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K. Jagadeeswara Reddy¹
K.T. Karunakaran²

Purification and characterization of hyaluronic acid produced by *Streptococcus zooepidemicus* strain 3523-7

Authors' addresses:

¹ Department of Biotechnology, Acharya Nagarjuna University, Guntur, India.

² MM Biolabs Private Limited, 274 Fortuna Glade B3/A, VB Layout, Bangalore, India.

Correspondence:

K.T. Karunakaran
MM Biolabs Private Limited
274 Fortuna Glade B3/A
VB Layout, 5th Main
Bangalore, India.
Tel: +91 8026484373
e-mail: mmbiolabsindia@yahoo.com

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ABSTRACT

Hyaluronic acid (HA) is a hydrated gel and comprises repeating units of glucuronic acid and *N*-acetylglucosamine. Production and recovery of HA has gained great importance due to its vast clinical applications. In pursuit of obtaining highly pure HA, we have developed a fed-batch fermentation process using *Streptococcus zooepidemicus* in a 25 L bioreactor that resulted in a maximum yield of 2.3 g/L HA. In addition, we have devised an efficient method for separation and recovery of hyaluronic acid from a highly viscous broth by treating with trichloroacetic acid (0.1%) and charcoal (1-2%), passing through filtration (0.45 μ m) and ultrafiltration that resulted in recovery of 72.2% of clinical grade HA with molecular weight of 2.5×10^6 Da. We have also characterized our purified HA using FTIR and NMR spectroscopy. These studies revealed the similarity in both the FTIR spectrum as well as NMR spectrum of both reference standard and purified HA from *S. zooepidemicus* indicating that the reported process is more efficient in terms of better yield and high quality (99.2%).

Key words: Hyaluronic acid, fed-batch fermentation, purification, FTIR analysis, NMR analysis, *Streptococcus zooepidemicus*

Introduction

Hyaluronic acid (HA) is a carbohydrate, more specifically a mucopolysaccharide occurring naturally throughout the human body. Hyaluronan is a polymer of disaccharides. HA is comprised of linear, unbranching, polyanionic disaccharide units consisting of glucuronic acid (GlcUA) and *N*-acetylglucosamine (GlcNAc) linked alternately by (β -1,3) and (β -1,4) glycosidic bonds. HA has been conventionally extracted from rooster combs and bovine vitreous humor. However, it is difficult to isolate high molecular weight HA at industrially feasible rate from these sources, because it forms complex with proteoglycans present in animal tissue. It is presently impractical to control the molecular weight of the biopolymer while it is synthesized in animal tissue. Moreover, the use of animal-derived biochemicals for human therapeutics has raised ethical issues, and is met with growing resistance. To overcome these disadvantages, the recent trend involves the usage of Lancefield's group A and group C *Streptococci*, which naturally produce a mucoid

capsule of HA. *Streptococci* are Gram-positive, nutritionally fastidious, facultative anaerobes, nonmotile, non-spore forming, catalase-negative cocci that occur in pairs or chains. Several studies have also reported on production of HA from *Streptococcus zooepidemicus* (Johns et al., 1994; Armstrong & Johns, 1997; Rangasamy & Jain 2008; Jagadeeswara Reddy et al., 2011).

The most significant clinical applications of HA are in the area of ophthalmology, orthopedics, and wound healing. Emerging uses include drug delivery, coatings, implants and therapeutics related application. Sodium hyaluronate injection is a viscoelastic, sterile solution of highly purified, high molecular weight hyaluronan in phosphate-buffered saline. Therefore, majority of the research has focused mainly on obtaining a highly pure HA suitable for clinical applications (Swann & Kuo, 1991). Several separation techniques such as protease digestion, HA ion-pair precipitation (with e.g., acetyl-pyridinium chloride), membrane ultrafiltration, HA non-solvent precipitation and lyophilization (Mendichi & Soltes, 2002; Soltes & Mendichi,

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2003) have been used to obtain a pure compound. Still an economical and a simple method is needed for high grade and pure HA production for medical applications.

Earlier we have reported on optimization of fermentation conditions for an enhanced HA production (1.89 g/L) by *S. zooepidemicus* mutant strain 3523-7 (Jagadeeswara Reddy et al., 2011). This study further describes on improving the HA production in a fed-batch fermentation process and a simple method of purification for high quality high molecular weight HA and its characterization.

