Original Article

Biotechnological Production and Purification of Hyaluronic Acid from *Streptococcus Zooepidemicus*

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DOI: 10.47750/pnr.2023.14.S02.13

Abstract

Historically, hyaluronic acid (HA) was extracted from animal sources, mainly rooster combs. However, there are some downsides to this technique of acquiring it. Consequently, the number of studies has expanded, and numerous microbial strains have been investigated for their ability to produce hyaluronic acid. Multiple types of bacteria are capable of manufacturing hyaluronic acid, most notably *Streptococcus zooepidemicus*, which may create up to 7 g/L of HA under optimal growth circumstances. The culture medium and ambient circumstances (pH, temperature, aeration, agitation) have a direct impact on the generation of hyaluronic acid, which may be minimised or maximised depending on these variables. Regarding recovery and purification processes, a series of techniques involving the precipitation of hyaluronic acid from the fermentation broth are often used, including organic solvents, surfactants, centrifugation, membrane separation, and others. Functions of hyaluronic acid include pharmacological, medicinal, and cosmetic applications, among others, resulting in an annual growth in commercial demand for this biopolymer, which justifies research into its bioproduction. This study involves that after 24–28 hours of growth in a 10 l bioreactor containing 50 g sucrose/l and 10 g casein hydrolysate/l, 5-6 g hyaluronic acid/l was produced by *Streptococcus zooepidemicus*. Purification of hyaluronic acid yielded a recovery of 65%, with the final material having a magnetic resonance of ~ 4 x 10^6 Da and containing less than 0.1% protein.

Keywords: *Streptococcus zooepidemicus*, Fermentation, Purification, Hyaluronic acid, Extraction.

Introduction

Hyaluronic acid (HA) is a linear glycosaminoglycan made up of 2,000–25,000 disaccharides of glucuronic acid and N-acetylglucosamine connected by alternating β-(1-3)- and β-(1-4)-glycosidic linkages. Hyaluronic acid is prevalent in human and animal tissues and occurs as a hydrated gel (Swann and Kuo 1991). It acts as a shock absorber and provides lubrication for the joints. Hyaluronic acid is also essential for embryogenesis, signal transduction, and cell motility, and is connected with cancer invasiveness and metastasis (Kogan et al. 2007). Its unique viscoelastic properties and lack of immunogenicity or toxicity have led to a wide range of cosmetic and pharmaceutical

applications, including skin moisturisers, osteo-arthritis treatment, ophthalmic surgery, adhesion prevention after abdominal surgery, and wound healing (Goa and Benfield 1994; Laurence and Fraser 1992).

Magnetic resonance values range from 10^4 to 10^7 Da for hyaluronic acid purified from a variety of sources (Shimada and Matsumura, 1975). Many therapeutic uses of hyaluronic acid rely on its molecular size, and a number of patents concentrate on this issue (Swann and Kuo, 1991). There is a growing demand for items derived from non-animal sources, despite the fact that hyaluronic acid is typically extracted from animal sources (Van Brunt, 1986). Physiological and genetic alterations are now being studied to increase the quantity and quality of hyaluronic acid synthesis by microorganisms. Group A or C *Streptococcus* strains are the most prevalently employed microorganisms for the synthesis of hyaluronic acid. Few studies have improved the culture conditions for manufacturing hyaluronic acid with a large molecular weight in *Streptococcus zooepidemicus* (Johns et al., 1994; Armstrong and Johns, 1997), and the bulk of the literature has concentrated on creating a very pure product suited for clinical applications. This research examines the impact of carbon source and medium composition on the magnetic resonance of hyaluronic acid. The centrepiece of this paper is an innovative and effective purification technique for medical-grade hyaluronic acid with a high molecular weight.

