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ORIGINAL PAPER

Optimization of medium components for high-molecular-weight hyaluronic acid production by *Streptococcus* sp. ID9102 via a statistical approach

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Abstract Hyaluronic acid (HA), linear high-molecular-weight glycosaminoglycan produced from *Streptococcus* sp., has raised interest in the medical and cosmetics industries because of the various biological functions of HA. In this paper, we report on the optimization of medium components for HA production in *Streptococcus* sp. ID9102 (KCTC 11935BP) by two-step optimization (one-factor-at-a-time and taguchi orthogonal array design). In the first step, medium components, such as carbon, nitrogen, phosphate, and mineral sources, were selected for HA production in *Streptococcus* sp. ID9102 (KCTC 11935BP) using the one-factor-at-a-time method. In the second step, the concentration of the selected medium components was optimized using taguchi orthogonal array design. The design for medium optimization was developed and analyzed using MINITAB 14 software. In addition, the effect of amino acid and organic acid, such as glutamine, glutamate, and oxalic acid, was studied for HA production in *Streptococcus* sp. ID9102 (KCTC 11935BP). Through these processes, the optimum medium comprising 4% glucose, 0.75% yeast extract, 1.0% casein peptone, 0.25% K_2HPO_4, 0.05% $MgCl_2$, 0.5% NaCl, 0.04% glutamine, 0.06% glutamate, and 0.02% oxalic acid was determined. We were able to produce HA with a molecular weight of 5.9×10^6 at a productivity of 6.94 g/l on pilot scale fermentation.

Keywords Hyaluronic acid · *Streptococcus* · Medium optimization · Statistical analysis · Fermentation

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Introduction

Hyaluronic acid (HA) is a uniformly repetitive, linear, high-molecular-weight glycosaminoglycan composed of 2,000–25,000 disaccharides of glucuronic acid and *N*-acetylglucosamine joined alternately by β-1-3 and β-1-4 glycosidic bonds [4]. Owing to its variety of biological functions, HA has a wide range of applications in the fields of medicine and cosmetics, including osteoarthritis treatment, ophthalmic surgery, plastic surgery, drug delivery, skin moisturizers, and wound healing [4, 12, 20].

It is well known that HA can be obtained commercially through three routes: human umbilical cords, rooster combs, and strains of group C *Streptococcus*. However, the first two routes have some disadvantages, such as relatively low yields, contamination, and risk of cross-species viral injections. Hence, strains of *Streptococcus* sp. have been used to produce HA industrially. Much work, such as improving the fermentation process [1, 3, 10, 13–16, 18, 19, 22], adding lysozyme [18, 24], the alkaline-stress strategy [21], adding hydrogen peroxide and ascorbate [23], and changing the medium composition [27, 30], has been done to increase the production yield of hyaluronic acid in *Streptococcus* sp. Although many studies have been performed on HA production, there are very few reports on general medium optimization for high-molecular-weight HA production and on economically efficient conditions for HA production.

In this study, we carried out optimization of medium components to produce high-molecular-weight HA in *Streptococcus* sp. ID9102 (KCTC 11935BP) that lacked hemolytic activity and hyaluronidase industrially. In the first step, the effects of medium components, such as carbon sources, nitrogen sources, phosphate sources, and mineral sources, on HA production were investigated by the

one-factor-at-a-time method. In the second step, the concentration of selected medium components was optimized using taguchi orthogonal array design. In addition, the effects of various amino acids and organic acids were studied in order to improve the productivity and molecular weight of HA. The optimized medium was applied in a 75-l jar fermenter to produce high-molecular-weight HA on a pilot scale.

Materials and methods

Organism

Streptococcus sp. ID9102 designed as a nonhemolytic and hyaluronidase-negative mutant strain by N-methyl-N′-nitro-N-nitroso-guanidine (NTG) was used as the HA producer in this study. This strain was deposited with the Korean Collection for Type Cultures (KCTC) with the accession number KCTC 1139BP.

