS exhibited 17 U/mg of catalase, 32 U/mg of superoxide dismutase, 58 U/mg of lipid peroxidation, 117 U/mg of malondialdehyde (MDA), and 129 U/mg of glutathione activity (GSH) (Figure 9).

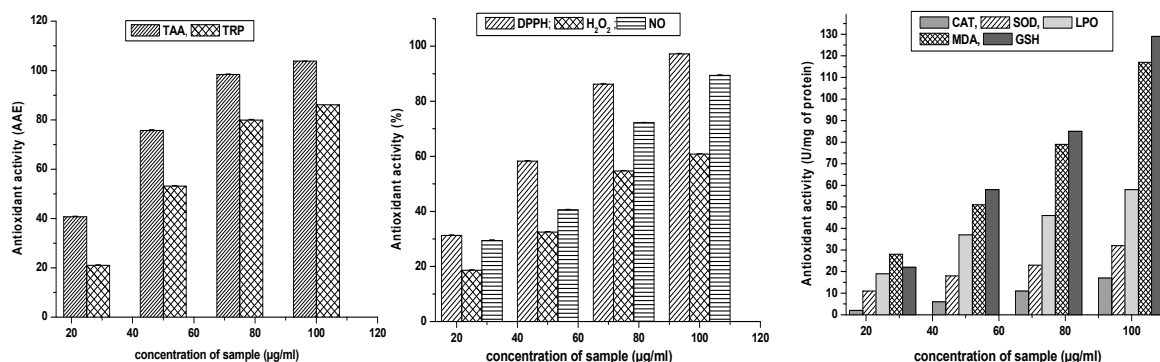


Figure 9. Antioxidant activity of marine archaeal EPS from *Halococcus* sp. AMS12.

3.7. Cell Viability (MTT) and DCFH-DA Assay

The 3T3 cell viability of EPS from the halo archaeal isolate was determined after 24 h incubation through the MTT assay (Figure 10). The results revealed that the EPS inhibited 13.19% of cell death against 3T3 cell lines with $86.11 \pm 4.16\%$ of cell viability at 100 $\mu\text{g}/\text{mL}$ of EPS concentration (Figure 11). The result suggested the archaeal EPS did not affect the noncancer cell lines.

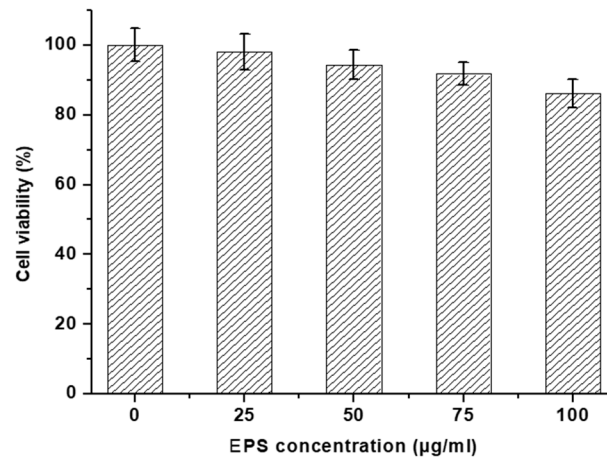


Figure 10. Cell viability assay of marine archaeal EPS obtained from *Halococcus* sp. AMS12.

