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Identification and antioxidant activity of hyaluronic acid extracted from local isolates of *Streptococcus thermophilus*

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ABSTRACT

The synthesis of hyaluronic acid (HA) by bacteria is a promising alternative to extracting the biopolymer from animal tissues. The production of HA by *Streptococcus thermophilus*, which belongs to the group of organisms generally recognized as safe (GRAS), is a well-studied viable alternative. The present study investigated five *S. thermophilus* strains (St1, St2, St3, St4, and St5) with markedly high HA productivity. The quantity of purified HA was different following the order St5 > St4 > St3 > St2 > St1. The characteristics of molecular mass analysis of HAs were evaluated by multi-technique (FTIR, NMR, and HPLC). The antioxidant activity of the purified HA was quantified by multi-technique (DPPH, iron chelating, reducing power, and hydrogen peroxide (H_2O_2) scavenging activity), over a range of relevant concentrations, i.e., 50, 100, 300, 500, 700, 1100, and 1300 μg/ml. They were tested for a concentration-dependent enhancement in their radical scavenging and lowering abilities. Significantly increased antioxidant activity was detected at concentrations of 1300 μg/ml, with 69.18%, 78.42%, and 73.74% of DPPH, hydrogen peroxide, and iron-chelating scavenging activities, respectively. Furthermore, HA was proven to have effective lowering power at the same concentration. When compared to the typical antioxidant Butylated hydroxytoluene (BHT), but these various antioxidant activities were low. It is suggested that HA have the potential to be the resource of natural antioxidant.

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1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan (GAG) that is a type of linear mucopolysaccharide. It's a naturally occurring molecule found in vertebrates' epithelial, connective, and neural tissues. The four primary groups of GAGs are classified based on their core disaccharide units and include heparin/heparan sulfate, chondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronic acid. HA, on the other hand, is unsulfated and is not generated in combination with proteins by Golgi enzymes [1–3]. HA is made up of the repeating components β-1,4-D-glucuronic acid (also called uronic acid) and β-1,3-N-acetyl-D-glucosamide (GlcNAc) [4].

From 1934 until the present day, Hyaluronic acid has been taken over many investigators' interests from its initial detection to extraction from different tissues. HA used several applications in bioengineering and biomedicine due to high biodegradation and biocompatibility [5,6]. In the commercial manufacture of HA, two processes are used: extraction of HA from rooster combs and microbial fermentation utilizing attenuated strains of pathogenic bacteria. It's difficult to separate very pure HA from rooster comb since it's found in low amounts and is complexed with proteoglycans. The use of animal-derived biochemicals is, however, restricted due to the possibility of viral infection [7].

Bacterial technology makes it possible to increase HA yield with low protein and endotoxin activity. At the same hand, streptococci fermentation raises concerns about safety, such as the presence of hyaluronidase or the danger of bacterial contamination [7–10]. In 2019, the global HA market was worth USD 9.1 billion, and by 2027, it is predicted to be worth USD 16.6 billion (https://www.grandviewresearch.com/press-release/global-hyaluronic-acid-market). HA is employed in a variety of applications, such as medicine, food, and cosmetics, due to its unique properties. HA is now widely utilized in medicine to treat a wide range of disorders, consider knee osteoarthritis pain relief [11]. skin moisturizers wound dressing materials [12], dry eye [13,14], otosclerosis sur-

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gery [15], non-reducing disc displacement [16], salivary gland tissue engineering [17] or even drug/gene delivery agent [18]. In the United States, the European Union, and Canada, HA is also utilized as a dietary supplement. Furthermore, in Japan and Korea, it can be used as a food ingredient [19].

HA could be utilized as a food ingredient to increase the water retention and rheological qualities of meat emulsions, as well as a replacement for polyphosphates, carrageenan, and soy protein, enabling for the creation of novel healthy meat products [20]. In recent years, a number of scientific investigations have demonstrated that In vitro and in vivo, HA exhibits antioxidant effects [21].