Materials and Methods**Bacteria and media**

Streptococcus equi subspecies *zooepidemicus* mutant strain 3523-7 derived from MTCC 3523 (Jagadeeswara Reddy et al., 2011) was used in this study. Both strains were maintained as freeze-dried cultures and stored at 4°C. Bacteria were sub cultured and grown by using one percent inoculums in Todd Hewitt broth (Difco) or a chemically defined medium (CDM) containing optimal carbon and nitrogen sources (Jagadeeswara Reddy et al., 2011).

Hyaluronic acid fermentation using *S. zooepidemicus* 3523-7

Fed batch culture experiments were performed in 25 L bioreactor (Scigenics, India) with a working volume of 12 L chemically-defined medium (CDM). The bioreactor containing CDM was autoclaved at 121°C 15 lb/in² for 20 min and allowed to cool at room temperature. Overnight CDM-grown *S.zooepidemicus* 3523-7 was used as an inoculum (1%). Agitation was provided by three four-bladed turbines, the pH of culture media was maintained as 7.2 ± 0.2 by automatic addition of 5N-sodium hydroxide and temperature at 36°C with automated regulation. The impeller speed (200-400 rpm) and aeration (0.4 to 0.5 vvm) in fed batch cultures of the bioreactor were optimized for defined culture conditions. As for the established conditions initial glucose concentration maintained by adding 20 g/L glucose at a flow rate of 1 ml/min from the stock (220 g/L). Fermentation was allowed to continue in a batch mode until the sugar level reaches 0.1% (nearly 8-10 hours) and if the concentration of the sugar is less than 0.1%, the dilution rate of glucose was increased. Dilution rate was calculated using the formula: $D=F/V$. Dilution rate of glucose (from 13-14th hour of the batch) was $F = 60$ ml/hr and $D = 0.005$ hr⁻¹. It was assured that the concentration of sugar in the batch should not exceed 0.1-0.2% with the dilution rate. Nearly at 13-14th

hour, dilution rate of glucose was increased to 2 ml/min to maintain residual sugar and then subsequently in fed-batch mode with the feeding of glucose at the rate of 1 ml/min. Cells were harvested after heat killing the bacteria at the end of 20 hrs of fermentation when the final OD₅₃₀ was at 4.7.

The growth of the culture was determined by measuring optical density (OD) at A₅₃₀ nm (Armstrong & Johns, 1997) with UV-Visible Spectrophotometer (Labomed Inc, USA) by using the medium without inoculation as reference blank. The culture samples were diluted with distilled water to give less than 1 OD at A₅₃₀ nm.

Isolation and purification of hyaluronic acid

HA produced by *S. zooepidemicus* in fermentation broth was purified as described in literature (Brown et al., 1994; Han et al., 2004; Carlino & Magnette, 2002) and some modifications in its purification steps were made to improve the recovery and purity of HA. Crude HA present in fermentation broth was estimated by the carbazole method (Bitter & Muir, 1962).

The fermentation broth (Van de Rijn & Kessler, 1980) containing >2.0 g HA/L was precipitated with isopropyl alcohol (1:3 v/v). The precipitated HA was redissolved in 0.15 M sodium chloride solution so as to reduce the viscosity and concentration of HA (preferably up to 0.01 g HA/L). The nucleic acids and bacteria derived proteins present in crude samples were removed by lowering the pH of the broth from 6 to 2 by addition of trichloroacetic acid (0.1%) and subsequent charcoal treatment (1-2%) for 1 h followed by centrifugation at 7000 rpm for 30 min at 4°C.

After removal of cells and charcoal, HA solution was passed through 0.45 µm filters (293 mm cassette holder, Millipore, USA). The filtered HA solution was fivefold diluted and further purified by ultrafiltration in diafiltration mode (Millipore, USA) using a 300 kDa cut off cassette (Sartorius). Finally, the retentate containing HA sample was concentrated to original volume (one liter and precipitated with isopropyl alcohol (1:3 v/v). This step of isopropyl alcohol will remove any residuals of endotoxins left at the filtration stage. White fibrous aggregates of pure sodium hyaluronate were precipitated and vacuum dried (Biotron, Korea). Endotoxin levels were measured using LAL reagent (Charles River Laboratories, SC, USA) according to the manufacturer's instruction.

Molecular weight (MW) of HA was determined by measuring intrinsic viscosity [η] using Cannon-Ubbelohdes Viscometer (Cannon Instrument Co, USA) followed by

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substituting the intrinsic viscosity in Mark Houwink Equation (Martin, 1953; Laurent et al., 1960). The intrinsic viscosity $[\eta]$ was assessed by British Pharmacopoeia method (2003).

