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Research Article
Physicochemical, Molecular and Functional Characteristics of Hyaluronic Acid as a Functional Food

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Abstract

Background: Hyaluronic acid (HA) is a natural carbohydrate that promotes proper joint function and tissue repair. Hyaluronic acid is an essential nutrient that effectively strengthens eyes, skin, collagen and immunity. So, elder people needs extra help and supplement with hyaluronic for maintaining collagen production, promoting skin elastin, hydrating damaged or chapped skin and reversing signs of aging. It is better to provide them their needs from natural and efficient ingredients or supporting some common foods with hyaluronic acid. **Materials and Methods:** To reialize the objectives of the present study the molecular characterization of HA produced bacteria was performed, followed by isolation and purification of HA and then the x-ray diffraction, scaning electronic microscope, HPLC analysis and antioxidant capacity were performed. **Results:** The molecular data emphasized that the ability of *Streptococcus thermophilus* BLM 58 to produce hyaluronic acid more than *Streptococcus thermophilus* TH-4. The FT-IR and x-ray diffraction confirmed the identity of purified HA with the profile obtained by standard which confirmed the non-crystalline structure of HA and its appearance as semi-hydrated fibers. Scanning electronic microscope indicated that the HA consists of many layers of cylindrical fibers with blined ended. Moreover, considerable antioxidant activity (IC_{50} = 493.58±0.48 μg mL^{-1}) with no cytoxicity of HA were observed. The hyaluronic acid-fortified yogurt showed a good overall acceptabilty without any adverse effect on the rheological properties. **Conclusion:** Hyaluronic acid can be safely used as a food supplement to support the nutritive value of food products (as a yogurt) without any negative effect on the rheological and organoleptic properties.

Key words: Hyaloronic acid, molecular characterization, chemical and functional properties, HA fortified yogurt

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Hyaluronic acid (HA) has been successfully produced on an industrial scale with *Streptococcus* species. It is the main necessarily solved by Meyer and Palmer[1], Kaur and Jayaraman[2] and Chauhan *et al.*[3], who found that hyaluronic acid consists of repeated D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) which joined by B-1,3 or B-1,4 glycosidic bond (Fig. 1)[4]. The HA is widely used in the cosmetic, medical and food industries, including as skin moisturizers, in orthopedic surgery, rheumatology, drug delivery and wound healing[3-5]. The HA is obtained from rooster combs or by microbial fermentation using certain attenuated strains of pathogenic bacteria[6]. In rooster comb, hyaluronic acid is complexed with proteoglycans making the isolation of highly pure HA costly[7]. Moreover, the use of animal-derived biochemicals for human therapeutics is being reduced because of the risk of cross-species viral and other adventitious-agent infections. Hence, microbial production is gradually replacing extraction as the preferred source of HA[8,9]. However, the pathogenic streptococci are also not ideal sources of HA because the risk of mutation of the bacterial strains and possible coproduction of toxins and pyrogens are difficult to eliminate entirely. Thus, at present, alternative sources of HA production is being explored. Exopolysaccharides (EPS) produced by certain strains named recognized as safe (GRAS) has received increasing attention in recent years[10]. Some researchers have reported that *Streptococcus thermophilus*, which is classified as GRAS, has the ability to produce a wide variety of EPSs[11,12]. In a previous study, it was found that *S. thermophilus*, a strain isolated from a dairy food product, can produce HA from milk[13]. Because of the safety of this bacterium, its direct use in yogurt and other fermentation products is expected. However, the fermentative yield of HA by *S. thermophilus* YIT2084 was very low (10 mg L^{-1}) as well as the small production of EPS by other lactic acid bacteria is the main drawback to its industrial use as a functional ingredient. Considering the direct use of HA that produced by YIT2084, enhancement of HA production by the fermentation method that can be applied to fermentation foods is essential. The EPS production by bacteria is affected by several factors, such as pH, temperature, agitation, aeration and medium composition. The effects of these factors on EPS production are dependent on the experimental conditions and the strain used. Izawa *et al.*[14] revealed that the optimal conditions for EPS production by *S. thermophilus* LY03 is at 42°C and pH 6.2[15,16] also found that the optimal temperature and pH for the production of EPS by *S. thermophilus* 1275 are 40°C and pH 5.5. Moreover, it has been shown that the nitrogen source, such as yeast extract, peptone and whey protein, plays a significant role in the enhancement of EPS production[10,13,14]. Moreover, several researchers[16-18] have reported that the aeration rate and agitation affect the HA production by *Streptococcus equi* subsp., *equi* zooepidemicus, which is a major bacterium for HA production. In this study, to develop industrial applications, we attempted to increase the HA yield by *Streptococcus thermophilus* TH-4 and *Streptococcus thermophilus* BLM 58 using skimmed milk-based medium by controlling pH, temperature, aeration rate and agitation. The present study is designed to investigate physicochemical, molecular as well as nutritional properties of hyaluronic acid to emphasize its potential use as a functional ingredient.

MATERIALS AND METHODS

Chemicals and instrumentation: Standards hyaluronic acid for HPLC analysis and carbazole was obtained from Sigma-Aldrich and all culture media used in this study were supplied by Merck Co.

Microorganisms: The *Streptococcus thermophilus* TH- 4 and *Streptococcus thermophilus* BLM 58 strains were brought from MERCEN (Faculty of Agriculture, Ain Shames University, Cairo, Egypt).