MATERIALS AND METHODS

Bacterial Strain and Media

Streptococcus equi subsp. zooepidemicus ATCC 39920 was cultured in a medium containing 25 g/l casein enzyme hydrolysate; 3.5 g/l yeast extract; 2 g/l K_2HPO_4; 1.5 g/l NaCl; 0.4 g/l $MgSO_4.7H_2O$; and 20 g/l carbon source; either in a 10 l bioreactor or in a (Bioengineering L-1523). The fermentor was run at 37^0C with 400 rpm and 2 vvm aeration for 28 hours. All experiments were conducted at least three times to guarantee repeatability.

Hyaluronic Acid Estimation

The carbazole test measuring uronic acid was frequently used to quantify hyaluronic acid in fermented broth (Bitter and Muir 1962). To prevent interference from media components in the test, the hyaluronic acid in the cell-free broth was precipitated with 1:1 2-propanol, redissolved in 3% (w/v) sodium acetate, and then quantified. After hydrolysis of hyaluronic acid with H_2SO_4, the test detects the glucuronic acid that is liberated.

Molecular Size Determination

Using size-exclusion chromatography on HPLC using Shodex OH-Pak SB805-804HQ columns coupled in series, the molecular size of hyaluronic acid was measured. 1 M $NaNO_3$ at 1 ml/min served as the mobile phase. An RI detector was used to monitor the effluent. Pullulan standards (Shodex P-82) of differing molecular weights were used to calibrate the column.

Hyaluronic Acid Purification

For the removal of cells, a very viscous fermented broth containing at least 5 g HA/l is diluted with pyrogen-free water and centrifuged at 17686g for 20 minutes at 4^0C.

As shown in Table 1, the hyaluronic acid in the clarified broth was purified.

Table 1: A Typical Hyaluronic Acid Batch's Purification Data

Treatment	Volume (ml)	HA yield (mg/ml)	Protein (mg/ml)	Total HA (mg)	Total Protein (mg)	% Protein w.r.t.HA
IPA[a]	100	3.5	0.57	342	57	16.6
Silica gel[b]	90	3.3	0.16	290	13.8	4.8
Carbon[c]	90	3.2	0.03	280	1.9	0.7
Diafiltration[d] **(5X)**	128	1.8	0.002	216.5	0.15	0.07
0.22 µm Filtration[e]	128	1.8	0.002	216.5	0.15	0.07

[a] Hyaluronic acid was precipitated from clarified broth with 1:1 2-propanol and resuspended in 3% sodium acetate.

[b] Two hours were spent treating the resuspended hyaluronic acid solution with 2% (w/v) silica gel in batch mode at room temperature and 150 rpm. By means of centrifugation (18000g for 20 minutes at 4^0C), the hyaluronic acid solution was clarified.

[c] At a flow rate of 14 ml/min, hyaluronic acid solution was filtered via a 0.45 µm charcoal filter assembly.

[d] After dilution by a factor of five with pyrogen-free water, the carbon-treated hyaluronic acid solution was further purified through ultrafiltration in diafiltration mode. A diluted hyaluronic acid solution was pumped at a rate of 15-20 ml/min into a cross-flow filter holder fitted with a polyether sulphone cassette with a 50 kDa cut-off. Concentration to original volume of hyaluronic acid-containing retentate.

[e] A 0.22 µm filter was used to sterilise the hyaluronic acid solution resulting from the diafiltration procedure.

Results and Discussion

Molecular Size Dependent Distribution of Hyaluronic Acid Throughout Development

As previously documented (Armstrong and Johns, 1997), the generation of Hyaluronic acid is a growth-associated phenomena, with low magnetic resonance HA (~ 5 kDa) occurring in the beginning (data not shown) and large magnetic resonance HA (>800 kDa) accumulating by the conclusion of 22 h of fermentation. As lactose or sucrose in the solution produced HA with a greater magnetic resonance (>800 kDa) than glucose, we used sucrose as a carbon source for all future tests.