As a result, focus has shifted to HA synthesis utilizing safe, non-pathogenic strains recognized as safe (GRAS), such as *Streptococcus thermophilus* strains with high productivity of beneficial exopolysaccharides (EPSs) containing HA that can be extracted from conventional dairy foods [9,22–25]. Hence, the present research aims to extract HA from a local isolate of *S. thermophilus* and determine its antioxidant activity in vitro.

2. Materials and methods

2.1. Materials

Hydrogen Peroxide (H_2O_2), Sulphuric acid (H2SO4), Dipotassium hydrogen phosphate (K_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), Sodium tetraborate·$10H_2O_2$ and Sodium Chloride (NaCl) were obtained from England (B.D.H), Trichloroacetic acid (TCA), Absolute Ethanol, Butylated hydroxytoluene (BHT), Ferric chloride ($FeCl_3$), Potassium ferricyanide (K4[Fe(CN)6].3H2O), Trichloroacetic acid (TCA), were obtained from Korea (Samchun), D-glucuronic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), were obtained from Sigma Aldrich (USA).

2.2. Organisms and culture conditions

Five local *Streptococcus thermophilus* isolates used in this study were obtained from the Dairy Technology Lab, Agriculture College, University of Basrah. It was grown in M17 agar medium for 48 h at 42 °C [26,27].

2.3. HA production and fermentation conditions

S. thermophilus produces HA in fermentation broth using the method described by [28], with some changes made to the production steps to improve HA production. Each strain of *S. thermophilus* was cultivated in M17 agar medium for a brief period of time. The cultivated cells were transferred to M17 broth medium at 42 °C and 150 rpm for 24 h. The amount crude HA in the fermentation broth was calculated by the carbazole method [29], with some modifications in its purification steps were enhance the yield of HA and its purity as described in the literature [30–33]. The fermentation broth was first precipitated with three volumes of ethanol. followed by centrifugation at 7000 rpm for 30 min. To remove the solid mass and reduce viscosity, The precipitate was collected by centrifugation re-dissolved in NaCl 0.15 M and centrifuged for 5 min at 3000 rpm. The precipitate was recovered and re-dissolved in deionized water, By decreasing the pH of the broth to 2 with 1% trichloroacetic acid, then treating it with charcoal (1–2%) for 1 h at 4 °C, followed by centrifugation at 7000 rpm for 30 min, the nucleic acids and bacteria-derived proteins in crude samples were eliminated. After removal of cells and charcoal, the solution was readjusted to a pH of about 6 with NaOH 0.1% and diluted with the equal volume deionized water. HA solution was passed through Millipore filters 0.45 μm. Then, the amount crude HA in the fermentation broth was calculated by the adding 0.025 M disodium tetraborate solution (in sulfuric acid sp. gr.1.84) and heated at 100 °C in a water bath for 10 min. The solution was cooled at room temperature. Then, 0.125% carbazole solution (in 95% ethanol) was added and boiled for 15 min in a water bath before cooling to room temperature. Finally, using D-glucuronic acid (Sigma G5269, USA) as the standard, read the optical density (OD) at A530 nm.

2.4. FTIR spectroscopy

In the range 4000–400 cm^{-1}, a Fourier transform infrared (FTIR) spectrometer (Perkin Elmer Spectrum One Nicolet 520) was utilized to characterize the generated HA from *S. thermophilus*. The experiment was carried out at room temperature, and the standard HA (Sigma 97616, USA) was employed for spectral collaboration.

2.5. NMR spectroscopy

The structural properties of the produced HA from *S. thermophilus* at 27 °C were determined using proton ^1H NMR (Bruker 400-MHz AV). As a solvent, deuterium water (D_2O) was employed. For the collaboration of the spectra, standard HA (Sigma 97616, USA) was employed.

2.6. High pressure liquid chromatography (HPLC)

All the samples were lyophilized, resuspended in 1 M NaCl, centrifuged, and filtered through a 0.22 μm filter membrane. An Agilent HPLC system with a C18 column, refractive index detector, and commercial hyaluronic acid as a reference was used to analyze the after-treatment solutions. A 6 mM sulphuric acid solution with a pH of 2.5 and a current speed of 0.4 mL/min was utilized as the mobile phase. The temperature in the column was maintained at 65 °C at all times.