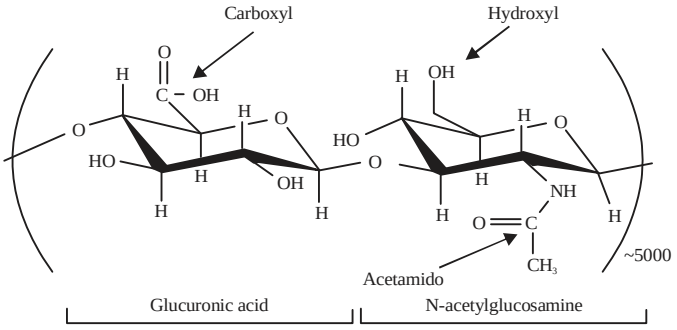

Fig. 1: HA consists of disaccharide repeats of D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) joined alternatively by β-1,3 and β-1,4 glycosidic bonds[2]

Molecular characterization of HA produced bacteria: Genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Germany). The extracted DNA was subjected to PCR amplification using two set of primers specific for detection of both of Has A and Has B genes. The forward primer of Has B was HsB F 5'-ATGTCGGACTCGC-3' and the reverse primer was HsB R 5'-ATATTTTAATCTCTCACAA-3' while the forward primer for Has A was 5'-AATTAATGGGGGAAGA-3' and the reverse primer was 5'-TAGTGTTATGGGTAA-3'. The PCR constituents, 2 µL of bacterial DNA were added to 2.5 µL Taq polymerase buffer 10x (Promega, Madison, USA), 2.5 µL of 25 mM $MgCl_2$, 2 µL of 2.5 mM dNTPs, 2 µL of 20 pmol μL^{-1} of each primer and 0.2 µL Taq polymerase (5 U μL^{-1}) in a final reaction volume of 25 µL. The PCR was performed on polymerase chain reaction cycler (Eppendorf, Germany). The reaction conditions, of 35 cycles, each cycle consisted of denaturation at 94°C for 20 sec followed by annealing at 42°C for 40 sec and extension at 72°C for 1 min. There was an initial delay for 15 min at 95°C at the beginning of the first cycle and 10 min delay at 72°C at the end of the last cycle as a post extension step[19]. The reaction mixture was analyzed by electrophoresis on 1.5% agarose in 1x tris-borate-EDTA buffer, stained with ethidium bromide to be visualized under ultra violet transilluminator[20]. The PCR product was stored at -20°C until use.

PCR product purification, sequencing and sequence analysis: The purification of PCR products was carried out by PCR clean up column kit (Maxim Biotech INC, USA) by following the manufacturer's instructions and then sequenced using a forward primer. Gene sequence was carried out using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and Genetic Analyzer model 3130xl (Applied Biosystems, Foster City, CA, USA). Analysis of nucleotide sequences was performed online using Blast bioinformatic program. In the same time, for sequence alignment, the obtained sequence was aligned with the published ones using Clustal W program (v1.83) (http://www2.ebi.ac.uk/clustalW[21] as well as for phylogenetic analysis MEGA4 program was used[22].

Total RNA extraction and cDNA synthesis: Total RNA was extracted from the bacterium strain TH4 using the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN, Germany). The RNA quantity and quality were determined spectrophotometrically (Eppendorf Biophotometer Plus, Eppendorf, Germany). Moreover, the integrity of the bacterial RNA samples was approached by agarose gel electrophoresis. For the first strand of cDNA synthesis a reaction with volume of 25 µL was performed and the reaction mixture consists of, 2.5 µL of 5x $MgCl_2$ buffer, 2.5 µL of 2.5 mM dNTPs, 4 µL of oligo (dT) primer (20 pol μL^{-1}), 2 µg RNA and 200 U reverse transcriptase enzyme (M-MLV, Fermentas, USA). The RT reaction was performed in a thermal cycler (Eppendorf, Germany) run at 42°C for 1 h and 72°C for 10 min. The cDNA was then stored at -20°C until further use.

RT-PCR and data analysis: The RT-PCR was performed using the SYBR Green PCR Master Mix (Fermentas, USA). The main constituent of the 25 µL mixture, contained 1 µL of 10 pmol μL^{-1} of each primer (HsB forward and reverse), 1 µL of template cDNA (50 ng), 12.5 µL of 2×SYBR green PCR master mix and 9.5 µL of nuclease-free water. Each sample was run in triplicate. The amplification program included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Data acquisition was performed during the extension step. The reaction was performed using a Rotor-Gene 6000 (Qiagen, ABI System, USA). After the 40 cycles, the melting curves were obtained to eliminate the inclusion of non-specific products. The relative expression ratio was accurately quantified and calculated according to Kumar *et al.*[23].

HA production by static flask culture and fermentation optimization: The stock cultures of the two bacterial strains were refreshed by cultivation on de Man Rogosa and Sharpe (MRS) agar medium according to Livak *et al.*[24]. For hyaluronic acid (HA) production, the bacterial strains *Streptococcus thermophilus* TH-4 and *Streptococcus thermophilus* BLM 58 were cultivated on MRS broth medium for 24 h, 200 rpm at 37°C according to Mozzi *et al.*[13] followed by re-inoculation on skim milk medium under the same conditions. For Hyaluronic Acid (HA) production, the bacterial strains *Streptococcus thermophilus* TH-4 and *Streptococcus thermophilus* BLM 58 were cultivated in MRS broth medium (dipotassium hydrogen phosphate 2 g L^{-1}, glucose 20 g L^{-1}, magnesium sulfate heptahydrate 0.2 g L^{-1}, manganous sulfate tetrahydrate 0.05 g L^{-1}, meat extract 8 g L^{-1}, peptone 10 g L^{-1}, sodium acetate trihydrate 5 g L^{-1}, triammonium citrate 2 g L^{-1}, yeast extract 4 g L^{-1} and pH 6.2±0.2). The strains cultivated for 24 h, 200 rpm at 37°C.

Isolation and purification: Isolation and purification of HA were performed using the method of Mozzi *et al.*[13]. Proteins were removed by using trichloroacetic acid, followed by collecting the HA from the supernatant using ethanol precipitation and the precipitate was dialyzed three times against ultrapure water.

Spectrophotometric analysis: According to the method reported by Izawa *et al.*[25], HA solution was analyzed using carbazole in absolute ethanol and calculated of HA based on a standard curve obtained using different HA concentrations (5, 10, 15, 20 and 25 μg mL^{-1}).

HPLC analysis: The HA was purified and dissolved in 0.1 M $NaNO_3$ according to Oueslati *et al.*[26]. The HPLC (Agilent 1000) analysis of hyaluronic acid was performed on a shodex OHpak SB-806MHQ×2 column (7.8×300 mm I.D), using mobile phase (0.1 M) $NaNO_3$ solution. A flow rate was 0.8 mL min^{-1} and the sample volume injected was 20 μL. The refractive index detector was used to detect the output signals at 280 nm.

