Chapter 162
Separation and Purification of Hyaluronic Acid from Fermentation Broth

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Abstract The objective of this research was to compare different methods for the separation and purification of hyaluronic acid from *Streptococcus zooepidemicus* ATCC39920 for their ease of use and reliability. The most appropriate conditions of pretreatment stage were heated to 70 °C for 1 h and kept at room temperature for 5 h. The fermented broth was diluted with the equal volume deionized water. Ten milligrams chitosan was slowly added to 1 L of solution with stirring for 30 min, followed by the addition 20 g diatomaceous earth-type filter aid. The mixture was stirring for 1 h. Then, the mixture was filtrated with diatomite, microfiltration (MF) membranes (5 μm in pore diameter), and ultrafiltration (UF) membranes (MWCO 50KD) to achieve the separation stage.The retentate was washed by the equal volume deionized water and when the volume concentration ratio (VCR) reached 4, the UF stage ended. Finally, the purification procedure adopted Sevag process to remove protein. The overall yield of HA could reach 62.53 % and the removal rate of protein could reach 95.22 %.

Keywords Hyaluronic acid · Fermentation broth · Pretreatment process · Separation process

162.1 Introduction

Hyaluronic acid (HA), is a high-molecular weight linear polysaccharide, composed of *D*-glucuronic acid and *N*-acetylglucosamine linked alternately by β-(1-3)- and β-(1-4)-glycosidic bonds. HA is typically found in the connective tissues of animals as well as in the capsules of streptococcal bacteria. The highest content of

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HA is found in rooster combs. Its molecular mass in human normal synovial fluid has been estimated to be 6–7 $\times$ 10^6 and in rheumatoid fluid 3–5 $\times$ 10^6 Dalton [1].

Traditionally, HA has been extracted from bovine eyes and rooster combs [2]. However due to limited tissue sources, risks of viral infection and high cost, HA production from microbial sources through the fermentation process has received increased attention especially when using the gram-positive bacterium *Streptococcus zooepidemicus* [3–7].

The separation and purification of HA involves the precipitation of HA from fermentation broth by repeatedly using large amounts of organic solvents such as ethanol, acetone, isopropanol, etc. [8–12]. However, the process is complicated and time-consuming, which leads to high cost. In order to improve the efficiency of the purification of HA a better understanding of each steps in the purification process is needed.

The purpose of this study was to compare different methods for the separation and purification of HA from *S. zooepidemicus* ATCC39920 for their ease of use and reliability. The yield of HA, the removal of protein and the transmittance of solution has been chosen to evaluate the step of the purification methods. The result presented can be used as a guide for the choice of purification method of HA from fermentation broth.

162.2 Materials and Methods

162.2.1 Bacterial Strain and Media

For the experiment, the broth used was produced by fermentation with *S. zooepidemicus* ATCC39920 (from College of Bioengineering, Tianjin University of Science and Technology).

The medium for seed culture contained (in g L^{-1} distilled water): glucose, 2.5; soy peptone, 3; tryptone, 17; K_2HPO_4, 2.5; NaCl, 5. After inoculum, the medium was incubated at 37 °C in a reciprocal shaker at 200 rpm for 12 h [13].

The production of HA was carried out in a 5 L fermentor (Biostat Aplus, Germany) with a working volume of 3 L. The fermentation medium contained (in g L^{-1} distilled water): sucrose, 50; yeast extract, 3.5; casein peptone, 10; K_2HPO_4, 2; NaCl, 1.5; $MgSO_4{\cdot}7H_2O$, 0.4. The fermentor was operated at 37 °C under shaking conditions at 400 rpm for 28 h at 2 vvm aeration [14].

162.2.2 Methods

162.2.2.1 General Separation and Purification Process

Figure 162.1 shows the general purification process of HA. Generally, the purification process included three steps, pretreatment procedure, separation procedure and purification procedure.

162.2.2.2 Pretreatment Procedure

Three pretreatment procedure including acidification, heating and dilution were treated to compare. Detailed methods see below.

1. Acidification: ① The fermentation broth was adjusted to a pH of about 4.5 with trichloroacetic acid and then rest for 1 h. ② The solution was readjusted to a pH of about 6 with NaOH and then rest for 5 h. ③ The solution was diluted with the equal volume deionized water.
2. Heating process: ① The fermentation broth was heated to 70 °C for 1 h. ② The solution was kept at room temperature for 5 h. ③ The solution was diluted with the equal volume deionized water.
3. Dilution process was that fermentation broth was diluted with the equal volume of deionized water directly.

Then, add 10 mg of chitosan to every 1 L of solution which were treated with three methods with stirring for 30 min. After that, add 20 g of diatomaceous earth-type filter aid to per liter of solutions with stirring for 1 h. The mixture was filtered by buchner funnel and filtrate was carried out with 5 μm in a continuous diafiltration mode. HA was recovered in the permeate solution.

