Journal of Research in Biology

An International Scientific Research Journal

ISSN No: Print: 2231 –6280; Online: 2231- 6299

Original Research

Comparative evaluation of hyaluronic acid production by *Streptococcus thermophilus* isolated from yoghurt

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ABSTRACT:

Hyaluronic acid (HA) is also known by the name hyaluronan. The necessity for using this fabulous material lead to investigate non-pathogenic strains which produce this material. The most non-pathogenic strain is *S. thermophilus*. The lack of literature on microbial production of this substance by the strain prompted us to examine the microbial production of HA from it and also to examine optimization of culture conditions where HA is produced. The bacteria *Streptococcus salivarius sub. thermophilus* was obtained from the Bank of Scientific and Industrial Research of Iran (PTCC 1738). To separate *S. thermophilus* strains from yogurts, three types of yogurts were used. They were cultured by pour-plate and surface methods on STA medium. To identify the isolated strains, biochemical tests and Polymerase Chain Reaction (PCR) were used. Bacterial strains isolated from yoghurts were identified as *S. thermophilus* MN-BM-A02, *S. thermophilus* JIM8232 and *S. thermophilus* MN-ZLW-002. To separate the capsule strains, each strain was cultured on STB medium and then they were centrifuged. In order to purify the samples, ethanol and charcoal were used. To optimize production, variety of sources of carbon, nitrogen, temperature and pH were studied.

Keywords:
Hyaluronic acid, *Streptococcus thermophilus*, FTIR.

Article Citation:
Diba Tabatabaei and Abbas Akhavan Sepahy
Comparative evaluation of hyaluronic acid production by *Streptococcus thermophilus* isolated from yoghurt
Journal of Research in Biology (2017) 7(6): 2342-2354

Dates:
Received: 09 July 2017 **Accepted:** 21 Aug 2017 **Published:** 27 Sep 2017

Web Address:
http://jresearchbiology.com/documents/RA0620.pdf

Journal of Research in Biology

INTRODUCTION

Hyaluronic acid (HA) is an unbranched polysaccharide with high molecular weight, composed of D-glucuronic acid and N-acetylglucosamine disaccharide units, linked alternately by β-1-3 and β-1-4 glycoside bonds. Owing to its unique hydrodynamic properties, HA has been widely applied in the cosmetic and medical fields (Boeriu *et al.,* 2013). *Streptococci* 'A' and 'C' are a group of prokaryotes which synthesize HA as an extracellular capsule (Naoki and Tomoko, 2008). *Streptococci* are gram-positive bacteria whose main fermentation product is lactate [Lactic Acid Bacteria (LAB)]. The homo lactic metabolism accounts for the conversion of more than 90% of sugars into lactate, while in the mixed acid metabolism, high amounts of formate (in an anaerobic environment), acetate, and ethanol are produced (Oliveira *et al.,* 2013). Common to all microbial syntheses of oligosaccharides and polysaccharides, the production of HA is a carbon-intensive and energy-intensive process (Necas *et al.,* 2008). Precursors for the HA synthesis (Uridine diphosphate–glucuronic acid and Uridine diphosphate–Nacetylglucosamine) are also precursors for the cell wall biosynthesis, specifically peptidoglycan, teichoic acids, and antigenic wall polysaccharides. Therefore, the HA synthesis competes with the cell growth for carbon source and energy.

Bacteria may alter cell metabolism and consequently, the direction of carbon flux in response to environmental fluctuations. Hence, the analysis of the metabolic changes represents a valuable tool, which potentially contributes to the understanding of how to manipulate the culture conditions of an organism, in order to improve the production of interesting substances (Kogan *et al.,* 2007). Since the 1990s, a myriad of metabolic studies have been focused on the effects which nutrition, and culture conditions exert upon the production of HA by *Streptococcus zooepidemicus*, such as carbon source, carbon-to-nitrogen ratio, initial glucose concentration, pH, agitation, aeration, and temperature (Liu, 2011).

HA properties

In physiological solution, HA showed conformational stiffness due to β-glycosidic bonds, internal hydrogen bonds, and solvent interaction. Thus, HA obtained an extended coil structure in solution possessing a very large domain. This, in combination with a high molecular weight, makes even weaken HA solution act in an exceedingly non-Newtonian, gel-like way. Customarily, the chain interactions in charge of this character were thought to be random coil entanglement. Recent information, recommended that HA molecules receive anti-parallel, β-sheet-like stable tertiary structure. The rheological properties depend emphatically on molecular weight and concentration (Necas *et al*., 2008).