2.7. Determination of the antioxidant activity of HA

2.7.1. DPPH radicals scavenging activity assay

A free radical of 2, 2-diphienyl-1-picrylhydrazile (DPPH) was utilized to assess free radical scavenging, according to the method given by [34,35] with minor modifications. In 0.2 mL of ethanol, DPPH was dissolved at a concentration of 0.04 μg/ mL. (50, 100, 300, 500, 700, 1100, and 1300 g/mL) of distilled water were utilized to dissolve the HA sample. Each sample received 2.0 mL DPPH and was shaken immediately before being maintained at room temperature in the dark for 30 min. The absorbance of the supernatant was measured at 517 nm against a blank after centrifugation at 4500 rpm for 15 min (ethanol as an alternative of the sample and DPPH solution). Increased free-radical scavenging activity is indicated by a lower absorbance of the reaction mixture. The scavenging % was estimated by the use of the equation below: **scavenging %age activity (%)** = [1 - (A_1 - A_2)/ $A_◦$], where A_0 is the sample's absorbance, A_1 is the sample's absorbance, and A_2 is the sample's absorbance under the identical conditions as A_1 but with water alternatively of DPPH* solution. At all doses examined, the HA's DPPH radical scavenging activity was observable, however it was lower than that of Butylated hydroxytoluene (BHT).

2.7.2. Reducing power scavenging activity assay

The reducing power of HA was calculated using [36], approach, including some modifications. 2 mL phosphate buffer (0.2 M, pH 6.6) and 2 mL 0.1% K_3Fe (CN) were added to each sample (50, 100, 300, 500, 700, 1100, and 1300 μg/ mL). After 30 min of incubation at 50°C, 1.5 mL of trichloroacetic acid solution (TCA, 10% w/ v) has been added to the mix. The supernatant (2 mL) was com-

bined with 2 mL distilled water and 0.5 mL $FeCl_3$ (0.1% w/v) after centrifugation, and at 700 nm, the absorbance was measured.

2.7.3. Hydrogen peroxide scavenging activity assay

The capability of the HA to scavenge hydrogen peroxide ((H_2O_2) was decided by employing the method of [37]. An aliquot of 0.1 mL of HA (50, 100, 300, 500, 700, 1100, and 1300 μg/ mL) was put into Eppendorf tubes, which were then filled to 0.4 mL with 50 mM phosphate buffer (pH 7.4) before adding 0.6 mL of H_2O_2 solution (2 mM). After 10 min of reaction time, the reaction mixture was vortexed and its absorbance was measured at 230 nm. As a positive control, BHT was used. The following calculation was used to calculate the HA's ability to scavenge H_2O_2:

Scavenging activity (%) = $[(A_1\text{-}A_0)/A_0] \times 100$ where: A_0- = Absorbance of the control, A_1 = Absorbance of sample.

2.7.4. Chelating of ferrous ion assay

The chelating effect of the produced HA on ferrous ions was calculated using the method of [38], with slight modifications. Using methanol, 1 mL of each test sample (50, 100, 300, 500, 700, 1100, and 1300 μg/mL) was increased to 3 mL. 0.74 mL methanol was mixed with 0.02 mL 2 mM $FeCl_2$ in a 0.02 mL flask. The reaction was started by adding 0.04 mL of 5 mM ferrozine to the mixture and letting it sit at room temperature, for 10 min. The mixture's absorbance was measured at 562 nm.

Chelating of Ferrous% = $[1\text{-}\ A_S/A_0] \times 100$ Where the absorbance of the control is A_0, and as is the absorbance of the iron chelator.

2.8. Statistical analysis

A factorial experiment with the complete randomized design (CRD) was used for analyzing data by SPSS software, version 18. The least-square design (L.S.D.) was used to compare among the means, and the treatments were performed in triplicate. *P* values of less than 0.05 were deemed statistically significant.

