

MICROBIAL PRODUCTION OF HIGH PURITY HYALURONIC ACID FROM STREPTOCOCCUS ZOOEPIDEMICUS

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ABSTRACT

Hyaluronic acid (HA) is a hydrated gel and comprises repeating units of glucuronic acid and *N*-acetylglucosamine. In pursuit of high purity and recovery of HA, *Streptococcus zooepidemicus* was grown in 22 l bioreactor for 20 h and gave yield of 2.3g/L HA. The optimized purification process in the present study includes 1% activated charcoal followed by centrifugation, filtration (0.45µm) and ultrafiltration in diafiltration mode. This method of purification process gave a recovery of 73 %, purity of 99.7 % with Mr of ~2.4 × 10⁶ Da. Endotoxins and proteins were efficiently removed by this process, the purified HA complies the specifications of clinical grade HA.

INTRODUCTION

Hyaluronic acid (HA) is a mucopolysaccharide, in all places expressed in human and animal tissues as hydrated gel, which comprises repeating units of glucuronic acid and *N*-acetylglucosamine (Swann and Kuo, 1991). It is widely applied in biomedical, healthcare, food and cosmetics because of its unique physico-chemical properties such as hydrophilicity, lubrication and biocompatibility (Kogan *et al.*, 2007). Since the isolation of HA is economically difficult from animal sources, technology has been developed to produce HA from microbial source through fermentation technology (O'Regan *et al.*, 1994). HA is synthesized by many strains of group A and C *Streptococci* (Wessels *et al.*, 1991). Several studies have optimized the culture conditions for producing high molecular size HA in *Streptococci zooepidemicus* (Johns *et al.*, 1994; Armstrong and Johns, 1997; Rangaswamy and Jain, 2008) and majority of the literature has focused mainly on obtaining a highly pure product suitable for clinical application. Many clinical applications of HA depend on its molecular size and there are several studies that focus on these aspects (Swann and Kuo, 1991). Number of separation procedures have been used to obtain a pure compound, such as protease digestion, HA ion-pair precipitation (with e.g., acetyl-pyridinium chloride), membrane ultrafiltration, HA non-solvent precipitation and lyophilization (Mendichi and Soltes, 2002; Soltes and Mendichi, 2003). The present study used simplified method for the purification process of HA where in efficient removal of endotoxins, proteins were achieved with

economically viable yield.

MATERIALS AND METHODS

Streptococcus equi subspecies *zooepidemicus* (NCTC 6177) obtained from National Collection of Type Cultures was grown as described by Johns *et al.* (1994). One percent inoculum was used to propagate *S. zooepidemicus* (Van de Rijn and Kessler, 1980) in chemically defined medium containing optimal carbon and nitrogen source. The fermentation process was carried out in 22 l fermentor (Scigenics, India), which was operated at 36 °C for 20 h, agitation 400 rpm; aeration 0.6vvm and pH 7.2.

Isolation and purification of hyaluronic acid

Hyaluronic acid produced by *S. zooepidemicus* in fermented broth was purified as described in literature (Han *et al.*, 2004; Rangaswamy and Jain, 2008) and some modifications in its purification steps were made to improve the recovery and purity of HA. Crude HA present in fermented broth during the growth and fermentation was estimated by the carbazole method (Bitter and Muir, 1962). The fermentation broth having ~2.3 g HA/L was precipitated with isopropyl alcohol (1:3 v/v). The precipitated HA was redissolved in 0.5 M sodium chloride solution so as to reduce the viscosity and concentration of HA (preferably up to 0.01 g HA/L). The above solution was treated with various amounts of activated charcoal (0.5-2%) and stirred for 1 h followed by centrifugation at 7000 rpm for 30 min at 4 °C (Kubota) for removing cells and charcoal

with impurities. The charcoal treatment was done to remove the nucleic acids and proteins present in crude samples.

After removal of cells and charcoal, HA solution was passed through 0.45µm filters (293 mm cassette holder, Millipore, USA). The filtered HA solution was diluted five times with pyrogen free water and further purified by ultrafiltration in diafiltration mode (Millipore, USA) using 300 kDa cut-off cassette (Sartorius). Finally, the retentate containing HA was concentrated to original volume (one liter). White fibrous aggregates of pure sodium hyaluronate were precipitated with isopropyl alcohol (1:3 v/v) and vacuum dried (Biotron, Korea). Endotoxin levels were measured using LAL reagent (Charles River Laboratories, SC, and USA) according to the manufacturer’s instruction. Protein was estimated using Bio-Rad Dc protein estimation kit.

Determination of hyaluronic acid molecular weight

Molecular weight of HA was determined by measuring intrinsic viscosity [η] using Cannon-Ubbelohdes viscometer (Cannon Instrument Co, USA) followed by substituting the intrinsic viscosity in Mark Houwink Equation (Martin, 1953; Laurent *et al.*, 1960). The intrinsic viscosity [η] was assessed by British Pharmacopoeia method 2003.

Characterization of hyaluronic acid

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).