Microbial HA production

HA can be obtained from rooster combs or by microbial fermentation. In rooster combs, HA is joined with proteoglycans making the confinement of high purity, high molecular weight HA costly (O'Regan *et al*., 1994). In addition, the utilization of animal based biochemicals for human therapeutics is being met with developing restriction in light of the danger of cross-species viral and opportunistic pathogen contamination. Consequently, microbial production is continuously replacing extraction as the favored source of HA. HA is produced as an extracellular capsule by pathogenic Lancefield group A and C *Streptococci*. Under the magnifying lens, these non-sporulating and non-motile microscopic organisms showed up as circular or ovoid cells that are normally typically spherical or chains encompassed by a broad extracellular capsule. Several *Streptococci*, such as *S. equisimilis*, *S. pyogenes* and *S. equi*, have HA synthase and produce HA (Pires *et al.,* 2010). Industrial HA production has been achieved using *S. equisimilis* subsp. *zooepidemicus*.

However, these strains, categorized as

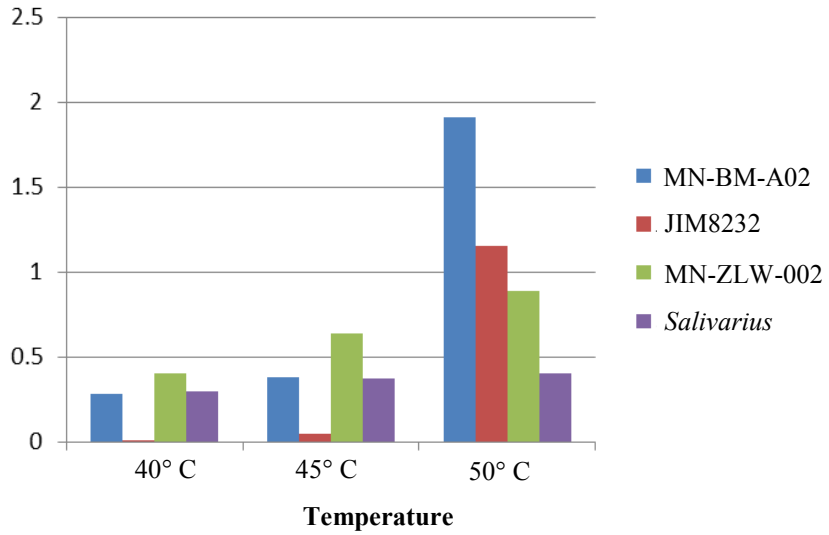

Figure 1. The effect of different temperature on EPS production

Lancefield groups A and C are pathogenic (Liu, 2011). *S. thermophilus* is traditionally used in the dairy food products, such as yogurt and cheese. *S. thermophilus* has been reported to produce various types of EPS with different monomer compositions (Vaningelgem *et al.,* 2004). LAB are broadly used for the production of fermented foods. In fermented dairy products, Exopolysaccharides (EPS) produced by LAB contribute to viscosity and decrease the susceptibility to syneresis. However, no clear correlation has been found between EPS concentration and viscosity of the fermented milk. Production of EPS by *Streptococcus thermophilus* is generally growth rate associated, and the influence of environmental factors such as temperature, pH, carbon source and its concentration, and carbon to nitrogen ratio, all are strain dependent (Naoki and Tomoko, 2008).

METHODS

Microorganisms

Streptococcus salivarius subsp. *thermophilus* (PTCC 1738) was obtained from the Ministry of Science, Research and Technology Organization of Scientific and Industrial Research of Iran (IROST) as a lyophilized culture kept in ampoules. The following strains isolated were isolated used throughout this study: *S. thermophilus* MN-ZLW-002, *S. thermophilus* JIM8232, *S. thermophilus* MN-BM-A02 isolated from home made yogurt and Persian industrial starter culture (Damdaran and Pak industries) which are located in Iran.

Gram's staining and biochemical tests

Gram staining was performed following the method of Cappuccino (2004). Gram positive cocci was observed in this study. In this experiment, the enzymatic properties such as the ability to ferment sugars were studied. To evaluate the ability to ferment sugars, Oxidative and Fermentative (OF) and Phenol red broth

Table 1. The primers used in this study (RD_1 and FD_1) for PCR

Name	Sequence	Temperature (°C)	Reference
RD_1	3'AGAAAGGAGGTGATCCAGCC5'	57	Weisburg *et al.* (1991)
FD_1	3'GAGTTTGATCCTGGCTCAG5'	57	