3. Result and discussion

3.1. Hyaluronic acid production by S. Thermophiles

After fermentation, five *S. thermophilus* isolates (St1, St2, St3, St4, and St5) were isolated based on acid production. The colorimetric carbazole approach was used to determine the concentration of HA generated by the bacterial strains, and the outcomes are presented in Table 1. The HA production was favored by strain St5 yielding 334.27 mg/L, while the lowest by strain St1 yielding was 114.28 mg/L. This difference in HA production among the strains during the fermentation may attribute to enzymic degradation and source of isolation.

Table 1
The production of hyaluronic acid from local isolates of Streptococcus thermophilus in M17 broth.

S. thermophilus isolates	Hyaluronic acid* (mg/L)
St1	$114.28^{f} \pm 10.38$
St2	$285.89^{d} \pm 11.43$
St3	$296.70^{c} \pm 13.97$
St4	$308.32^{b} \pm 12.68$
St5	$334.27^{a} \pm 13.52$

* The mean of three replicates. [a-f] Different small letters of hyaluronic acid production samples were significantly different ($p < 0.05$). ± SD: standard deviation

3.2. FTIR characterization of hyaluronic acid produced by S. Thermophilus

FTIR spectroscopy is a useful instrument for discovering functional groups and organic compounds because it examines transitions between vibrational states of bonds inside the molecule. The FTIR spectrum of the HA sample and the reference standard, as well as their waves, are similar, as shown in Fig. 1.

The location of the peaks in terms of wavenumber (cm^{-1}) of both the reference standard and the HA produced by *S. thremophilus* are identical and associated under ideal conditions. In this investigation, the FTIR spectra of both the reference standard and pure HA matched their characterization (Fig. 3). Multiple strong peaks (cm^{-1}) in conventional HA were discovered by FTIR at 3348, showing the presence of OH stretching and N-H stretching vibrations in the N-acetyl side chain [39]. Due to symmetric methyl C-H stretch, glucuronic acid is represented by two bands at 2926 and 2927 cm^{-1}. The amide I group of C=O carboxyl and primary aromatic amine CN stretching are ascribed to the peaks at 1637 and 1421 cm^{-1}, respectively. The major alcohol C-O stretch reached a high of 1023 cm^{-1} [40].

3.3. 1H NMR spectroscopy

Fig. 2 shows ^1H NMR spectra of both test HA sample and HA standard. The peaks at 0.999 and 1.25 ppm in ^1H NMR spectrum (Fig. 2). At 1.79, 3.50, 3.72, and 4.4 ppm, protons from the CH_3, CH_2, NH, and OH groups were detected. Protons of the CH group were measured at 3.22–3.70 ppm. In the literature, the same peaks were shown in the NMR spectra of a HA standard [41]. Methylene groups were identified in the protected region, which is consistent with prior findings from proton NMR spectra of HA derived from bacterial sources [42]. The peak between 4.6 and 4.8 strongly suggests the presence of glycosidic linkages in the isolated HA. The peak responsible for the formation of N-acetyl glucosamine was measured between 1.8 and 1.7 ppm [43]. The noisy peaks in the bacterial hyaluronic acid NMR spectrum were thought to be generated by impurities added during the manufacturing or purification processes.

3.4. High-Pressure liquid chromatography (HPLC)

The results of HPLC analyses using the refractive index (RI) detector are shown in Fig. 4. The HA standard and S. thermophilus HA extraction had the same retention time. The chromatogram shows only one distinct peak, showing that the isolated material was not contaminated with other glycosaminoglycans. The typical hyaluronic retention time was 4.91 s in Fig. 3A. The extraction time from *S. thermophilus* was 4.90 min (Fig. 3B). These results are in agreement with [44]. As a consequence, the HA extraction from *S. thermophilus* included no additional polysaccharide from the culture media that might have been removed during the purification procedure.