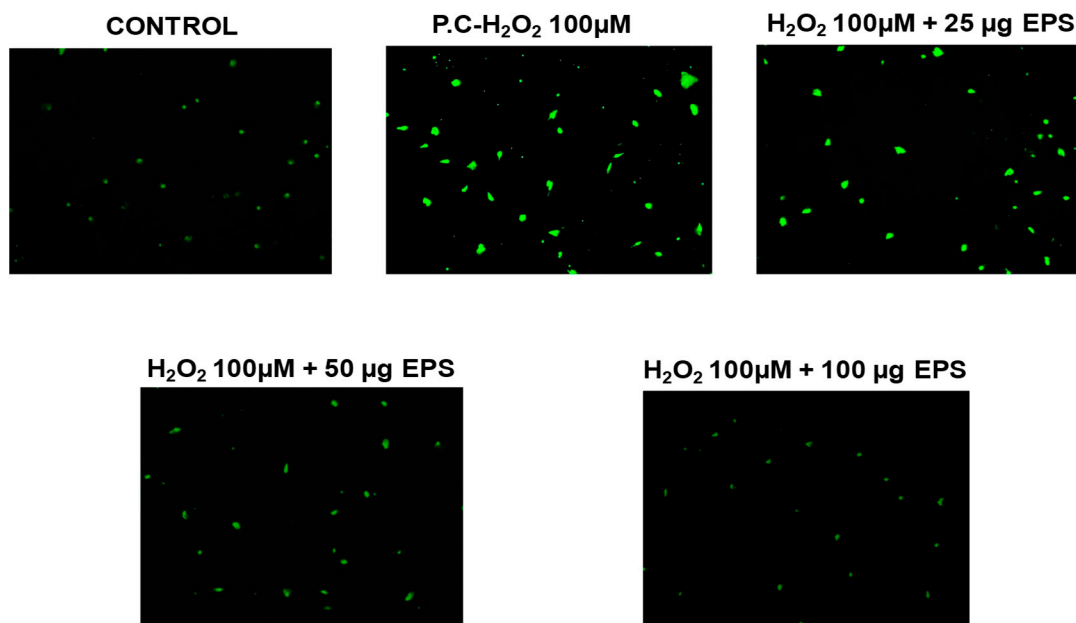


Figure 11. ROS inhibition assay using EPS obtained from *Halococcus* sp. AMS12.

4. Discussion

4.1. Isolation and Identification of Marine Archaea

In the marine environment, archaeal community has a key role in sulfur metabolism, methane oxidation, and nitrification [33]. Thus, the account of the archaeal community in coastal water, estuarine, basins mangrove, and the open sea is investigated to determine the role in marine habitats. However, the archaeal diversity in marine coastal sediment was little known [34]. Hence, the present study pays attention to the admittance of archaeal diversity from coastal sediment samples. Red-pigmented archaeal isolates, such as *Haloarcula vallismortis* and *Haloarcula qudrata*, were isolated from salter soil, which have optimal growth at 25% of salt concentration with a wide range of antibiotic resistance [35].

Similarly, the maximum growth of *Halococcus* sp. AMS112 was also observed in the medium with 25% of salt content. Archaeal genera, such as *Pyrococcus*, *Pyrodictium*, *Igneococcus*, *Thermococcus*, *Methanococcus*, *Archaeoglobus*, and *Methanopyrus*, were isolated from deep-sea hydrothermal systems, and aquifex were isolated from hydrothermal vents around 3500 m below the seabed of the North Sea of Alaska [36]. Similarly, archaeal communities were isolated from poly-aromatic hydrocarbon and poly-chlorinated sand samples from the Johnston atoll [37] and geothermal stream vent aerosols [38]. Methanogenic archaea were isolated from marine sediment samples collected at 800 m depth at the hydrated ridge in Oregon [39]. Unculturable *Euryarchaeota* and *Crenarchaeota* were isolated from the Skan Bay of Alaska. In this study, both culture-dependent and culture-independent analyses were used to explore the methanogens from the sediment sample [40]. The phylogenetic diversity of archaea from a sediment sample collected from the tidal creek of the coastal salt marsh was studied by Munson et al. [30] with archaeal-specific primers. Likewise, the archaeal community from polychlorinated biphenyl and polycyclic aromatic hydrocarbon contaminated sand samples were collected from the Johnston atoll by a molecular technique (SSU rDNA) [41]. The *Euryarchaeota* phylum was reported from an oil-contaminated shoreline sediment sample, which has taken a major part (94%) of total microbial diversity [42].