Effect of Sucrose and Casein Enzyme Hydrolysate Concentration on the Synthesis of Hyaluronic AcidMolecules of High Molecular Weight

Increasing the content of sucrose in the medium from 20 to 50 g/l while simultaneously decreasing the concentration of casein enzyme hydrolyzate from 25 to 10 g/l caused a substantial rise in the viscosity of the fermentation broth due to increased hyaluronic acid synthesis (Table 2). These circumstances favour a slower growth rate and more hyaluronicacid production, resulting in a yield greater than 5 g/l (Fig. 1). In contrast to a previous study in which lysozyme wasadded to the culture broth to boost the magnetic resonance to 3.8×10^6 Da (Kim et al., 1996), our technique produceshyaluronic acid with a magnetic resonance of around 3.5×10^6–3.9×10^6 Da in 24 h at 37^0C, pH 7.0, 400 rpm, and 2vvm aeration without substantial modification.

Table 2: Impact of Casein Enzyme Hydrolysate on the Synthesis of Hyaluronic Acid

Casein Enzyme Hydrolysate (g/l)	Hyaluronic Acid yield (g/l)
2	1.2
3.3	2.6
10	5.2
25	2.5

All of these studies were conducted with a 50 g/l concentration of sucrose in the medium.

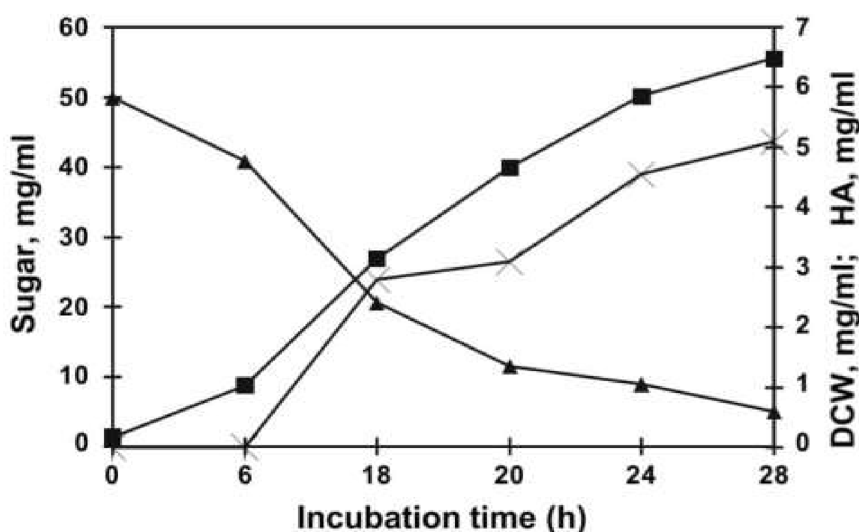

Figure 1: 10 litre batch fermentation medium adjustment for enhanced HA production. During the procedure, the sucrose concentration (m), the HA concentration (mg/ml) (X), and the dry cell weight (j) are given.

Hyaluronic Acid Purification

Production and purification of high magnetic resonance hyaluronic acid that fulfils the requirements stipulated by the pharmacopoeia for medicinal purposes has been a problem, and quality rather than quantity has been the emphasis of strain and method research in hyaluronic acid production patents. During fermentation and purification, the intrinsic viscosity of hyaluronic acid, which rises with an increase in magnetic resonance, poses a significant challenge. Important fermentation parameters are those that balance the formation of hyaluronic acid, which is inversely linked to growth.

For the purification of hyaluronic acid, numerous solvent precipitations, cationic detergent treatment, diafiltration, anion exchange resin treatment, and protease digestion have been used in a number of prior separation processes. Table 1 outlines an unique purification technique for hyaluronic acid including silica gel filtration in conjunction with active carbon treatment and diafiltration. In contrast to other procedures, the utilisation of a single solvent precipitation phase dramatically minimises the amount of solvent used (Brown et al. 1994; Han et al. 2004). Treatment using silica gel and active carbon instead of detergents, which need repeated post-treatment washes to remove protein impurities