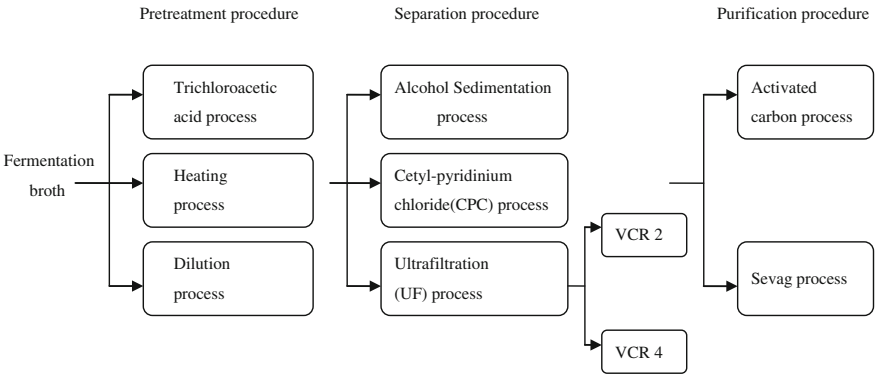

Fig. 162.1 The flow chart of experiment

162.2.2.3 Separation Procedure

The fermentation broth was investigated to determine the suitable operating condition for the good pretreatment process. Then pretreatment fermentation broth was treated, respectively, with alcohol sedimentation, cetyl-pyridinium chloride (CPC), and ultrafiltration (UF).

1. Alcohol sedimentation process: The HA solution was precipitated from the fermentation mixture by addition of 2 volumes of absolute ethanol and the mixture was kept at 4 °C for 1 h. Then the precipitate was separated by centrifugation and redissolved in pure water.
2. Cetylpyridinium chloride process: A 4 % solution of CPC was added to the pretreatment fermentation broth. The amount of CPC added is 2 times in weight than HA and the mixture was kept at 4 °C for 1 h. The precipitated cetylpyridinium salt was separated by centrifugation, and then redissolved in 2 M sodium chloride.
3. Ultrafiltration process: The experimental run was stopped upon achieving the desired volume concentration ratio (VCR). The UF processing used ultrafiltration membranes (MWCO 50KD) with pure water as diafiltrate. The transmembrane pressure (TMP) for UF was determined to be 0.1 MPa under different operation modes. HA product was collected from the retentate of UF. To investigate the effects of different operation modes on the performance of UF, two operation modes were examined, including ① UF stage ended when VCR reached 2. ② The equal volume deionized water was added to the retentate and made VCR reached to 4, after the process of UF was ended.

162.2.2.4 Purification Procedure

Activated charcoal process: The solution was treated with 10 g L^{-1} of activated charcoal and the mixture was shook in a reciprocal shaker at 200 rpm for 3 h.

Sevag process: The solution was treated with a quarter of the volume chloroform-normal butanol (volume ratio is 4:1), and shook fully for 30 min. Then, water phase and organic phase were separated by centrifugation. Water phase was collected and added a quarter of the volume chloroform-normal butanol, repeated many times.

162.2.3 Analytical Methods

The analysis of HA concentration, the raw fermentation broth sample was treated with the equal volume of 0.1 % (w/v) sodium dodecyl sulfate for 10 min to liberate the capsular HA and to facilitate the separation of the cells [15]. After the

cells were removed by High speed refrigerated centrifuge (CR 22G, Hitachi, Japan) at 12,000 rpm for 15 min, the supernatant was then subjected to HA precipitation by mixing it with three volumes of ethanol. The precipitate was collected by centrifugation at 5,000 rpm for 15 min, redissolved in deionized water, and analyzed for the HA concentration by the method of Bitter and Muir [16]. The optical density was measured at 530 nm by spectrophotometer (TU-1810, Purkinje General, China) with D-glucuronic acid used as the standard.

The concentration of protein was measured by the Bradford protein assay using BSA (bovine serum albumin) as standard.

The transmittance of solution was determined by spectrophotometer at 600 nm, and the transmittance of pure water was 100.