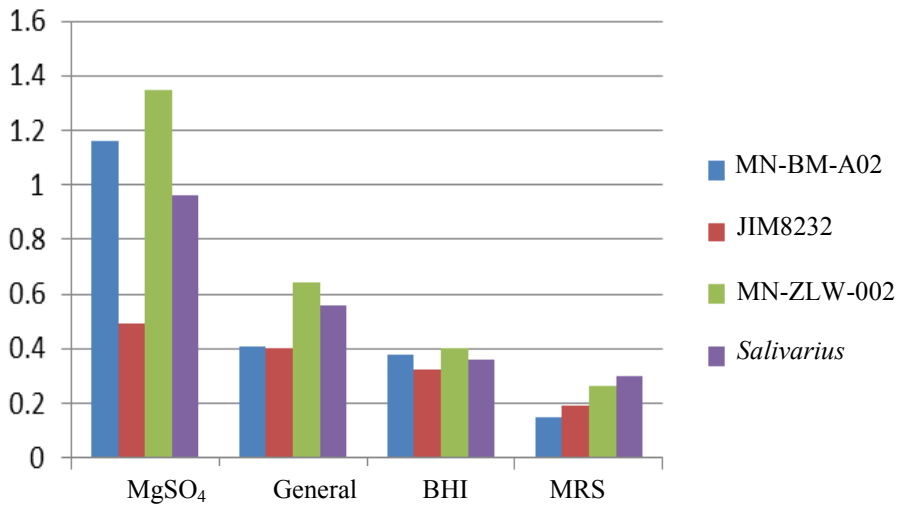

Figure 2. The effect of different media on EPS production

media were used (Murray *et al*., 2003). *Streptococcus* strains that were grown at 42°C, were able to produce acid from fructose, glucose, lactose, mannose and saccharose. None of these strains were able to produce acid from arabinose, maltose, mannitol, rhamnose, sorbitol and glycerol (Murray *et al*., 2003).

Arginine hydrolysis

To evaluate the ability of arginine hydrolysis, the samples were inoculated in the media containing 1% amino acid arginine (Murray *et al*., 2003). None of the strains were able to produce ammonia from arginine.

Salt tolerance

Mannitol salt agar was used for this test (Vos *et al*., 2009). The results showed that none of these strains were able to grow with 6.5% salt. Based on the above preliminary studies, the strains isolated were identified as *Streptococcus thermophilus*. To identify at the subspecies/strains level. PCR amplification was done.

PCR

Bacterial samples were isolated from home made and industrial yogurts (Chekide, Sade and Probiotic) and were identified by Polymerase Chain Reaction (PCR) and gel electrophoresis. Table 1 indicates the primers used for amplifying samples on PCR. Based on the results of PCR, the organisms were identified up to the strain level of *Streptococcus thermophilus*.

Culture maintenance and inoculum preparation

The stock culture was maintained frozen in Trypticase soy broth containing 10% glycerol and glass beads. Stock culture was grown in *Streptococcus thermophilus* broth and were stored at -18°C in 50% glycerol until it is used (Kirsop and Doyle, 1991). To obtain fresh cultures for the experiments, the bacteria were propagated at 42°C for 24h, first in Trypticase Soy broth (Oxoid), followed by two sub cultures in the medium used later (*Streptococcus thermophilus* broth). The inoculum for the sub culture and fermentations experiments were always 1.0 %(v/v).

Isolation of EPS

Grown culture of *S. thermophilus* was centrifuged (8000 rpm, 20 min, 4°C) in order to eliminate the solid biomass. Cell-free culture was filtered first under 0.20 µm. The last retentate was supplemented with ethanol (70%) (Merck, Darmstadt, Germany). After 24h, at 4°C the precipitates were eliminated by centrifugation (12,000 rpm, 30 min, 4°C). One volume of chilled ethanol (99.7%) was added to the supernatant. The solution was kept for 24h at −18°C. Precipitates were recovered by centrifugation (12,000

Table 2. Molecular weight of isolated polymer from the bacterial strains

S. No	Strain	K	a	Solvent	Molecular weight
1	*S. salivarius* subsp. *thermophilus*	0.016	0.841	0.1 M Nacl	1255 Da
2	*S. thermophilus* MN-BM-A02	0.016	0.841	0.1 M Nacl	4307 Da
3	*S. thermophilus* JIM8232	0.016	0.841	0.1 M Nacl	1119.55 Da
4	*S. thermophilus* MN-ZLW-002	0.016	0.841	0.1 M Nacl	6901 Da

rpm, 30 min, 4°C) and dissolved in the hot distilled water. Exopolysaccharide solution was neutralized in 1 M NaOH. Then one volume of sodium acetate (3%) was added to the solution. For more purification, one volume of 80% ethanol was added and kept for 12h on the shaker. The solution was centrifuged (4000 rpm, 30 min, 4°C) and then the precipitates were dissolved in one volume of sodium acetate (3%). Active carbon (charcoal 3%) was added and the solutions were kept on the shaker for 3h. After three hours, the solutions were filtered under 0.20 µm to remove active carbon. For the last step, 85% ethanol was added to the solution for 12h. Precipitates were recovered by centrifugation (4000 rpm, 40 min, 4°C) and dissolved in sodium acetate (3%) and finally freeze-dried (Shene *et al.,* 2008).