4.2. Optimal Growth Condition of *Halococcus* sp. AMS12

Halophilic archaeal isolates, such as *Haloarcula*, *Natrinema*, *Halorubrum*, *Natronomonas*, and *Halovivax*, were isolated from the Salt Lake which has optimal growth at 20% of salt concentration, and they are incapable of growing in a salt concentration lower than 15% [43]. Similar results were observed from the halophilic archaea isolated from Lonar Lake, which have optimal growth at pH range between 8 to 10 and with 25% salt; no growth was observed when the concentration was less than 15% [16]. The maximum growth of *Halobacterium* sp. from fish sauce was noted at the range of 15–20% salt concentration, and the growth pattern greatly depends on the concentration of NaCl [44]. The optimal growth for *Halorhabdus* sp., AMS6 was recorded at pH 8.2 for 168 h. Halo archaeal isolates from Thai fish sauce grew well in 20–25% of salt concentration, which exhibited an enzyme with food and biotechnological applications [45].

4.3. Production and Characterization of Marine Archaeal EPS

The carbohydrate content in the EPS may perform the inhibition process of pathogen and host [46]. Other protein and lipid components might be involved in cell adhesion, receptor function, and cell recognition [47]. Halophilic and thermophilic archaeal EPS have been considered as stable sources for gelling and emulsifying agents in food industries [11,48]. Popescu and Dumitru [49] reported that halophilic archaeal EPS may reduce the contamination in an extreme halophilic environment. Halo archaeal EPS have been considered as base material for petrochemical plastic [50].

4.4. Biosurfactant Activity and Cell Membrane Protection

The amphiphilic surface-active agent (bio-surfactant from archaea) has a high stability with exclusive composition and thereby plays a vital role in biofilm formation [51]. Earlier studies reported the bio-surfactant producing halophiles from a hypersaline environment. The halotolerant bacteria *Bacillus* spp. produced biosurfactants that have improved the oil revival [52]. Similarly, bio-surfactants from extreme thermophilic microbes, such as *Methanobacterium thermoautotrophicum*, are stable and active in a wide range of salt (up to 20%) and pH range (5–10) [53]. The bio-surfactant-producing microbes are harmless and biodegradable [54]. Additionally, the extracellular production of the bio-surfactant may be limited to a small area on the surface coupled with the cell membrane [55]. Desai et al. [16] reported the stable emulsifying agent from *Halobacterium* sp. has simultaneous anti-freezing and anti-dedication properties. These surface-active substances improve the stability of microbes and biofilm formation [56]. In addition, surface-active molecules on the cell surfaces improve the hydrophobicity of the cell [57]. In 1998, Watanabe et al. [58] reported

that the intra and extra cellular biopolymers protected the membrane of RBCs. These biopolymers improved the survival of organisms from the unfavorable environment and provide resistance to tolerate pollutants [59]. In addition, the EPS from *Bacillus circulans* have potential antimicrobial activity and cell-membrane-protecting activity [60]. In addition, Shivani et al. [61] reported that the bacterial exopolysaccharides can protect the cells and make them resistant to antimicrobial agents. Similar results were found in the present study which provided evidence of archaeal EPS having significant membrane protecting activity towards human RBCs.

4.5. Enzymatic Activity of Marine *Halococcus* sp. AMS12

Halophilic protease enzyme-producing archaea have been reported by Zorgani et al. [44]. Similarly, hydrolytic enzyme-producing archaea *Natrinema* sp. was isolated from Lonar Lake at 20% of salt concentration at pH 9.5 [62] and isolated from solar slattern. The halo archaeal protease is applied to accelerate the fish sauce fermentation, which minimizes the time of fish sauce maturation [63]. Likewise, Singh and Singh [64] reported extracellular protease, amylase-producing archaea, and poly (hydroxy butyrate) (PHB). In addition, hydrophobic protease and thermostable enzymes were reported from haloarchaea [65]. Promising enzyme activity of EPS was very important, and it can be precise to predict active bioactive fragments [66].