Detection of hyaluronic acid by TLC: Hyaluronic acid standard solution (1 mg mL^{-1}) was prepared in 1 mL water and spotted on the silica gel-60 TLC 3×5 cm aluminum plates (Merck, Germany). Spotting volume of standard and samples was always 1 μL, the solution was diluted when necessary. The plate was air-dried for approximately 30 min and then irrigated with the solvent system that consisted of n-butanol/formic acid/water, 4:8:1 (v/v) in the ascending direction in a tight container. The solvent was allowed to move upward about 12.5 cm for approximately 90 min. The plate was removed from the tank, air-dried for 30 min, sprayed with diphenylamine-aniline-phosphoric acid reagent (1 mL of 37.5% HCl, 2 mL of aniline, 10 mL of 85% H_3PO_3, 100 mL of ethyl acetate and 2 g diphenylamine) for 3 sec and finally heated at 110-150°C for 10 sec. The location of each spot on the plate is then represented numerically by calculating a retention factor (Rf)[27]:

$$Rf = \frac{a}{b}$$

where, a is measurement of the distance from the starting point to the center of the spot on the TLC plate (distance a) and b is measurement of the distance from the starting point to the solvent front (distance b).

Fourier transform infrared spectroscopy: The FT-IR analysis of HA was carried out[28]. Dry powder of HA was subjected to IR spectroscopy (Shimadzu, Japan), using the KBr pellet technique. Sample was mixed with 99 parts of dried KBr and then compressed to prepare a salt disc (3 mm diameter). The absorption was read between 500 and 4500 cm rang, at a resolution[29] of 4 cm.

Scaninning electronic microscope: The morphology of the HA was visualized using scanning electron microscopy with 15 kV (JEOL SEM, GSM-6610LV, Japan) and the cross-sections of HA were coated with gold, then observed and photographed on a QUANTA 200 Scanning Electron Microscope (SEM)[30].

X-ray diffraction: Characterization was carried out with the x-ray diffraction (Shimadzu xRD 7000 x-ray diffractometer, Japan) using a DRON 2.0 diffractometer with Cu tube (Kα radiation). The data was collected[31] at 2θ between 0-40°.

Antioxidant capacity: The antioxidant activity of HA production was detected according to Huang *et al.*[32] with slight modifications. Solution of DPPH was freshly prepared by dissolving 1 mg mL^{-1} of HA and followed by different dilutions to get the concentrations of 800, 700, 600, 500, 400, 300, 200 and 100 μg mL^{-1}. Equal volumes of each dilution and 1 mg mL^{-1} methanolic solution of DPPH (1,1-diphenyl-2-picryl-hydrazyl) were mixed together and incubated for 30 min at 25°C in the dark. The absorbance was measured at 517 nm using UV-visible spectrophotometer. The inhibition percentage was calculated using equation:

$$\text{Inhibition (\%)} = \frac{\text{A of control-A of sample}}{\text{A of control}} \times 100$$

Whilst IC_{50} values were estimated from the percentage of inhibition versus concentration plot, using a non-linear regression algorithm.

Preparation of HA fortified yogurt: Pasteurized milk (90°C/20 min), cooled to 43°C then inoculated with yogurt starter culture; Yo-Mix 495 containing (*S. thermophilus* and *L. bulgaricus*) obtained from Danisco to be used as (control). Different concentrations from HA was added (15, 25 and 50 mg/100 mL) as T15, T25, T50, respectively. The inoculated milk was incubated in water bath at 43°C until pH reached 4.55 (Approximately 5 h). The product was kept refrigerated (4°C) for further analysis.

Texture analysis of HA fortified yogurt: Instrumental Texture Analysis (TPA) has been applied as a useful method to evaluate mechanical properties in a wide range of foods[33]. The TPA compresses a piece product twice imitating the conditions in the mouth[34]. Five basic textural parameters may be obtained in only one test (hardness, cohesiveness,

springiness, adhesiveness and gumminess), extracted from the resulting force-time curves. Texture profile analysis was carried out by the method described by Szczesniak[35]. The data obtained from typical texture profile curve was used for the calculation of the textural parameters[36,37] as follows:

Cohesiveness = Area under 2nd compression (area 2)/ area under 1st compression (area 1)
Hardness (g) = Maximum force of the 1st compression
Adhesiveness (g.s) = Negative area in the graph (area 3)
Springiness = Length 2/length 1
Gumminess (g) = Hardness × cohesiveness

Sensory evaluation: The sensory evaluation was carried out by a panel consisting of 10 graders, including members of Food Technology Department, Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technological Applications (SRTA-City). Each panel member assessed the yogurt samples separately, taking into account the following features; flavor (odor and taste), color, texture and appearance. The scale was used from 1-10, where 10 is the best. The grader was asked to give the functional yogurt products an overall acceptance grade out of (10) as well. The sensory evaluation procedure was modified from the method described by Bourne[38] and the taste panel was carried out on the second day of the yogurt manufacturer. The average of sensory evaluation data with standard deviations was determined.

Statistical analysis: The data of this study were analyzed by Duncan's analysis with $p<0.05$ to identify the significant differences among all parameters on the study.

RESULTS AND DISCUSSION

Molecular characterization of HA gene: *Streptococcus thermophilus* TH-4 and *Streptococcus thermophilus* BLM 58 strains were subjected to PCR amplification for detection of HasB gene using specific primers. Data presented in Fig. 2 revealed that, amplicon of 400 bp was amplified with strain *S. thermophilus* TH-4 and no PCR product was obtained with the strain *S. thermophilus* BLM 58. Ogden[39] amplified two fragments from *S. thermophilus* with molecular sizes 750 and 320 bp. Moreover[40], researchers amplified Has A and Has B from *S. zooepidemicus* and reported that mutation in these genes affect the production of HA and also on the bacterial growth as well. The same observation was recorded by Zhang *et al*.[41] that HA produced by an operon in *S. zooepidemicus*[42-44] previously discovered the same operon in *A. streptococci*.