Molecular mass estimation - Intrinsic viscosity

A glass capillary viscometer (Cannon-Fenske 75) immersed in a constant temperature water bath at (25°C) was used. Stock solutions (0.5 g of the EPS/l) were prepared by dissolving the powder in NaCl (0.01, 0.05 and 0.1 M) solutions. Twenty milliliters of the EPS solution filtered under 0.20µm were loaded into the viscometer. Relative viscosities (η_{rel}) were calculated by dividing the flow time of the EPS, solutions by that of the solvent. Elution time of each solution was taken as the average of five concordant readings. Dilutions to yield at least five lower concentrations were made by adding the appropriate aliquots of the solvent. Intrinsic viscosity was determined from Huggins (1942).

$$\frac{\eta_{sp}}{C} = [\eta] + K_H[\eta]^2 C$$

$$[\eta] = k(Mw)^a$$

where '*C*' is the mass concentration of the macromolecule in solution

$$\eta_{sp} = \frac{\eta}{\eta_s} - 1 = \eta_{rel} - 1$$

where, 'η_s' is the viscosity of the solvent and 'K_H' is the Huggins' constant and 'η_{sp}' is the specific viscosity of a

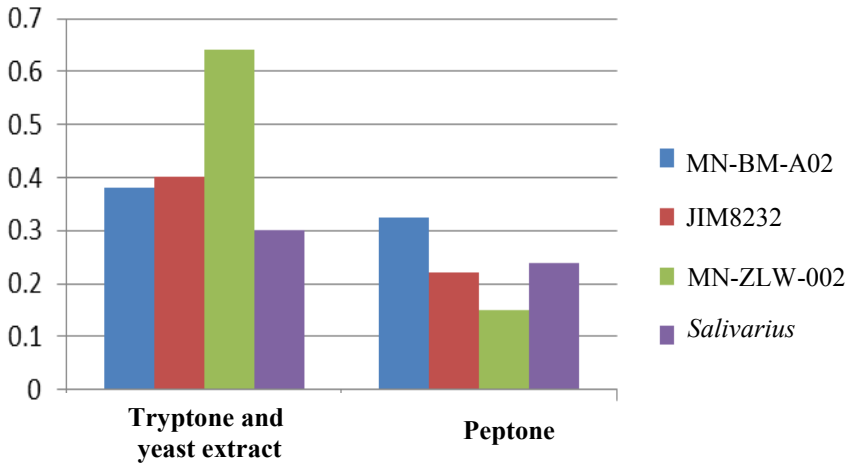

Figure 3. The effect of different nitrogen sources on EPS production

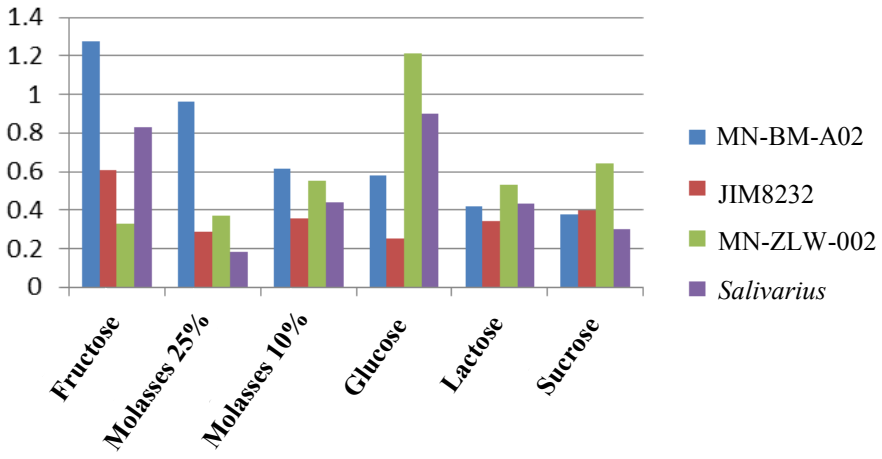

Figure 4. The effect of different carbon sources on EPS production

solution of concentration.

Impact of various factors on the production of EPS

In this study, factors such as temperature, different kinds of media, nitrogen sources, carbon sources and pH on the production of extracellular polysaccharide were studied. All fermentations were carried out in triplicate independent experiments (Zhang *et al*., 2011).

The effect of temperature on EPS production by *S. thermophilus* strains was investigated with *Streptococcus thermophilus* broth at 40, 45 and 50°C (Zhang *et al*., 2011).

The effect of different media on growth and EPS production was analyzed using STA enriched by $MgSO_4.7H_2O$, Brain Heart Infusion broth media (BHI) MRS and nutrient broth (General) following the method of Ashraf and Shah (2011).

The effect of peptone, yeast extract and tryptone as different nitrogen sources on the growth and EPS production was also determined following the method of Vaningelgem *et al*. (2004).

The effect of different carbon sources such as glucose fructose, sucrose, lactose and molasses (10% and 25%) concentration was studied following the method of Vaningelgem *et al*. (2004).