4.6. Antioxidant Activity of EPS Obtained from *Halococcus* sp. AMS12

Generally, antioxidant activity should not conclude with a single assay, and various in vitro and in vivo models have been observed to have antioxidant potential from particular samples [67]. Therefore, in the present study, in vitro and in vivo analyses of EPS from archaea were conducted. Total antioxidant activities are the method to determine the presence of total antioxidants in the sample, which are evaluated by the discoloration. The total reducing power of archaeal EPS was maintained by increasing the concentration, which shows significant reducing capacity and prevention of free-radical chain reaction [68]. Antioxidants are used mostly to prevent oxidative tissue damage from processed food. Most synthetic antioxidants are accountable for carcinogenesis and liver damage [69]. In the present study, antioxidant activity was evaluated at various concentrations. Sodium nitroprusside will oxidize at alkaline pH (7.2) to synthesize nitric oxide (NO) free radicals. These NO radicals are converted into nitrate and nitrite by the oxygen supply from the archaeal EPS [70].

Metabolites from microbes have various bioactive potentials, such as antioxidant, immunosuppressive, enzyme inhibitory, anti-inflammatory, and other therapeutic uses [71]. In the present observation, archaeal EPS has the capacity to form polyoxometalates from molybdate and convert the trivalent ferric iron into divalent ferrous iron by the oxidation process [72]. Similarly, ROS formation of the cell membrane was generated by the reaction of hydrogen peroxide with divalent ferrous, and copper ions were inhibited by the archaeal EPS [73]. Superoxide and hydroxyl radical scavenging capacities of the three marine EPS were reported by Mao et al. [74]. Reductase activity was found in the cytoplasmic membrane of *Halobacterium salinarum*, and further research revealed that oxidoreductase was associated with the membrane as a loose bounded enzyme [75]. Similarly, Wakagi et al. [76] reported the oxidoreductase catalyzes from *Sulfolobus* sp. Likewise, Wang et al. [77] reported oxidase encoding genes from *Metallosphaera* species and 19 other genera of archaea [78]. Chelating activity of oxidoreductase on ROS was very significant, which was proven by Wheaton et al. [79] and Mesle et al. [80]. The reaction of sodium nitroprusside with oxygen forms nitrite and further reaction of oxygen will form nitric oxide and peroxy nitrate, which damage the nucleic acids, other proteins, and lipids in the cell (73).

4.7. Cell Viability (MTT) and DCFH-DA Assay

Haloarchaeo biusiranensis has no effect on noncancer cell lines and the significant reduction of cancerous cell viability against the prostate cancer cell line DU145, the breast cancer

cell line, MDA-MB468, and the lung cancer cell line, A549 [81]. The halophilic archaeal strain, *Halogeometricum limi*, produced carotenoid, which has antioxidant, antihemolytic, and anticancer activity [82]. Previous studies demonstrated the archaeal inhibition of cancer cell proliferation by their bioactive metabolites. Similarly, *Halobacterium halobium* inhibited the proliferation of cancer cells [83]. In addition, DCFH-DA assay has been conducted to reveal the potential intracellular ROS inhibition of EPS against the 3T3 cell line. The microscopic results indicated that the inhibition of intracellular ROS by the reduction of fluorescence (Figure 11). ROS-induced positive control has bright fluorescence which was reduced by the EPS-treated samples, and the activity was improved at 100 µg/mL of *Halococcus* sp. AMS12 EPS.

5. Conclusions

EPS from marine archaea *Halococcus* sp. AMS12 have multifunctional bioactivity in terms of hydrolytic activity of protein substrate, bio-surfactant activity, cell membrane protection, and enzymatic and nonenzymatic antioxidant activities. Furthermore, archaeal EPS are more stable and less toxic, and the current study proved low toxicity effects in fibroblast cells. The present study showed that archaeal EPS have bioactive potential, which points to their use in the biomedical, cosmetic, and food preservation industries.

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