3.5. Antioxidant activities in vitro of bacterial hyaluronic acid (HA)

3.5.1. DPPH radicals scavenging activity of HA

Because of the harmful effects of free radicals in nutritional and biological systems, the DPPH radical test is essential for determining antioxidant activity. Excessive free radical production accelerated the oxidation of lipids in food and caused significant damage to nearby biomolecules [45]. When compared to the conventional BHT (98.09%), the isolated HA had the lowest activity (26.22%) at 100 μg/ml and the highest activity (69.18%) at 1300 μg/ml (Fig. 4). The scavenging effect enhanced when the concentration was increased up to 1300 μg/ml. The presence of car-

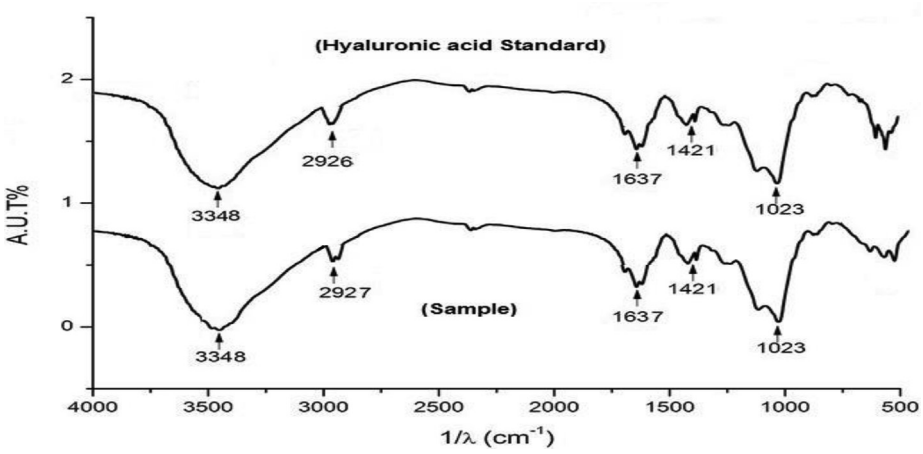

Fig. 1. Infrared spectra (FT-IR) of Standard hyaluronic acid bacterial hyaluronic acid (HA-sample).

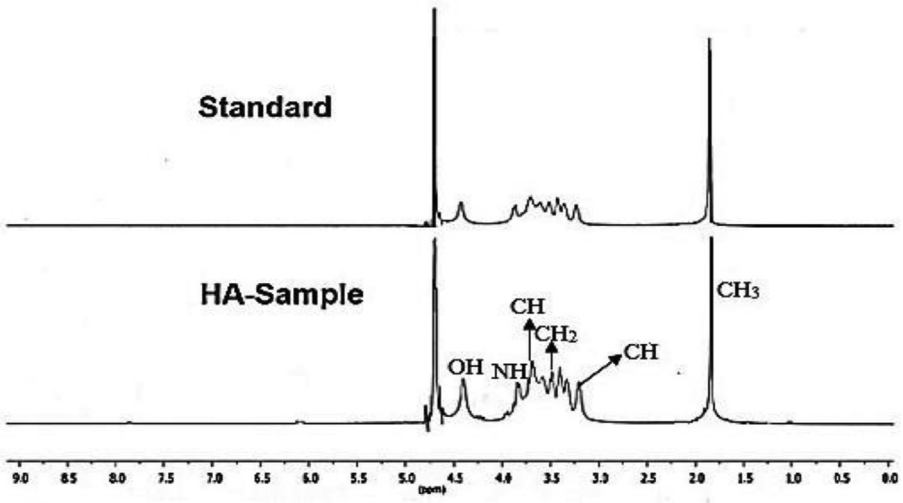

Fig. 2. ^{1}H NMR spectra of HA-standard and bacterial hyaluronic acid (HA-sample).

boxylic groups in HA is most likely responsible for its antiradical properties. Free radicals may interact with these organizations [23]. This outcome was consistent with previous research by [21], where the DPPH scavenging capability of HA (1050 kDa) and LMWHA-1 (145 kDa) were 53.63%, 59.38%, respectively, at 1.6 mg/ml.