Effect of different pH values (5.5, 6.2 and 7.0) on the growth and EPS production was detected following Zhang *et al*. (2011).

FTIR Spectroscopy

Fourier Transform Infrared spectrum for the polymer was attained in transmittance mode with Thermo Nicolet, Avatar 370 spectrometer to examine diverse functional groups. Compressed discs of 3 mm diameter were prepared by mixing with 2 mg of lyophilized polymer with 200 mg of KBr, and the spectrum was adjusted for KBr background. The pellets were then scanned in the range of 4000–500 cm^{-1} with a resolution 4 cm^{-1} and using 32 scans (Kanamarlapudi and Muddada, 2017).

Statistical analysis

Comparative analysis of the results were done using Microsoft excel software.

RESULTS

PCR amplification

Based on preliminary identification, the strains were identified as *Streptococcus* and from PCR the strains were identified as *S. thermophilus*. In relation to sample of chekide yogurt, the bacteria identified was *Streptococcus thermophilus,* JIM8232, whereas in probiotic yogurt, *S. thermophilus* MN-ZLW-002 and in sade yogurt, *S. thermophilus* MN-BM-A02 were

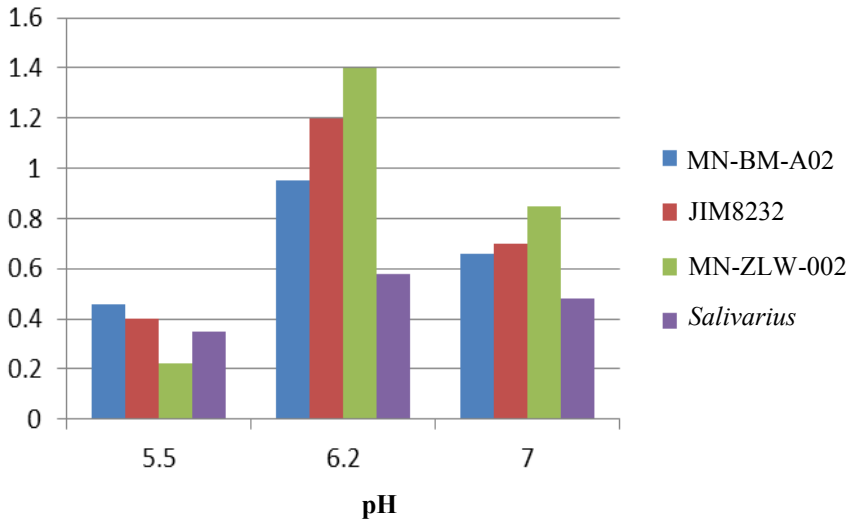

Figure 5. The effect of different pH on EPS production

identified.

Molecular mass estimation

The molecular weight of the polymer isolated from different strains of *S. thermophilus* were determined using a viscometer. High molecular weight was found in *S. salivarius* subsp. *thermophilus* followed by *S. thermophilus* JIM8232 with 1119.55 Da. The least molecular weight was found in *S. thermophilus* MN-BM -A02 (4307 Da) (Table 2).

Effects of temperature, different media, nitrogen and carbon sources and pH on EPS production

The results showed that at the optimal temperature (50°C) for growth, all strains produced the highest amount of EPS with the maximal yield. The strains produced much less EPS at 40 and 45°C (Figure 1).

Data listed in Figure 2 indicated that variable amounts of EPS could be achieved using different media. Relatively low EPS yield were maintained using STA enriched by $MgSO_4.7H_2O$ and BHI. However, the maximum EPS yield was recorded in case of fortified STA medium. On the other hand, the other tested media, namely MRS and nutrient broth failed in supporting EPS production. The cell growth was also influenced significantly by the different cultivation media. However, fortified STA is the best medium for EPS production and cell growth, consequently there is a relation between cell growth and EPS production and levels of EPS were greatly dependent on the composition of the used medium. Composition of the growth medium has an important influence on EPS production (Ashraf and Shah, 2011).

The high EPS titer in the selected medium (fortified STA) may be due to their content of glucose and minerals, respectively, which were found to be preferred by the organism. The highest EPS titer may possibly be due to the presence of simple monosaccharide and minerals in the nutrient medium (Figure 2).

Results of this study showed that in all the four strains, the initial concentration of 1.5g per 100 ml in fortified STA culture medium with tryptone and yeast extracts, have the greatest effect on growth and production of hyaluronic acid in comparison with peptone (Figure 3).