RESULTS AND DISCUSSION

Purification process of hyaluronic acid

The optimization of fermentation process has resulted in the yield of 2.3 g/L (Fig. 1) at 20h of culture duration. Several purification procedures have been employed previously for isolation and purification of HA (Brown *et al.*, 1994; Han *et al.*, 2004; Rangaswamy and Jain, 2008). 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 increased 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

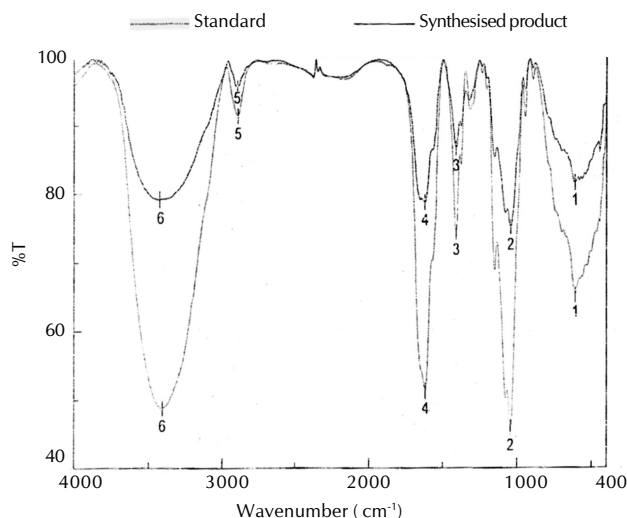


Figure 1: Pattern of time duration for HA production with *S. zooepidemicus* in a 22 l fermentor.

purification process for highly pure HA had been developed. The optimized purification process includes 1% activated charcoal followed by centrifugation, filtration (0.45µm) and ultrafiltration in diafiltration mode. The concentrated HA was precipitated with isopropyl alcohol 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 of much expensive materials specifically used for the endotoxin removal. The quality of HA obtained in this process complies with the specifications of British Pharmacopoeia 2003 for clinical use with a recovery of 73 %. HA from the clarified broth was purified as described

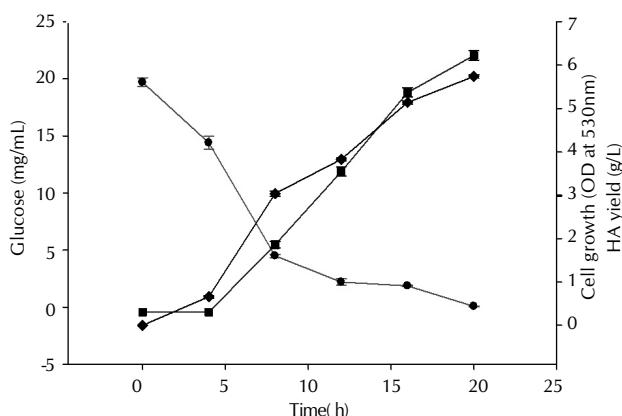


Figure 2: Characterization of hyaluronic acid by FTIR

Table 1: Purification and recovery of hyaluronic acid (HA) from fermentation broth

Charcoal(%)	Before charcoal Treatment ^a			After centrifugation ^b			After 0.45µm filtration ^c			After ultrafiltration ^d		
	HA g/L	O.D ₂₆₀ (20 fold diluted)	Protein mg/L	HA g/L	O.D ₂₆₀	Protein mg/L	HA g/L	O.D ₂₆₀	Protein mg/L	HA g/L	O.D ₂₆₀	Protein mg/L
0.5%	2.30	0.192	358	1.53	0.625	83	1.45	0.522	72	1.47	0.112	9.32
1%	2.30	0.192	358	1.94	0.412	48	1.81	0.401	46	1.68	0.027	1.22
1.5%	2.30	0.192	358	1.96	0.402	42	1.80	0.394	41	1.71	0.025	1.17
2%	2.30	0.192	358	1.95	0.398	41	1.82	0.389	37	1.70	0.024	1.15

HA from clarified broth was precipitated with Isopropyl Alcohol (1:3 v/v) and suspended in 0.15 M sodium chloride; The resuspended HA solution was treated with various amounts of activated charcoal (0.5-2%) and centrifugation; After centrifugation, HA solution was sterilized by passing through a 0.45 µm filter; The filtrate sample was connected to cross flow filtration and concentrated upto original volume

in Table 1.

Characterization of hyaluronic acid

The six different peaks obtained in the HA sample share a relatively similar position when compared to that of the standard (Fig. 2). Standard HA showed a sharp band at 1043.3 cm^{-1} whereas in case of HA sample, the band was observed at a wave number of 1041.37 cm^{-1} due to the C-O-C stretching (Alkrad *et al.*, 2002). Similarly, a peak was observed at a wave number of 1411.64 cm^{-1} for standard HA while HA sample showed the peak at 1413.57 cm^{-1} which corresponds to the presence of C-O group with C=O combination. Standard HA showed another peak at 1616.06 cm^{-1} and with regards to HA sample, the peak was observed at 1619.91 cm^{-1} which indicates the presence of amid II group. Another sharp band at 2892.7 cm^{-1} was observed for standard HA while a similar band at 2896.51 cm^{-1} was shown by HA sample due to the C-H stretching. A final peak at 3407.6 cm^{-1} was given by standard HA and at a wave number of 3424.96 cm^{-1} by sample HA which confirms the presence of O-H stretching. These results revealed the similarity of standard HA and test sample HA and the peak positions also provide ample testimony to this observation.

CONCLUSION

The purification method reported in this study could efficiently remove exothermic material, proteins, nucleic acids and metal impurities, compared to the conventional method. The yield, recovery and purity achieved by this method are ~ 2.3 g/L, 73 % and 99.7% respectively. HA purified by simplified method complies with the specifications of British Pharmacopoeia 2003 and this method could be used for industrial purification of clinical grade HA.

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