3.5.2. Reducing power

The dosage response curves for the reduction forces for all isolating HA concentrations (50–1300 μg/ml) are illustrated in the Fig. 5. As the concentration grew higher, the reducing power of all isolate HA increased. At a concentration of 1300 g/ml, isolate HA had a reducing power (absorbance at 700 nm) of 1.1, which was lower than BHT (Fig. 5). Because of its carboxylic groups, HA has an antioxidant function [23]. Transition metal ions like the Cu + 2 or Fe + 2 may interact with this charged group, resulting in the commencement of Fenton's reaction. Several writers have extensively reported on the ability of HA to chelate various ions and transition metals [23,46,47].

3.5.3. Hydrogen peroxide scavenging activity assay

As demonstrated in Fig. 6, the scavenging of HA on hydrogen peroxide was concentration-dependent (50–1300 μg/mL) and was compared to that of BHT, a reference molecule. The outcomes showed that HA had an effective hydrogen peroxide scavenging activity, and various concentrations (50, 100,300,500.700,1100, and 1300 μg/ml) exhibited 6.33%, 29.15%, 41.15%, 56,78%, 61.08%, 77.15% and 78.42% hydrogen peroxide scavenging activity respectively, while, at the same concentration BHT showed 60.12%, 81.01%, 89.88%, 92.15%, 98.16%, 98.61 and 98.62% activity respectively·H_2O_2 is not extremely reactive in and of itself, but it can be hazardous to cells when it produces hydroxyl radicals [48].

3.5.4. Chelating of ferrous ion assay

Iron can be found in nature as either ferrous or ferric ions, with the latter form of ferric ion being more common in foods. Chelation of ferrous ions (Fe^{+2}) may have significant antioxidant benefits by delaying metal-catalyzed oxidation [49]. Fig. 7 shows the chelating activity of HA and BHT for ferrous ions. The chelating power of isolate HA was 7.11%, 21.28%, 48.93%, 57.79%, 64.77%, 71.98%, and 73.74%, respectively. The metal chelating effects of HA are summarized in Fig. 7. That could be owing to the existence of hydroxyl and carboxylate groups, which could interact with the ferrous ions, according to our theory. Total antioxidant capacity (14.02%), reducing power (18.18%), DPPH radical-scavenging (5.57 kmol TE/g), and hydroxyl radical-scavenging activity of HA produced by *Strepetococcus zoepidemicus* CCT 7546 were all reported to be active in a recent study (28.39%) [50].