Characterization of hyaluronic acid by FTIR

Characterization of HA was performed by FTIR Spectrophotometer (Jasco, Japan) as described in British Pharmacopoeia (2003). HA obtained by extraction and purification from the fed batch cultures of *S. zooepidemicus* was compared with a standard sample of HA (Sigma chemicals, USA).

Dried potassium bromide was taken into mortar and grinded with a pestle. 2 mg of standard HA (as a reference substance) was then added and grinded to get a uniform mixture. The sample was evenly spread on the pelletier, and the pelletizing bit kept over it with the smooth surface facing towards the sample. The pelletier (Kimaya Engineers, India) was fixed and tightened by the screw head. Pressure was then applied slowly by using the lever to 10 tons (not exceeding 10 tons). The pelletizer was depressurized after one minute with the help of the knob. The pellet was carefully collected from the pelletizer and placed in the sample holder for measurement in the FTIR spectrophotometer. In a similar manner, HA sample obtained from *S. zooepidemicus* was also prepared for FTIR spectrophotometer. The HA sample to be examined and the reference standard were both prepared by the same procedure and the spectra recorded was between 4000-400 cm^{-1} (2.5-25 μm) under the same operational conditions against air as blank. All organic molecules can absorb FTIR radiation between 4000 cm^{-1} and 400 cm^{-1} , which corresponds to absorption of energy between 11 kcal/M and 1 kcal/M. This amount of energy initiates transitions between vibrational states of bonds contained within the molecule. The transmission minima (absorption maxima) in the spectrum obtained with HA sample to be examined corresponds to the position and relative size to those in the spectrum obtained with the reference substance.

Characterization of hyaluronic acid by NMR spectroscopic analysis

For determining the chemical structure of hyaluronic acid, analysis of the C^{13} nucleus present in the compound (hyaluronic acid) was studied by Nuclear magnetic resonance (NMR) spectroscopy (Bruker, Germany) as described below.

Ten milligrams of HA sample obtained from *S. zooepidemicus* was taken into a clean, dry 5 mm NMR tube (Wilmad glass). The compound was dissolved in 500 μl of

D_2O inside the NMR tube. Similarly the standard reference (10 mg of HA in 500 μl of D_2O) was prepared for the analysis.

The isotropic solution for both the test HA and reference standard has been prepared in D_2O . All experiments were performed using the BRUKER AMX 53 400 NMR spectrometer operated at 9.7 Tesla using 5 mm QNP probe. Standard Zgdc pulse program has been used to acquire the C^{13} NMR spectra with Waltz-16 decoupling. The temperature was maintained at 300 K using standard BRUKER BVT temperature unit and the sample was not spun during the time of experiment. The spectra were recorded and the obtained spectrum for the sample was compared with reference standard spectrum.

Statistical analysis

Data were expressed as mean \pm SD obtained from at least three independent experiments. Statistical significance of the obtained results was verified by Student's t-test and one way ANOVA using a commercial package (Sigma Plot 5.05). $p < 0.05$ in comparisons to controls was considered as significant.

Results and Discussion**Hyaluronic acid production by *S. zooepidemicus* 3523-7**

S. zooepidemicus strain 3523-7 has been shown to produce ten times more HA compared to its parent strain (Jagadeeswara Reddy et al, 2011). We have employed the same strain in the study in order to get better yields. During fed-batch fermentation growth, HA production and total sugar concentration were continuously monitored (Figure 1). At the end of 18 hrs, both the final OD_{530} of the culture (4.7) and HA production (2.34 g/L) reached the maximum. On the contrary, total sugar concentration started to decrease during the growth and HA production till the end of fermentation. Thus, the fermentation process has resulted in higher yield of HA compared to our previous study (Jagadeeswara Reddy et al, 2011).

Isolation and purification of hyaluronic acid produced by *S. zooepidemicus* 3523-7

Isolation and downstream purification process are crucial for efficient recovery of HA from the fermentation broth. Several purification procedures have been employed previously for isolation and purification of HA (Brown et al., 1994; Han et al., 2004).