by 96% (Nimrod et al. 1988; Brown et al. 1994), removes 96% of protein impurities. Ultrafiltration in the diafiltration mode eliminates further contaminants, resulting in a product containing 0.06% protein relative to hyaluronic acid. Although diafiltration has been employed in prior publications (Carlino and Magnette, 2002), our method is more efficient, requiring very little dilution with solvent and producing a higher grade of hyaluronic acid. A final 0.22 µm filtering makes the product sterile and increases the yield by 65%. The acquired hyaluronic acid meets the standards established by the British Pharmacopoeia (BP 2018) for hyaluronic acid of medical grade (Table 3). Consequently, the procedure described here is straightforward, cost-effective, and repeatable, yielding a high output of high magnetic resonance hyaluronic acid of medical quality.

Table 3: Hyaluronic Acid Characteristics for a Typical Batch

Test	BP Specifications [a]	Sample
Appearance of	Clear	Clear
SolutionIR spectra [b]	$A_{600\ nm} = \leq 0.01$	$A_{600\ nm} = 0.004$
pH	5.0 - 8.5	6.65
Nucleic acids	$A_{260\ nm} = \leq 0.5$	$A_{260\ nm} = 0.033$
Protein	≤ 0.1%	0.056%
Chlorides	≤ 0.5%	Complies
Loss on	≤ 20% by weight	18.2%
drying		99.2%
% Na-hyaluronate [c]		3.9×10^6 Da

[a] British Pharmacopeia 2018.
[b] The spectrum of the test material matches the spectrum of sodium hyaluronate used as a reference.
[c] Not less than 95% and not more than 105% of sodium hyaluronate, based on the percentage of sodium hyaluronate in the dried material.

Acknowledgements

The authors significantly acknowledged the Lincoln University College, Petaling Jaya, Selangor, Malaysia for providing the learning and resource facilities for executing the present comprehensive research work.

References

1. Armstrong D, Johns MR (1997). Culture conditions affect the molecular weight properties of hyaluronic acid produced by Streptococcus zooepidemicus. Appl Environ Microbiol 63:2759–2764.
2. Bitter T, Muir M (1962). A modified uronic acid carbazole reaction. Anal Biochem 4:330–334.
3. British Pharmacopeia (2018).
4. Brown KK, Ruiz LLC, van de Rijn I et al (1994). Method for the microbiological production of non-antigenic hyaluronic acid. US Patent 5,316,926, 31 May 1994.