162.2.4 Calculation Methods

162.2.4.1 Symbol Meaning

C_0 HA concentration in raw fermentation broth (g L^{-1})
$C_{i,HA}$ HA concentration in treated fermentation broth (g L^{-1})
C_p Protein concentration in raw fermentation broth (μg mL^{-1})
$C_{i,p}$ Protein concentration in treated fermentation broth (μg mL^{-1})
V_o Raw fermentation broth volume (mL)
V_i Treated fermentation broth volume (mL)
Y_i The yield of HA in different processes
R_i The removal of protein in different processes

162.2.4.2 Equations

During the experiments, the yield of HA in different processes was calculated by using the following equations.

$$Y_i = \frac{C_{i,HA} \times V_i}{C_o \times V_o} \times 100\,\% \tag{1}$$

The removal of protein in different processes:

$$R_i = \frac{C_p \times V_o - C_{i,p} \times V_i}{C_p \times V_o} \times 100\,\% \tag{2}$$

162.3 Results and Discussion

162.3.1 Comparison of Different Pretreatment Procedures

Sterilization and degerming were considered first in the downstream process. In this section, the study compared the removal of protein impurities, the transmittance of solution at 600 nm, and the yield of HA after fermentation broth was treated with three pretreatment procedure methods. The data was shown in Table 162.1.

As can be seen in Table 162.1, trichloroacetic acid process should be eliminated due to its low yield of HA (69.59 %). The yield of HA (92.46 %) by heating process was the highest in three pretreatment processes. What's more, heating process had an obvious advantage on the transmittance of solution when compared with dilution process. Moreover, heating process without adding other substances will not contaminate environment potentially and lead to low cost. Therefore, heating process was more suitable for the pretreatment procedure.

162.3.2 Separation Procedures

The separation and purification of HA is one of the keys in HA production, and the method of separation and purification not only affect the quality of products, but also the cost of production. In this section, after fermentation broth was treated with three separation procedure methods, the removal of protein impurities, the transmittance of solution, and the yield of HA were determined. The data was shown in Table 162.2.

The experimental data of separation procedure under the four conditions were shown in Table 162.2. The CPC process and alcohol sedimentation process were lower than UF process in the content of HA. In UF process, the VCR 4 process showed higher the yield of HA and the removal of protein about 7.78 % and 39.87 % than the VCR 2 process, respectively. In addition, the transmittance of the solution of the VCR 4 process was higher, which showed lower impurity and pigment. In order to realize high degrees of yield and purification during HA

Table 162.1 The comparison of three pretreatment procedures

Operating mode	HA		Protein		T_{600nm}
	$C_{i,HA}$	Y_i	$C_{i,p}$	R_i	
Raw fermentation broth	4.11	100	3.16	0	41.52
Trichloroacetic acid process	2.86	69.59	2.32	26.58	92.88
Heating process	3.80	92.46	2.96	6.33	94.13
Dilution process	3.34	81.27	3.04	3.80	69.63

Table 162.2 The comparison of three methods for separation procedure

Operating mode		HA		Protein		T_{600nm}
		$C_{i,HA}$	Y_i	$C_{i,p}$	R_i	
Raw fermentation broth		4.11	100	3.16	0	41.52
Alcohol sedimentation process		1.50	36.50	1.87	40.82	96.26
Cetylpyridinium chloride (CPC) process		1.85	45.01	1.2	62.03	13.95
Ultrafiltration (UF) process	VCR 2	2.53	61.56	2.5	20.89	95.57
	VCR 4	2.85	69.34	1.24	60.76	97.25

Table 162.3 The comparison of two methods for purification procedure

Operating mode	HA		Protein		T_{600nm}
	$C_{i,HA}$	Y_i	$C_{i,p}$	R_i	
Raw fermentation broth	4.11	100	3.16	0	41.52
Activated charcoal process	2.41	58.64	0.222	92.97	68.46
Sevag process	2.57	62.53	0.151	95.22	99.13

separation, the VCR 4 process was adopted to effectively recover HA and washed out more small soluble molecules.

162.3.3 Purification Procedures

In this section, the main objective was to remove protein. The data of the two different methods were shown in Table 162.3.

As shown in Table 162.3, Sevag process had an obvious advantage of higher transmittance at 600 nm. What's more, the yield of HA (62.53 %) and the removal of protein (95.22 %) by Sevag process was slightly lower than the activated charcoal process, which were 58.64 % and 92.97 %, respectively. Therefore, Sevag process was more favorable to purification procedure.

162.4 Conclusions

The optimal procedure for purification was as follows: the fermentation broth was heated for 1 h at 70 °C and then kept at room temperature for 5 h and diluted with the equal volume deionized water. Ten milligrams of chitosan per liter of solution were added slowly to the fermentation broth by stirring for 30 min, 20 g L^{-1} of diatomaceous earth-type filter aid were added to the solution with stirring for 1 h. The mixture was filtered by buchner funnel and the filtrate was carried out with 5 μm in a continuous diafiltration mode. Then the filtrate used UF and the retentate

was washed with the equal volume of deionized water. The UF process did not finish until VCR was four. Finally, the Sevag process was used to remove protein in the purification procedure. The overall yield of HA could reach 62.53% and the overall removal rate of protein could reach 95.22 %.

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