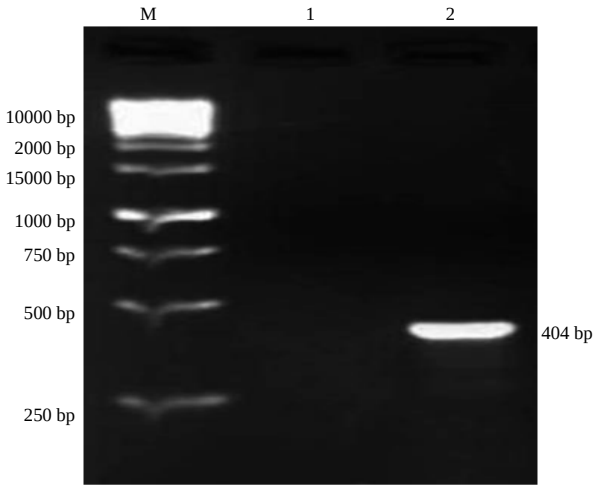

Fig. 2: PCR amplification of hyaluronic acid (Has B) gene using specific PCR primers. M: 1 kbp DNA ladder, Lane 1: *Streptococcus thermophilus* BLM 58, Lane 2: *Streptococcus thermophilus* TH-4

Sequencing and sequence analysis: When the PCR product was subjected to DNA sequencing and by using the DNA Blast N alignment, results showed that the sequence is Has B with similarity 90% (Fig. 3b). The obtained DNA sequence was deposit in GenBank under accession number (KT069220). The phylogenetic tree was constructed for the obtained DNA sequence in comparison with Has A, Has B and Has C genes belong to another *Streptococcus* sp., strains (Fig. 3a). Results presented in Fig. 3 revealed that the isolated gene was highly closely similar to Has B and Has C genes (cluster I). Moreover, in the case of phylogeny which constructed based on the deduced amino acids, the isolated gene was closely related to Has A of *S. equi*. The Has A gene was aligned with what we obtained and the results presented in Fig. 3a and b showed a high similarity between the two genes which confirm that ours is an interface between Has A and Has B genes. It was reported that bacteria has HA synthase in an operon called (has), this operon is responsible for the production of HA precursors and there are two families of (has) operon, family from *Streptococcus equi* subsp., *zooepidemicus* and *Streptococcus equi* subsp., *equi*[45,46] isolated and sequenced the HA from the group of *Streptococcus pyogenes* and they found that significant homology between the HA synthase and the rhizobium nodC gene. This finding agrees with results obtained in this study and explained the high similarity between the Has gene of *S. thermophilus* with Has A of *S. pyogenes.*

For detection of the amount of gene expression of the Has B gene in the selected bacterium, real time PCR was performed using the specific Has B primers and the results

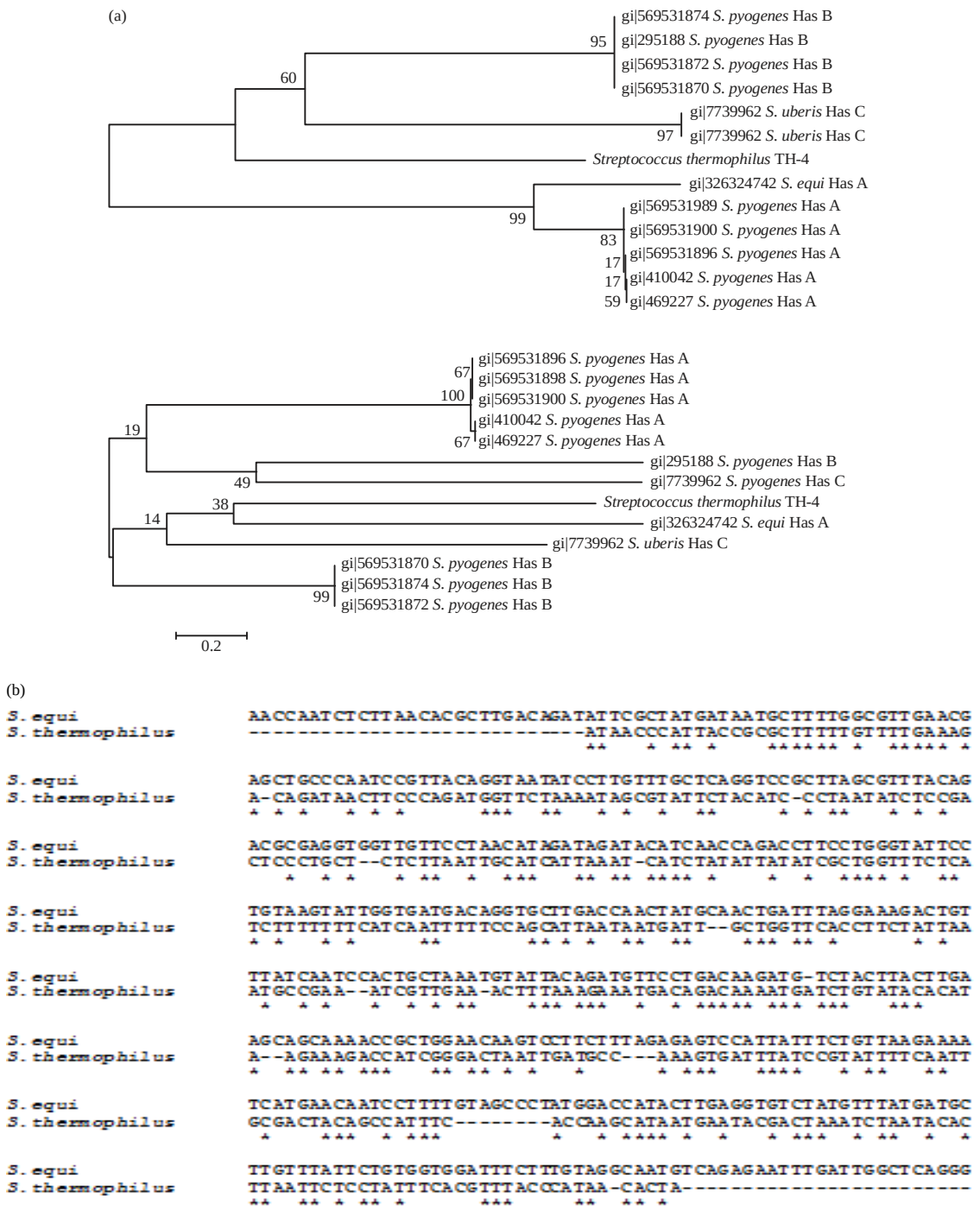

Fig. 3(a-b): (a) Phylogentic tree for the obtained Has B gene compared with the others Has A, B and C genes amplified from different strains of *Streptococcus* sp. The phylogeny was constructed based on the deduced DNA sequence by Mega4 program and (b) Sequence alignment between the Has B gene of *Streptococcus thermophilus* TH-4 and *Streptococcus equi* Has B gene. The sequence was performed based on the DNA nucleotide sequence and using Clustal W program 1.6

were compared with the expression of the 16S rRNA gene as a reference gene. Data revealed that the expression of the Has B gene was high when compared to the housekeeping gene. The expression rate of the Has B gene was 42% more