Fructose was proved to be the best carbon source for EPS biosynthesis, followed by glucose and molasses 25%. However, sucrose, lactose and molasses 10% were not found to be a suitable carbon sources for cell growth and EPS production. Consequently, the type

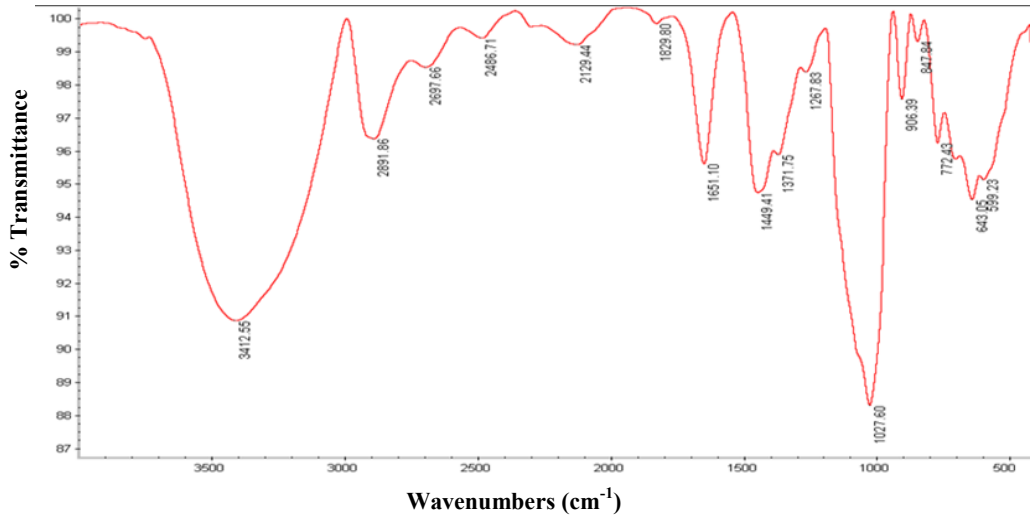

Figure 6. FTIR results showing polymer derived from *Streptococcus salivarius* subsp. *thermophilus*

of the carbon source in the fermentation medium plays an important role in EPS biosynthesis by *S. thermophilus* strains (Vaningelgem *et al*., 2004). It had been concluded that it reached the maximal yield of hyaluronic acid when fructose is used. On the other hand, a lower yield of hyaluronic acid was recorded upon using lactose as a carbon source (Figure 4).

Results of this experiment indicated that the maximum level of EPS was reached at pH 6.2. Also, a marked increase in the cell growth was observed at pH 6.2. Exopolysaccharide (EPS) production by *Streptococcus thermophilus* MN-BM-A02, JIM8232, MN-ZLW-002, *Streptococcus salivarius* subsp. *thermophilus* increased to the maximum value when adjusted to a pH of 6.2 (Zhang *et al*., 2011) (Figure 5).

FTIR Spectroscopy

FTIR is an effective technique that works on the principle that group of bonds vibrates at characteristic frequencies. It can be employed to detect functional groups and for characterizing covalent bonding.

FTIR results showed the polymer derived from *Streptococcus salivarius* subsp. *thermophilus*. Several sharp peaks (cm^{-1}) such as at 1371.75, 3312.55 that could be due to the stretch N-H bond, at 2891.86, 2697.66, 2486.71 that corresponded to the presence of stretch O-H bond, at 643.05, 1651.10, 2129.44 which

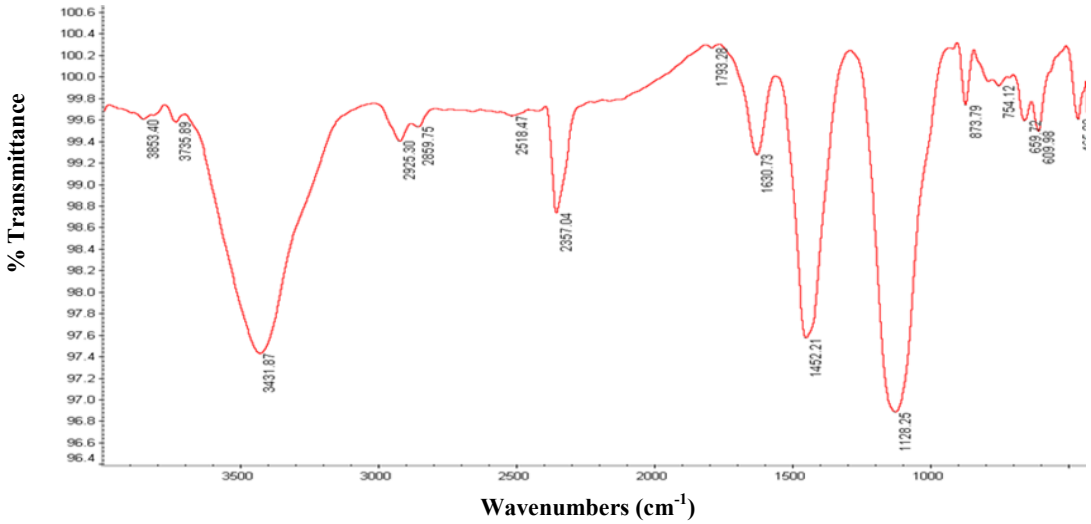

Figure 7. FTIR results showing polymer derived from *Streptococcus thermophilus* MN-ZLW-002