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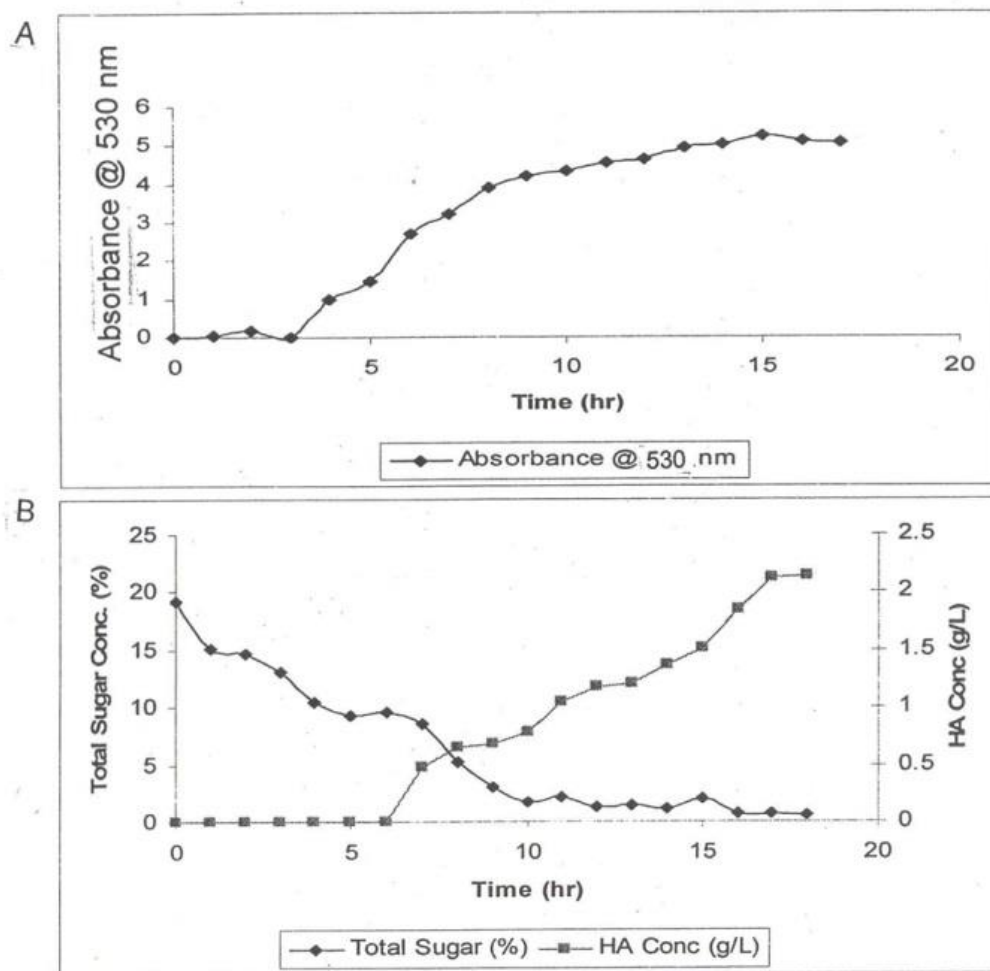


Figure 1. Hyaluronic acid production by fed-batch fermentation by *S. zooepidemicus* strain 3523-7. **A)** Change in growth of *S. zooepidemicus* is shown by \blacklozenge as a function of absorbance at OD_{530} and **B)** The change in hyaluronic acid production is shown as \blacklozenge along with total sugar in CDM indicated by \blacksquare are shown in line graph.

The release of HA from the complexes with other polysaccharides and proteins is usually achieved by using enzymes, organic solvents and detergents (lauryl sulfate, hexadecyltrimethyl ammonium bromide etc.) and anion exchange resins (Han et al., 2004). However the major disadvantage associated with these processes are higher production cost and it becomes difficult to completely remove exothermic material, proteins, nucleic acids, etc., hence they are not preferred for scale up processes. In the present study, a novel, cost-effective purification process for highly pure HA has been developed.

The optimized purification process (Table 1) in the present study includes trichloroacetic acid (0.1%) and activated charcoal (1-2%) treatment followed by centrifugation. Charcoal treatment yielded a high purity HA

and efficiently removed impurities like proteins and nucleic acids. Clarified HA solution thus obtained was fivefold diluted and passed through filtration (0.45 μ m) and ultrafiltration in diafiltration mode. The retentate containing HA was concentrated to half of the original volume with the recovery of 72.2% and purity of 99.2% (free of DNA and protein). The isopropyl alcohol precipitation step in the presence of 0.5 M sodium chloride, has efficiently removed the endotoxins from the final step of the purification (data not shown), which is very important step and reduced the cost very expensive materials specifically used for the endotoxin removal. Using this process, we achieved 99.2% purity of HA with better purity compared to the previous studies (Brown et al., 1994; Rangaswamy & Jain, 2008).