Table 1: Determination of hyaluronic acid in *Streptococcus thermophilus* TH-4 and *Streptococcus thermophilus* BLM 58

	Concentration of hyaluronic acid			
	Control (mg mL^{-1})		Fermentation (mg mL^{-1})	
Strains	Colorimetric	HPLC	Colorimetric	HPLC
Streptococcus thermophilus TH-4	0.430±0.12	0.461.20±.007	1.34±0.02	1.73±0.01
Streptococcus thermophilus BLM 58	0.556±0.14	0.583.32±0.07	2.45±0.01	2.95±0.02

All samples were carried in triplicates, the numbers were presented as the mean of triplicates ±SD

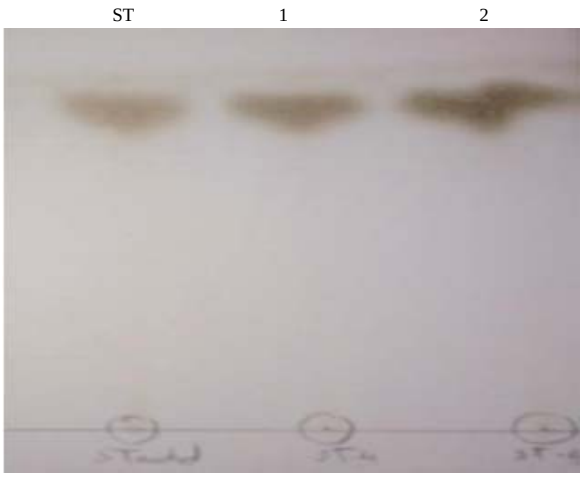

Fig. 4: TLC chromatogram of hyaluronic acid produced by the two examined bacteria. Lanes: ST: Standard hyaluronic acid, Lane 1: HA from *Streptococcus thermophilus* TH-4 and Lane 2: HA from *Streptococcus thermophilus* BLM 58

than the housekeeping gene. Falaleeva *et al.*[47] used the real time PCR to study the expression of Has B and Has C gene compared with the HA production by *Streptococcus pyogenes*. They found a correlation between the expression of the Has operon genes and the HA production[48].

Detection of hyaluronic acid using TLC and colormetic methods: The TLC chromatogram presented in Fig. 4 revealed that both of the bacterial strains capable producing a reasonable amount of HA. The band thickness on TLC for the two examined strains showed *Streptococcus thermophilus* BLM 58 produce more HA than *Streptococcus thermophilus* TH-4 compared to the standard which showed faint band. These data are highly matched and subsequently, confirm the data obtained by colorimetric and HPLC detection which revealed the same results.

Production conditions of HA from bacterial strains: The stock cultures of the two bacterial strains were refreshed by cultivation on de Man Rogosa and Sharpe (MRS) agar medium for 24 h according to Livak and Schmittgen[24]. The concentration of HA produced by the two bacterial strains was determined using the colorimetric carbazole method and the results revealed that; *Streptococcus thermophilus* TH-4 produced HA with a concentration of 0.430±012 mg mL^{-1}. However, *Streptococcus thermophilus* BLM 58 produced the HA with a concentration of 0.556±0.14 mg mL^{-1} (Table 1). It is emphasizing that two strains able to produce the considerable amount of HA, whereas, the *Streptococcus thermophilus* BLM 58 producing HA more than *Streptococcus thermophilus* TH-4. On the other hand, the fermented bacteria showed high production of HA when compared with the control, this productivity which analyzed with HPLC was increased with percentage 74 and 80% by *Streptococcus thermophilus* TH-4 and *Streptococcus thermophilus* BLM58 bacterial strains, respectively (Fig. 5). Kakehi *et al.*[49] reported that HPLC method is very efficient and can determine HA in the range 0.5-50 μg with high reproducibility. These results are by Vazquez *et al.*[50], who obtained 90.5 mg L^{-1} when cultivated the *Streptococcus thermophilus* on the skim milk medium. It is reported that high production of HA was obtained by complete residual media (2.46 g L^{-1}) and they found that using marine media could reduce the production costs by more than 50%[24,51]. The medium constituents also affect the production HA; this finding was obtained by Vazquez *et al.*[52], who reported that 3.03 g L^{-1} had been achieved in commercial (medium) this amounts of HA was decreased when different peptone sources were used in the batch medium. On the other hand, researchers used the oxygen with the lactic acid bacteria and found that the HA production was increased[53] 0.73%. However, when these strains were treated with N-methyl-N'-nitro-N-nitroso-guanidine (NTG), the production of HA was maximized many folds than the control.