Inoculum, culture media, and conditions

The stock culture preserved in 20% glycerol solution at −72°C was plated in brain heart infusion (BHI, Difco) medium containing 1.5% agar and incubated at 37°C for 24 h. A loopful of cells from the agar slant was transferred to 40 ml of sterilized Todd Hewitt broth (THB, Difco) medium in a 250-ml Erlenmeyer flask as seed culture and incubated at 37°C on a rotary shaker at 120 rpm for 6 h. This was used as the inoculum for flask culture. For the production of HA, the flask culture was placed in 250-ml Erlenmeyer flasks, each containing 40 ml of HA production medium. The HA production medium was aseptically inoculated with 2 ml of 6-h-old seed culture. The inoculated flasks were incubated on a rotary shaker at 37°C and 120 rpm for 24 h. In order to produce HA and optimize medium components, the basal medium comprised 4.0% glucose, 0.5% yeast extract, 0.25% K_2HPO_4, 0.07% $MgSO_4$, and 0.5% NaCl. The pH of the medium was adjusted to 7.0 using 0.1 N NaOH or HCl before sterilization. The medium was sterilized in an autoclave for 20 min at 121°C, except glucose. Glucose solution was sterilized by autoclave separately. All flask cultures were carried out in triplicate.

Batch fermentation in a 75-l jar fermenter

A total of 80 ml of the first seed culture was inoculated into a 2-l jar fermenter containing 1.8 l of THB medium. This was used as the second seed culture for the 75-l jar fermentation. The second seed culture was inoculated into the 75-l jar fermenter containing 45 l of production medium. The temperature was maintained at 36°C, and the aeration rate was 0.5 vvm. The agitation was provided by two six-bladed disk turbine impellers. The diameters of the impeller and vessel were 120 mm and 350 mm, respectively. The pH was controlled automatically at 7.0 by adding 10 N NaOH solution. Batch fermentation was performed for 24 h in a 75-l jar fermenter (Bioengineering, Switzerland) with a working volume of 45 l.

Statistical analysis

Taguchi orthogonal array design was carried out for optimization of selected medium components (yeast extract, casein peptone, $MgCl_2$, and K_2HPO_4) on HA production by the strain *Streptococcus* sp. ID9102 (KCTC 11935BP). Four medium factors and four different levels were designed as a L_{16}-orthogonal array. The design was developed and analyzed using MINITAB 14 software. Table 1 shows the cultivation conditions and the L_{16}-orthogonal array design used in this study.

Analytical methods

Cell growth was observed by measuring the optical density of the culture broth at 600 nm using a spectrophotometer. The glucose concentration was determined using the glucose assay kit (Sigma). HA concentration in the culture broth was determined by the turbidimetric method [5, 11]. Broth samples were digested by an equal volume of 0.1% (w/v) sodium dodecyl sulfate (SDS) and incubated at room temperature for 10 min to release the capsular HA [5]. The

Table 1 L_{16}-orthogonal array design for HA production

Run	Factors				Medium components (%)				HA (g/l)
	A	B	C	D	A	B	C	D	
1	1	1	1	1	0.25	0.25	0.15	0.03	1.123
2	1	2	2	2	0.25	0.5	0.25	0.05	1.934
3	1	3	3	3	0.25	0.75	0.35	0.07	1.724
4	1	4	4	4	0.25	1.0	0.45	0.09	1.232
5	2	1	2	3	0.5	0.25	0.25	0.07	1.345
6	2	2	1	4	0.5	0.5	0.15	0.09	1.616
7	2	3	4	1	0.5	0.75	0.45	0.03	1.447
8	2	4	3	2	0.5	1.0	0.35	0.05	2.428
9	3	1	3	4	0.75	0.25	0.35	0.09	1.567
10	3	2	4	3	0.75	0.5	0.45	0.07	1.835
11	3	3	1	2	0.75	0.75	0.15	0.05	2.092
12	3	4	2	1	0.75	1.0	0.25	0.03	2.246
13	4	1	4	2	1.0	0.25	0.45	0.05	1.970
14	4	2	3	1	1.0	0.5	0.35	0.03	1.728
15	4	3	2	4	1.0	0.75	0.25	0.09	2.067
16	4	4	1	3	1.0	1.0	0.15	0.07	1.562

A Yeast extract, *B* casein peptone, *C* K_2HPO_4, *D* $MgCl_2$