5. Carlino S, Magnette F (2002). Process for purifying high molecular weight hyaluronic acid. US Patent 6,489,467 B1, 3 Dec 2002.
6. Das, P., & Janahiraman, V. (2019). Analytical Study on Heterocyclic Anticancer Compounds. Research Review International Journal of Multidisciplinary, 4(5), 172-175.
7. Das, P., & Janahiraman, V. (2019). Study on New Chalcones and Nitrogen Containing Heterocyclics. International Journal of Scientific Research And Review, 8(5), 1069-1080.
8. Das, P., & Srivastav, A. K. (2014). Phytochemical Extraction and Characterization of the Leaves of Andrographis paniculata for Its Anti-Bacterial, Anti-Oxidant, Anti-Pyretic and Anti-Diabetic Activity. International Journal of Innovative Research in Science Engineering and Technology, 3(8), 15176-15184.
9. Das, P., & Srivastav, A. K. (2015). A Comparative Study on Nutritive Values of Several Vegetables from West Bengal, Eastern India. International Journal of Pharmaceutical Research and Bio-Science, 4(1), 381-390.
10. Das, P., & Srivastav, A. K. (2015). In-vitro Micropropagation of The Miracle Plant Aloe vera - A Method of Rapid Production. International Journal of Pure and Applied Research in Engineering and Technology, 3(11), 12-27.
11. Das, P., & Srivastav, A. K. (2015). Phytochemical Extraction And Characterization of the Leaves of Aloe vera barbadensis For Its Anti-Bacterial And Anti-Oxidant Activity. International Journal of Science and Research, 4(6), 658-661.
12. Das, P., & Srivastav, A. K. (2015). To Study the Effect of Activated Charcoal, Ascorbic Acid and Light Duration on In-vitro Micropropagation of Aloe vera L. International Journal of Innovative Research in Science Engineering and Technology, 4(5), 3131-3138.
13. Das, P., & Srivastav, A. K. (2021). A Study on Molecular Targeted Approaches To Cancer Therapy And The Role of Chalcones In Chemoprevention. European Journal of Molecular & Clinical Medicine, 8(3), 3254-3267.
14. Goa KL, Benfield P (1994). Hyaluronic acid: a review of its pharmacology and use as a surgical aid in ophthalmology and its therapeutic potential in joint disease and would healing. Drugs 47:536–566.
15. Han HY, Jang SH, Kim EC et al (2004). Microorganism producing hyaluronic acid and purification method of hyaluronic acid. WO 2004/016771, 26 Feb 2004.
16. Johns MR, Goh LT, Oeggerli A (1994). Effect of pH, agitation and aeration on hyaluronic acid production by Streptococcus zooepidemicus. Biotechnol Lett 16:507–512.
17. Kim JH, Yoo SJ, Oh DK et al (1996). Selection of a Streptococci equi mutant and optimization of culture conditions for the production of high molecular weight hyaluronic acid. Enzyme Microb Technol 19:440–445.
18. Kogan G, Soltes L, Stern R et al (2007). Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. Biotechnol Lett 29:17–25.
19. Laurent CT, Fraser JRE (1992). Hyaluronan. FASEB J 6:2397–2404.
20. Nimrod A, Benjamin G, Dov K et al (1988). High molecular weight sodium hyaluronate. US Patent 4,784,990, 15 Nov 1988.
21. Shimada E, Matsumura G (1975). Viscosity and molecular weight of hyaluronic acids. J Biochem 78:513–517.
22. Srivastav, A. K., & Das, P. (2014). Phytochemical Extraction and Characterization of Roots of Withania somnifera for Its Anti-Bacterial, Anti-Oxidant, Anti-Inflammation and Analgesic Activity. International Journal of Innovative Research and Development; 3(7), 22-33.
23. Srivastav, A. K., & Das, P. (2014). To Study the Formulation of Niosome of Ofloxacin and Its Evaluation for Efficacy of Anti-Microbial Activity. International Journal of Innovative Research in Science Engineering and Technology, 3(12), 17958-17965.
24. Srivastav, A. K., & Das, P. (2015). A Comparative Study of the Effect of Ampicillin and Tetracyclin on Bacterial Culture by Measuring the Zone of Inhibition. International Journal of Pharmaceutical Research and Bio-Science, 4(1), 277-285.
25. Srivastav, A. K., & Das, P. (2015). Phytochemical Extraction and Characterization of Acorus calamus, Moringa oliefera, Cucurbita maxima, Hibiscus rosa sinensis and Chrysanthemum leucanthemum For Their Anti-Bacterial and Anti-Oxidant Activity. International Journal of Pharmaceutical Research and Bio-Science, 4(3), 356-377.
26. Srivastav, A. K., & Das, P. (2015). To Study the Production and Standardization of Veterinary Vaccines. International Journal of Science and Research, 4(6), 2331-2337.
27. Srivastav, A. K., & Janahiraman, V. (2019). Microbial Synthesis of Hyaluronan and Its Biomedical Applications. International Journal of Scientific Research And Review, 8(5), 852-863.
28. Srivastav, A. K., & Janahiraman, V. (2019). Study on Microbial Production of Hyaluronic Acid. Research Review International Journal of Multidisciplinary, 4(5), 231-233.
29. Swann DA, Kuo JW (1991). Hyaluronic acid. In: Byrom D (ed) Biomaterials-novel materials from biological sources. Stockton Press, New York, pp 286–305.
30. Van Brunt J (1986). More to hyaluronic acid than meets the eye. Bio/Technology 156:1059–1065.