FT-IR: The FTIR spectroscopy is a powerful method for the identification of functional groups and organic compounds by evaluating the transitions between vibrational states of bonds contained within the molecule. The spectrum of FT-IR showed the presence of 7 different peaks (Fig. 6). These peaks ranged

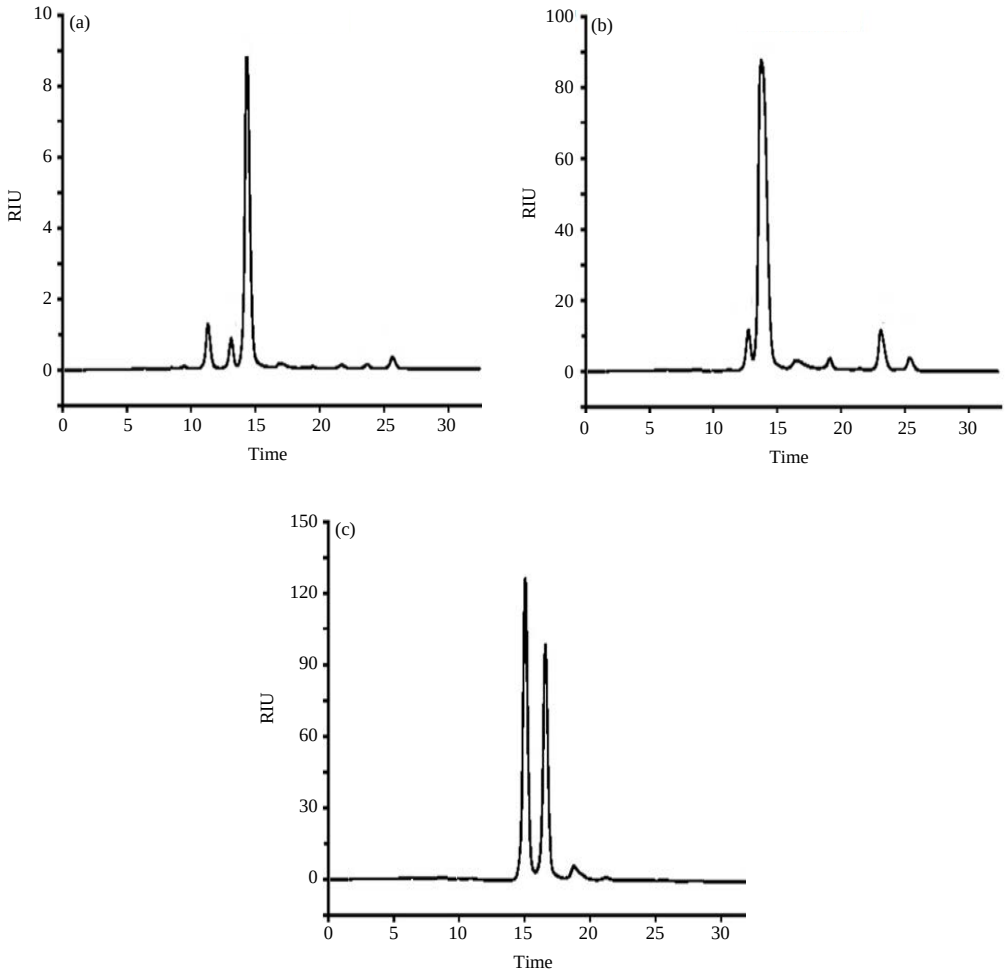

Fig. 5(a-c): HPLC chromatogram of the hyaluronic acid (HA), (a) Standad HA, (b) HA produced by *Streptococcus thermophilus* TH- 4 and (c) HA produced by *Streptococcus thermophilus* BLM 58. The peak of HA was identified by comparison of retention time

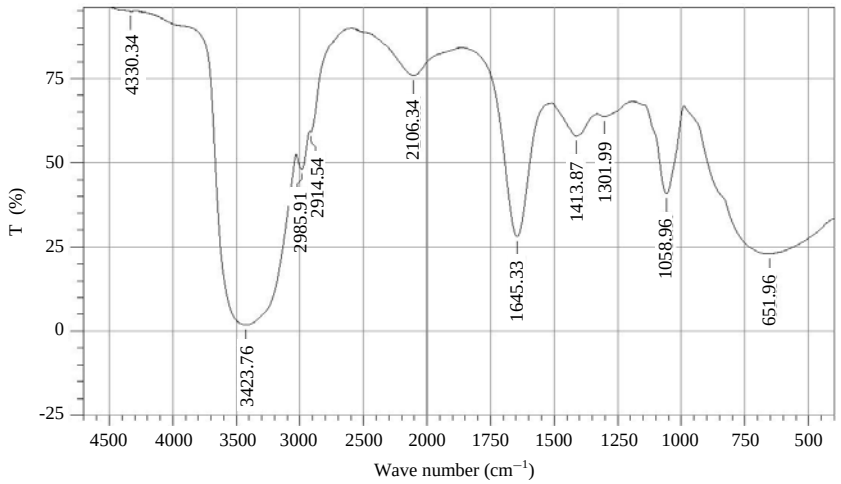

Fig. 6: FT-IR chromatogram of HA

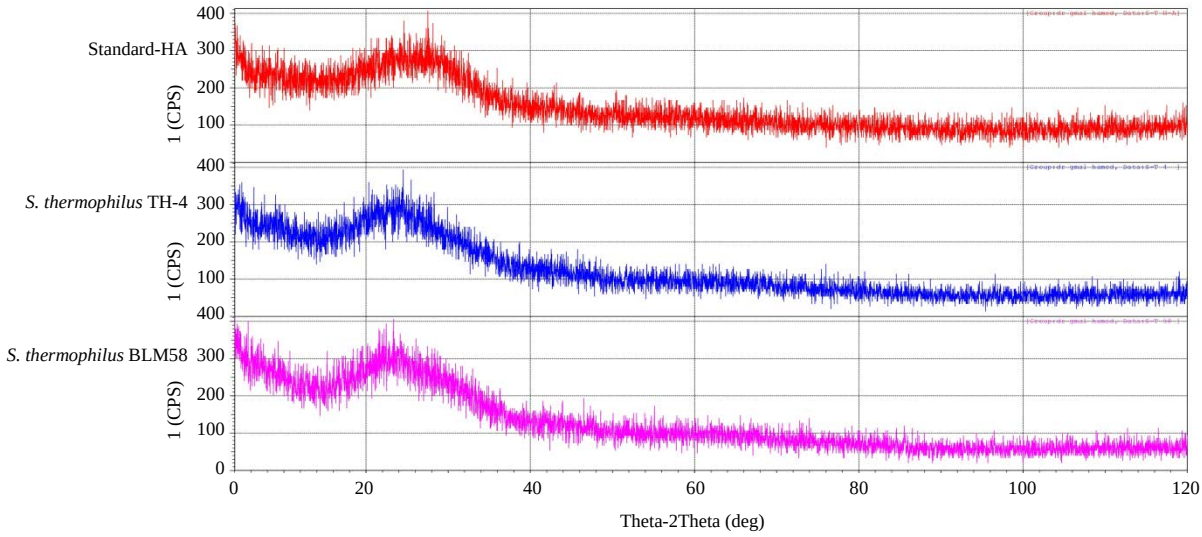

Fig. 7: X-ray diffraction patterns of the three HA: Standard, *Streptococcus thermophilius* TH-4 and *Streptococcus thermophilius* BLM58