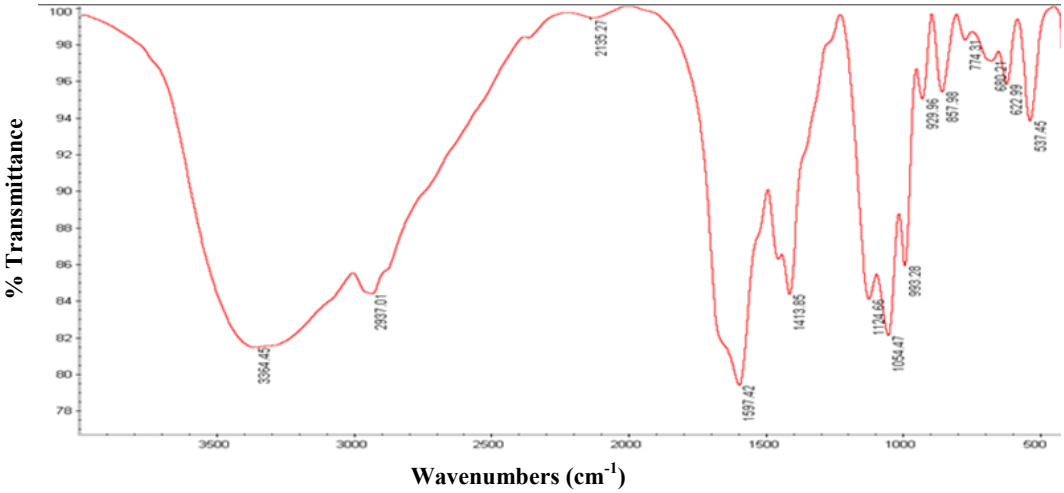

Figure 8. FTIR results showing polymer derived from *Streptococcus thermophilus* JIM8232

indicated the presence of stretch C=C, at 1449.41, 772.43 due to the C-H bond and at 1027.60, 1267.83 due to the C-O stretch, at 906.39 that confirms the presence of N-H bond that was similar to standard hyaluronic acid (Figure 6).

FTIR results showed a polymer derived from *S. thermophilus* MN-ZLW-002. Several sharp peaks (cm^{-1}) such as at 2357.04, 1128.25 that could be due to the C-N Stretch, at 1452.21 corresponding to the presence of N-H bond, at 3431.87, 2925.30 indicated the presence of O-H stretch bond, at 873.79, 659.72, 609.98 due to the C-H bond (Figure 7).

FTIR results showed polymer derived from *S. thermophilus* JIM8232. Several sharp peaks (cm^{-1}) such as at 1124.66, 1054.47 that could be due to the stretch C-N bond, at 1597.42 that corresponds to the presence of N-H bond at 2937.01 indicated the presence of C-H stretch, at 993.28, 857.98 due to the C-H bond, at 1413.85 that confirms the presence of stretch C-C, at 3364.45, 929.96 that indicated the presence of O-H stretch and O-H bonds respectively (Figure 8).

FTIR results of the polymer derived from *S. thermophilus* MN-BM-A02 showed several sharp peaks (cm^{-1}) such as at 1120.25,1052.52 that could be due to the stretch C-N at 3295.29, 2934.01 which corresponded to the presence of C-H stretch, at 992.13 indicating the presence of C-H bond, at 1596.90,

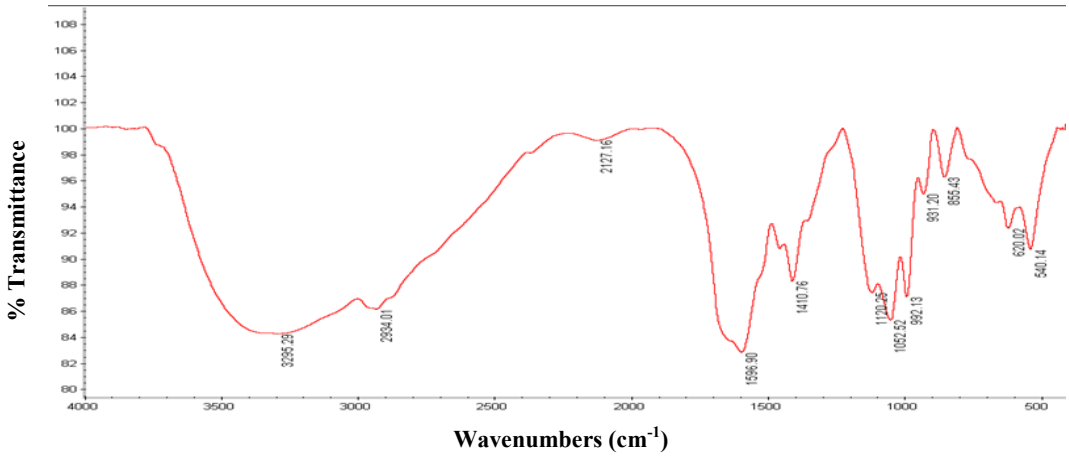

Figure 9. FTIR results showing polymer derived from *Streptococcus thermophilus* MN-BM-A02

1410.76 confirming the presence of stretch C-C/C=C (Figure 9).