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The quality of HA obtained in this process complies with the specifications of British Pharmacopoeia (2003) for medical use with a yield of 2.3 g/L during the process (Table 1). Accordingly, HA obtained by a simple, economical purification method of the present invention has a high purity HA (free of DNA and is of medical grade. The molecular weight of HA isolated from fed batch cultures was found to be 2.5×10^6 Da.

Table 1. Purification of hyaluronic acid produced by *S. zooepidemicus* 3523-7.

	Before purification	After purification
HA (g/L)	2.320 ± 0.091	1.675 ± 0.075
OD ₂₆₀	3.800 ± 0.060	0.032 ± 0.008
Protein (mg/L)	356.00 ± 1.701	1.181 ± 0.142

HA from clarified broth was precipitated with isopropyl alcohol and suspended in sodium chloride. Then HA solution was treated with charcoal for 1 h. After centrifugation HA solution was passed through a 0.45 μ m filter, subjected to cross flow filtration and finally concentrated up to original volume.

FTIR characterization of hyaluronic acid produced by *S. zooepidemicus* 3523-7

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 FTIR spectrum of both test HA sample and reference standard and their waves are similar as shown in Figure 2 and Table 2.

The position of the peaks in terms of wave number (cm^{-1}) of both the reference standard and HA produced by *S. zooepidemicus* under optimal conditions are identical and correlated. This FTIR spectrum of both reference standard and purified HA in this study complied with similarity in their characterization (Figure 2). Standard HA showed several sharp peaks (cm^{-1}) such as at 611.32, at 1043.3 that could be due to the C-O-C stretching (Alkrad et al., 2002), at 1411.64 that corresponds to the presence of C-O group with C=O combination, at 1616.06 that indicates the presence of amid II group, at 2892.7 due to the C-H stretching and at 3407.6 that confirms the presence of OH stretching. Similar peaks (cm^{-1}) are seen with the test HA as indicated in Table 2. These six different peaks obtained in the HA sample share a relatively similar position when compared to that of the

standard. These results revealed the similarity of standard HA and test sample HA and the peak positions.

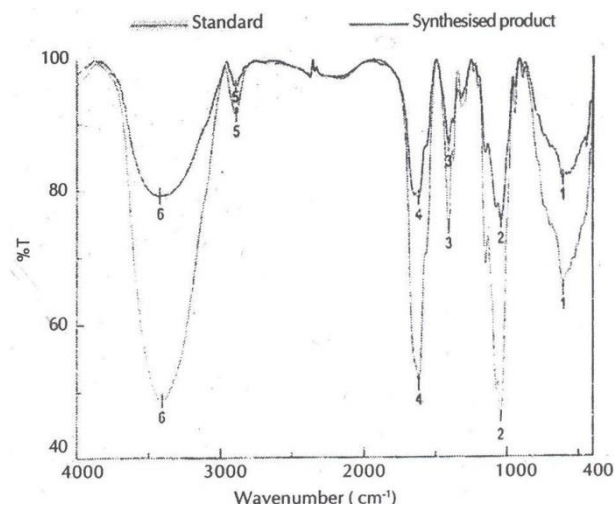


Figure 2. FTIR characterization of hyaluronic acid produced by *S. zooepidemicus* strain 3523-7.

Table 2. The peak number and position of the peaks (in terms of wave number, cm^{-1}) of both the reference HA standard and test HA produced by *S. zooepidemicus* 3523-7 using FTIR spectroscopy.

Peak No	Wave length (cm^{-1}) Reference HA standard	Wave length (cm^{-1}) Test HA sample
1	611.32	613.25
2	1043.30	1041.37
3	1411.64	1413.57
4	1616.06	1619.91
5	2892.70	2896.51
6	3407.60	3424.96

NMR spectral analysis of hyaluronic acid produced by *S. zooepidemicus* 3523-7

¹³C NMR spectroscopy is used to identify the resonances associated with the solvent used for the NMR sample. Peaks in the ¹³C NMR spectra (Figure. 3) corresponding to the 86 deuterated solvent molecules show a unique spin coupling patterns and the magnitude of the coupling depends on the