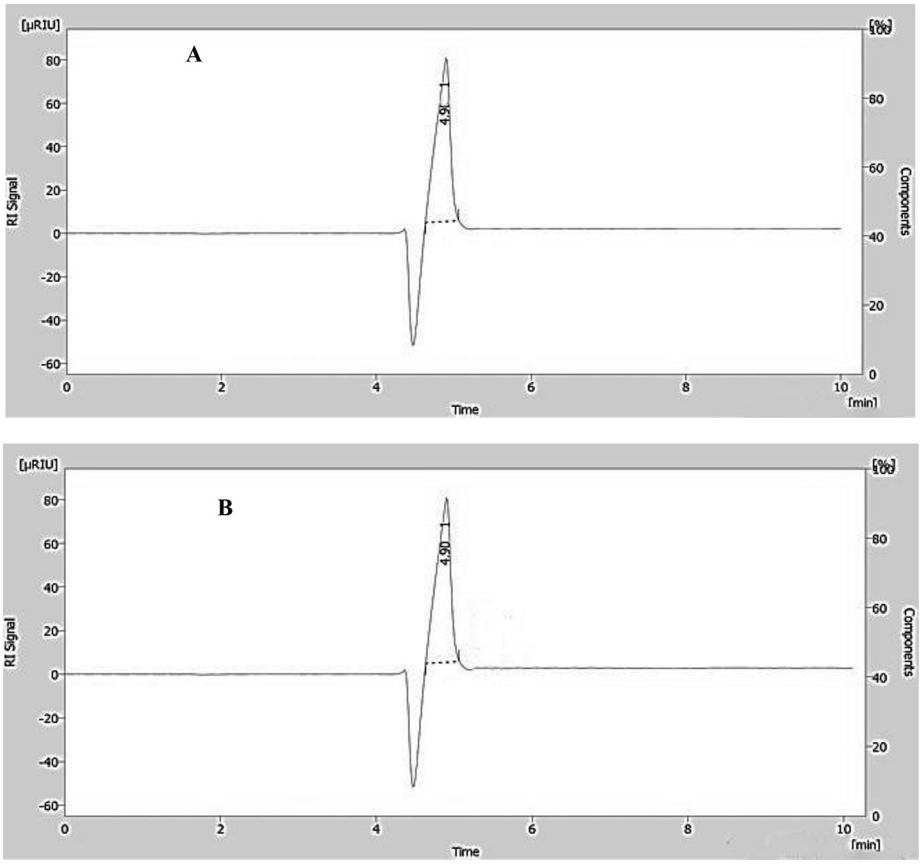

Fig. 3. HPLC of hyaluronic acid, (A): HA-Standard and (B): bacterial hyaluronic acid (HA-sample).

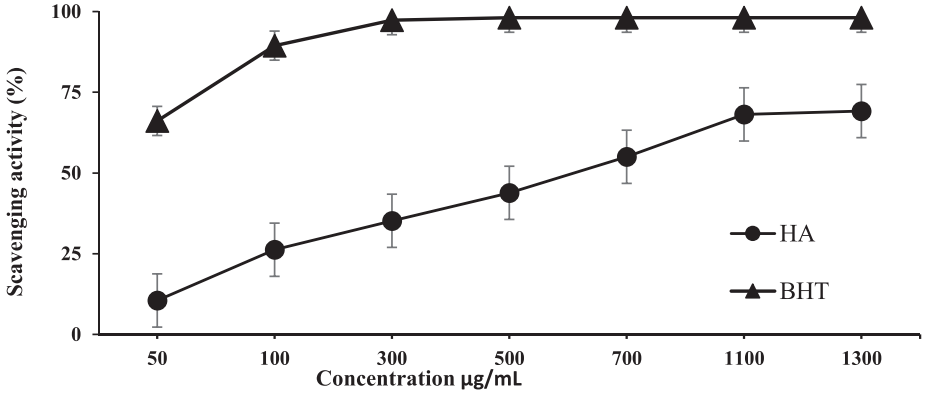

Fig. 4. DPPH radical scavenging activity of BHT and bacterial hyaluronic acid.

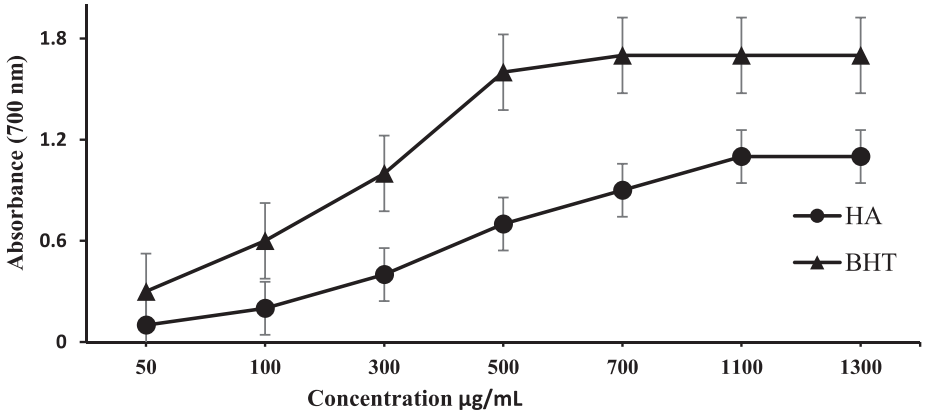

Fig. 5. Reducing power scavenging activity of BHT and bacterial hyaluronic acid.