DISCUSSION

The results of this study showed that the sources of carbon, nitrogen, temperature, pH and types of medium are effective on producing hyaluronic acid by the strains. This study indicated that fructose and glucose as carbon sources have efficient effects on the growth of strains and contributed to the production of the hyaluronic acid with the use of glucose or fructose in the medium, turbidity, growth of strains and subsequently, the production of the material has been also increased.

Im *et al.* (2009) optimized the medium components for the production of hyaluronic acid with *Streptococcus* sp. ID 9102. They observed that the level of glucose as a carbon source, on growth and HA subsequently produced by the strain was highest. This research study is methodologically very similar with the exception that types of strains were different. Their study and our study confirmed that the highest growth and production of hyaluronic acid by strains in the medium were by glucose and fructose (Im *et al.,* 2009).

Among the most important aspect of the research was the use of cost-effective carbon sources. In this study, molasses 10% and 25% were used as a carbon source. It was seen that all strains were grown greatly with the carbon source of molasses. Tu and Trang (2013) concluded that the production of hyaluronic acid with inexpensive carbon sources could be increased.

Our research study was conducted with different sources; sugar cane molasses was the important component used in this study as an inexpensive carbon source. Results showed that each strain had a good pattern of growth and hyaluronic acid production. So, it is recommended that replacing these carbon sources will be suitable choice for hyaluronic acid production in the large scale industrial production (Tu and Trang, 2013).

The study also found that probably the best temperature and pH, for the growth of four strains and production of hyaluronic acid is the 50°C and a pH of 6.2 respectively. Vaningelgem *et al.* (2004) showed the effect of temperature and pH on the extracellular polysaccharide production by *S. thermophilus* ST111. The optimum temperature for production of hyaluronic acid by this strain was in the range of 32-42°C and pH between 5.5 -6.5 was reported. Difference in results is due to the application of different types of genus of strains (Vaningelgem *et al.,* 2004).

This study also examined the different nitrogen sources such as, peptone, yeast extract and tryptone in the medium. Results of this study showed that, growth for all four strains and production of hyaluronic acid was more in STA medium with initial concentration of 5.1 g per 100ml of tryptone and yeast extract than peptone.

Degeest and Vuyst (1999) investigated the effect of nitrogen sources on the production of hyaluronic acid by *S. thermophilus* LY03. The results showed that the production of polysaccharides in the culture medium containing yeast extract and tryptone as nitrogen sources were more than using peptone, that is similar with our investigation. The results showed that glucose and fructose, yeast extract, tryptone, temperature at 50°C, pH 6.2 and STA-rich medium could be used to increase growth and HA production in each strain and was found to be effective.

Kanamarlapudi and Muddada (2017) reported a broad stretching at 3448 cm^{-1}, 2578 cm^{-1}, 2131 cm^{-1} and 2095 cm^{-1}, 1673 cm^{-1}, 1219 cm^{-1}, 927 cm^{-1}, 1500 cm^{-1}, 836 cm^{-1} and 1700–1770 cm^{-1} in the FTIR spectra of the polymer characterized from *S. thermophilus* CC30. The stretching at 3448 cm^{-1} is characteristic of a carbohydrate ring (Kumar *et al*., 2011). Similarly, the absorption band at 2578 cm^{-1} can be assigned to the C-H stretching of methyl or methylene groups, usually present in hexoses

like glucose or galactose, or deoxyhexoses like rhamnose or fucose (Ismail and Nampoothiri, 2010), the two peaks at 2131 cm^{-1} and 2095 cm^{-1} correspond to the presence of free carboxylic groups (Osman *et al*., 2012), the region 1673 cm^{-1}, usually represents the stretching vibration of C=O group (Shen *et al*., 2013), the peak at 1219 cm^{-1} could be assigned to C-O stretching in ether or alcohol groups (Botelho *et al*., 2014), the main absorption band at 927 cm^{-1} indicates the vibrations of the glycoside link C-O-C (Dyk *et al*., 2012). Wang *et al*. (2015) showed that the band at 836 cm^{-1} is characteristic of α-D glucan. The absence of characteristic absorption peak around the region of 1700–1770 cm^{-1} suggests that neither glucuronic acid nor diacetyl ester is present in the EPS**.**

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