Table 2: Antioxidant capacity of HA

HA (μg mL^{-1})	Inhibition (%)	IC_{50} (μg mL^{-1})	Ascorbic acid (μg mL^{-1})	Inhibition (%)	IC_{50} (μg mL^{-1})
100	16.88±0.37	493.58±0.48	10	38.32±0.42	25.46±0.36
200	24.91±0.52		20	39.76±0.39	
300	33.89±0.58		30	58.92±0.28	
400	44.55±0.29		40	82.75±0.36	
500	50.65±0.53		50	93.75±0.29	
600	54.49±0.48		60	129.82±0.24	
700	68.26±0.56		70	130.06±0.38	
800	79.16±0.37		80	132.57±0.42	

All samples were carried out in triplicates; the numbers were presented as the mean of triplicates ±SD

from 651-3423 with intensities ranged from 1.843 into 75.822. Also, the band width differed from band to another based on their position and the band area ranged from 25.174-700.315. The HA showed several peaks such as at 551.96 and 1058.96 that could be due to the C-O-C stretching[46]. The peak at 1413.87 is corresponding to the presence of C-O group with C=O combination and the peak at 1645.33 is indicating the presence of amid group. The peak at 2914.54 is presenting the presence of C-H stretching, 3423.76 presenting O-H stretching. The FT-IR analysis confirm the structure of HA as consists of disaccharide repeats of D-glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc) joined alternatively by β-1,3 and β-1,4 glycosidic bonds. The FT-IR band pattern of the produced HA the same profile obtained by standard[54,55]. While all of the small hydrogen atoms occupy the less sterically favorable axial positions. Thus, the structure of the disaccharide is energetically very stable[56].

X-ray diffraction: Data presented in Fig. 7 showed the x-ray diffraction of the HA, produced from *S. thermophilus* TH4 and *S. thermophilus* BLM58 strains against the standard. The x-ray diffraction profiles revealed the identity structure of the examined two samples and standard which confirms the non-crystalline structure of HA and its appearance as semi-hydrated fibers. The obtained results agreed with the results of Dumitriu *et al.*[31].

Scanning Electron Microscope (SEM): The HA appeared in scanning electron microscope as a sponge with large porous. Moreover, the porosity of the surface due to the presence of huge numbers of fibers, these fibers are swollen and blinded ended and are similar to the fungus hyphae (Fig. 8). The SEM indicated that the HA consists of many layers of fibers, these fibers are cylindrical and have blind ended. The same observation was reported by Necas *et al.*[57].

Antioxidant capacity and cytotoxicity: The HA showed an acceptable level of antioxidant capacity with IC_{50} (493.58 μg mL^{-1}) compared to ascorbic acid (as standard) (IC_{50} = 25.46 μg mL^{-1}) (Table 2). The antioxidant capacity of hyaluronic acid declared its health benefits and the possibility of its use as a food supplement.

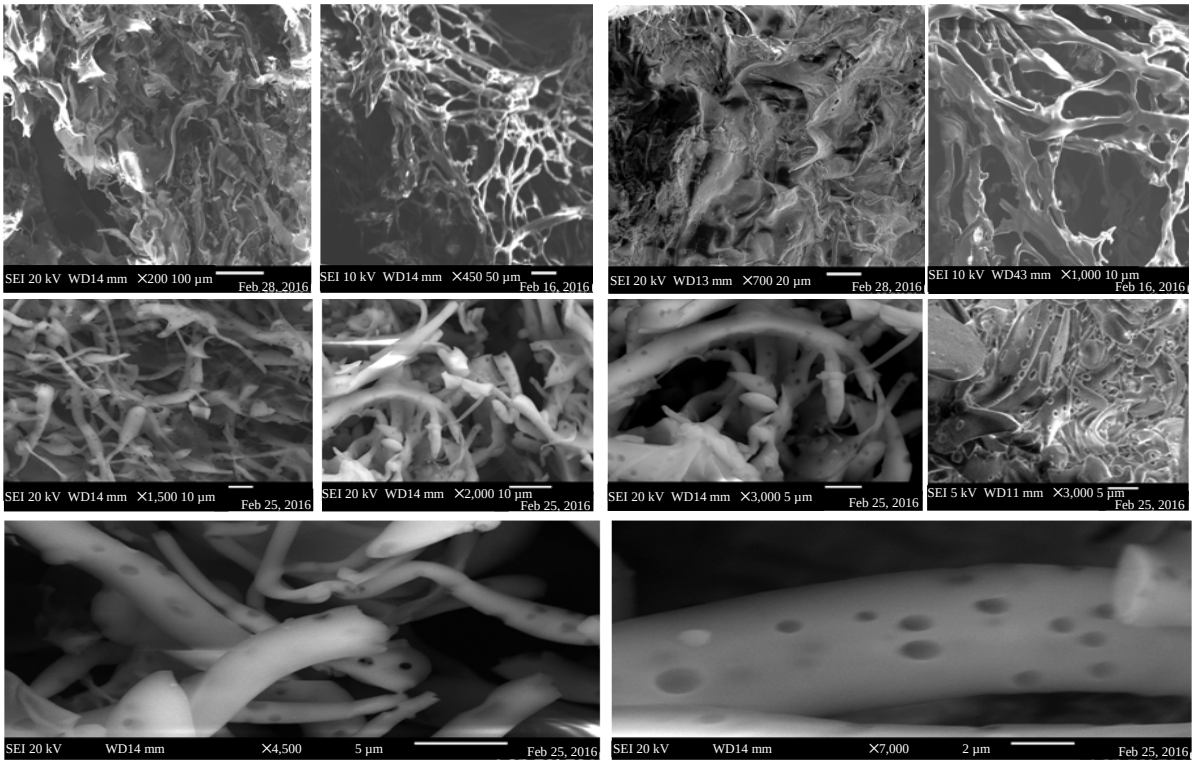

Fig. 8: Scanning Electron Microscope (SEM) examination of HA using different magnification starting from 200-7000x