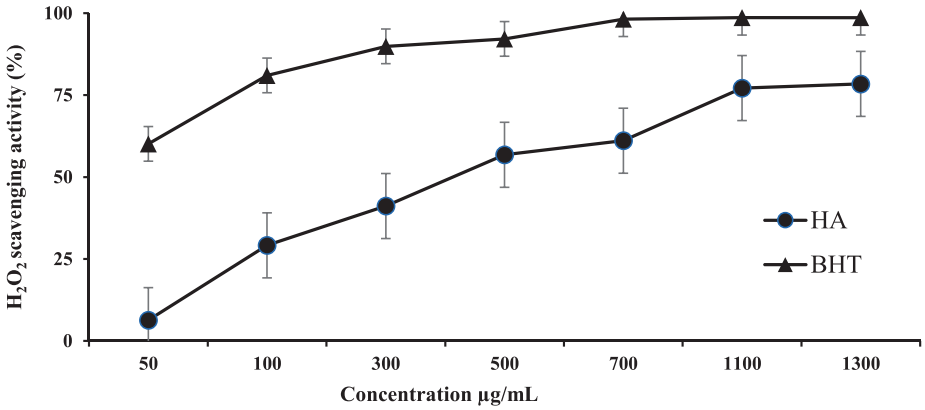

Fig. 6. Hydrogen peroxide scavenging activity of BHT and bacterial hyaluronic acid.

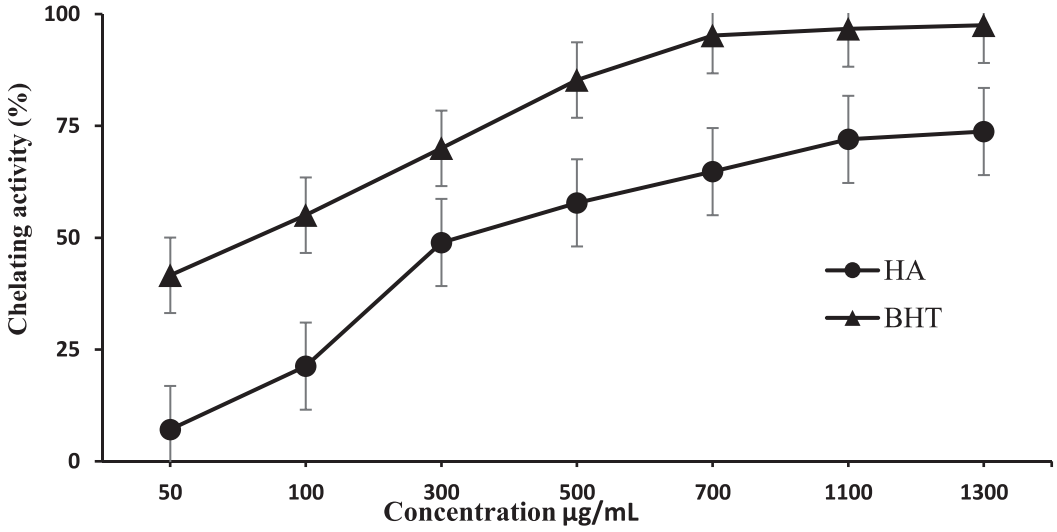

Fig. 7. Chelating of ferrous ion scavenging activity of BHT and bacterial hyaluronic acid.

4. Conclusions

Local isolates of *Streptococcus thermophilus* were used in this investigation. The bacterium *S. thermophilus*, which belongs to the GRAS (generally regarded as safe) group, may generate EPSs such as HA. As a result of its safety, we believe that this bacterium is ideal for HA synthesis. In an in vitro antioxidant test, HA showed much higher antioxidant activity. Consequently, it's possible that this substance will be beneficial in medicinal, cosmetic, and food applications.

CRediT authorship contribution statement

Ameer A. Mohammed: Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization. **Alaa Kareem Niamah:** Software, Investigation, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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