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number of bonds separating the atoms and the geometry of the bonds relative to each other. In present study, the NMR spectroscopic results of HA produced in fed batch cultures of *S. zooepidemicus* exhibited similarity in chemical shift of ^{13}C as that of the reference standard at the same operational conditions. The chemical shift of ^{13}C at 25 ppm, 57 ppm, 63.2

ppm, 71.2 ppm, 76.1 ppm, 78.9 ppm, 82.6 ppm, 85 ppm, 103.2 ppm, 105.9 ppm and 176.6 ppm are similar in both test HA sample and reference standard as shown in Figure 3. In the present study the exhibited chemical shifts of ^{13}C was correlated with the earlier reports (Alkrad et al., 2002; Bjarne et al., 1992; Scott et al., 1984; Cowman et al., 1984).

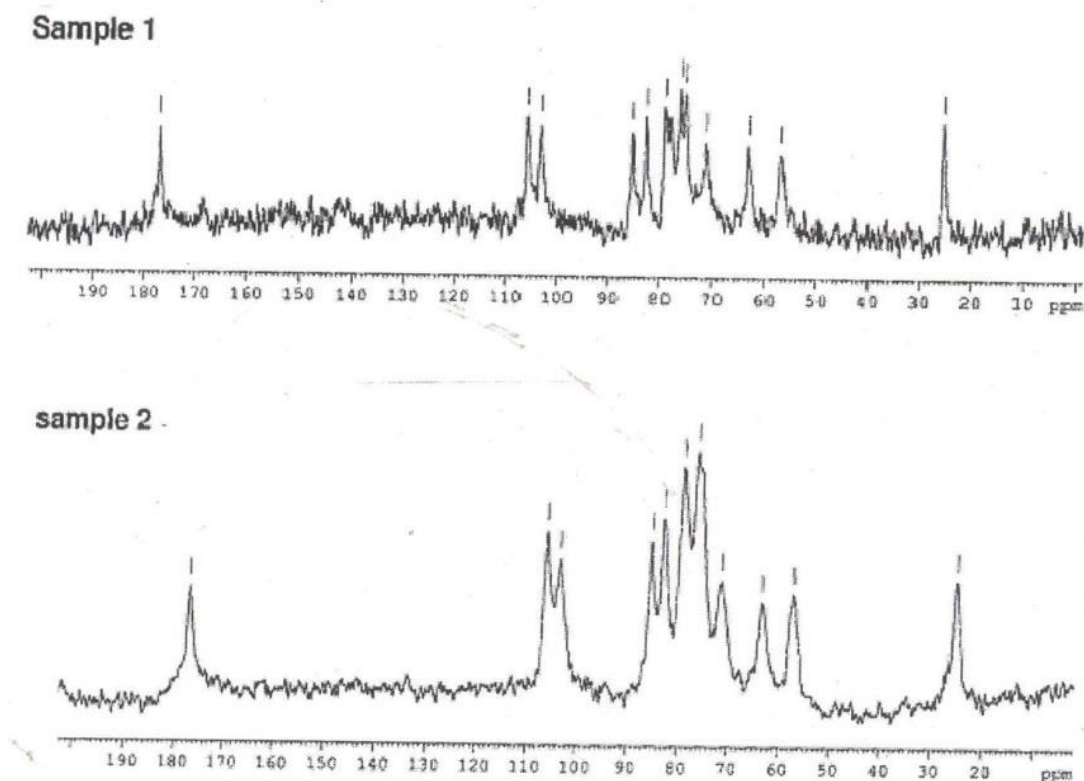


Figure 3. NMR spectral analysis of hyaluronic acid produced by *S. zooepidemicus* strain 3523-7 Sample.1: Reference Standard from Sigma Aldrich and Sample.2: In-house test sample.

Conclusion

S. zooepidemicus has produced 2.3 g/L of high molecular weight HA (2.5×10^6 Da) under optimal conditions in fed-batch fermentation. The purification method developed in present study efficiently removed exothermic material, proteins, nucleic acids and other impurities compared to the conventional method and hence high recovery (72.2%) of HA was obtained. The recovery process used in this report is simple and economical by use of trichloroacetic acid (0.1%), charcoal treatment, filtration and diafiltration. HA purified by this purification procedure yielded a high purity (99.2%) HA and this method could be useful for industrial purification of

clinical grade HA. Further analysis by FTIR and NMR spectroscopy have revealed that HA produced in this study exhibited chemical properties similar to that of the reference standard HA. Hence, this HA could be used for medical applications.

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