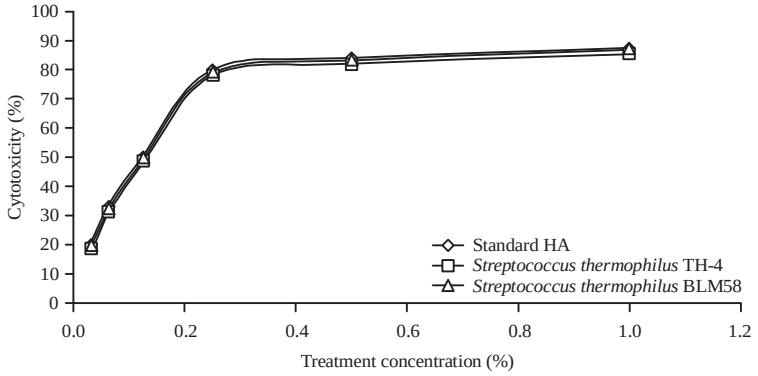

Fig. 9: Cytotoxicity of hyalorunic acid

The cytotoxicity experiment did not show any toxic effect of HA on the living cells which is confirm the safety of using it as a food supplement without any health risk (Fig. 9). Rangaswamy and Jain[58] investigated the possible cytotoxic effects of HA and found that a hyaluronic acid is not cytotoxic and showed an excellent biocompatibility. These results in agreement with what obtained by Jansen *et al.*[59]. These obtained results emphasize the functional properties and health benefits of HA. It is encouraging the use of HA as a food supplement in food processing and industry.

Rheological properties of HA fortified yogurt: The HA-fortified yogurt in different concentrations (Fig. 10) was investigated n the level of overall acceptance and rheological properties. Yogurt rheology is described not only regarding viscosity but is also described concerning how hard, brittle, elastic and cohesive it is. Textural characteristics and rheological properties of coagulated dairy products are affected by their structural characteristics. The structural arrangement of the network determines the textural characteristics of these coagulated products and is affected by

Fig. 10: Prepration of yogurt treatment with or without HA to test fortified yogurt: Control (yogurt without HA treatment), T-15 (yogurt with HA treatment 15 mg/100), T-25 (yogurt with HA treatment 25 mg/100), T-50 (yogurt with HA treatment 50 mg/100)

Table 3: Texture analysis of HA-fortified yogurt

Samples	Hardness (g)	Adhesiveness (g.mm)	Cohesiveness	Springiness (mm)	Gumminess (N)	Chewiness (mJ)
C	29	45.62	0.60	3.61	17	62.81
T15	27	31.64	0.72	3.85	19	73.75
T25	34	30.66	0.62	3.83	21	81.34
T50	49	49.48	0.41	3.97	20	79.99

Table 4: Organoleptic properties of HA-fortified yogurt

Samples	Color (10)	Odor (10)	Taste (10)	Texture (10)	Appearance (10)	Overall acceptance (10)
Control	5.0±0.115^{c}	6.0±0.145^{c}	5.5±0.145^{c}	4.3±0.115^{c}	4.8±0.057^{c}	4.9±0.057^{d}
T15	6.5±.0.145^{b}	7.0±0.088^{b}	6.6±0.088^{b}	4.9±0.115^{b}	6.4±0.057^{b}	7.1±0.057^{b}
T25	7.5±0.230^{a}	7.6±0.120^{a}	7.5±0.152^{a}	6.6±0.176^{a}	6.5±0.088^{a}	7.7±0.115^{a}
T50	6.5±0.185^{b}	7.0±0.057^{b}	7.0±0.057^{ab}	5.3±0.057^{b}	6.2±0.057^{b}	6.0±0.115^{c}

Means with the same letter are not significantly different according to Duncan's-each value represented the mean of three replicates ±SD

factors such as composition and manufacturing processes[60]. Force measuring instruments like the texture analyzer to allow food materials to be evaluated in such terms and have been used to assess yogurt texture[61,62]. Hardness is a commonly evaluated parameter when determining yogurt texture. A hardness of yogurt increased with growing of HA concentration from 27 for 15 mg to 49 for 50 mg HA (Table 3). The hardness of the yogurt increased with increasing of HA concentration and has been attributed to sulfhydryl groups of HA[63]. The presence of HA enhanced the formation of gel during fermentation of milk, thus increased the hardness. Adhesiveness is the force necessary to remove the material that adheres to the mouth during eating. The maximum adhesiveness was appeared with the 50 mg of HA. An important characteristic of texture properties is the negative area between the first and second compression cycle which corresponds to adhesiveness of product. Cohesiveness represents how well the product withstands a second deformation relative to how it behaved under the first deformation. The results showed that the cohesiveness of HA-fortified yogurt was decreased with increasing of HA (from 0.72-0.41). Springiness is the rate at which the sample returns to its original shape when the deforming force is removed. The texture data not appeared any significant differences in springiness, gumminess and chewiness (Table 3). From the texture results it can conclude that the fortification of the yogurt with HA has no any adverse effect on the rheological properties.

Sensory evaluations: As illustrated in Table 4, fortification of yogurt with hyaluronic acid enhanced all sensory parameters; color, odor, taste, texture and appearance that positively

affected on overall acceptance, where the three treatments achieved significantly higher scores compared to the control. Thse obtained results showed the dueal benefits of hyaluronic acids when improved the nutritional and functional properties of yoguat and overall acceptance for the consumers.

CONCLUSION

The present study emphasized that Hyaluronic Acid (HA) can be naturally produced in considrible amount from *Streptococcus thermophilus* BLM 58. The HA showed high antioxidant activities (IC_{50} = 493.58 μg mL^{-1}) with no cytotoxicity. The fortification of yogurt with HA do not appear any adverse affect the rheological and organoleptic properties. Finally, we can conclude that the hyaluronic acid can be safely used to develop the nutritive value of food products (such as yogurt) as a functional food supplement.

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