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Production and optimization of hyaluronic acid extracted from *Streptococcus mutans*

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Abstract
Historically, hyaluronic acid (HA) was extracted from animal sources, mainly rooster combs. Coupled with the frequent occurrence of animal epidemics, source cross-infection events lead to increased health and safety concerns, thus limiting the application of HA in biomedicine and clinical practice. 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 mutans*, which may create up to 7 g/L of HA under optimal growth circumstances.

This study investigated the optimal conditions for producing hyaluronic acid from the *Streptococcus mutans* bacterial strain. The isolated *Streptococcus mutans* were cultured on MRS broth, Skim milk, and M17 broth with an addition of 1% lactose. The bacterial strains were grown in 100 ml of culture media, placed in volumetric flasks of 250 ml capacity, and incubated at 42˚C for 24 hours, pH 6.8, inoculum volume 1%, and a vibrating incubator at 150 rpm. After the end of the fermentation period, the isolation and purification of HA have performed accordingly: proteins were removed using 1% trichloroacetic acid (TCA), and HA in the supernatant was collected by isopropanol precipitation. The collected HA was dialyzed against ultrapure water and lyophilized. The amount of acid produced was estimated. The results show that the best production of hyaluronic acid was from the *S. thermophilus* bacterial strain grown on the alternative medium containing whey at a ratio of 450 ml/L and7.5 g/L yeast extract at 40 ˚C, with a 3% of inoculum volume and 102×10^8 colony-forming units/ml of bacterial cells, in pH 6.8 and agitation speed of 150 rpm for 18 h, which had the most significant effect on the fermentation process and gave the highest value of HA production of 0.598 g/L and biomass of 6.08 g/L. These results showed the best production method for HA to achieve maximal production yield.

Keywords: Hyaluronic acid, *Streptococcus mutans*, fermentation optimization, microbial production, microbial production

Introduction

Glycosaminoglycans (GAGs) are a class of straight-chain acidic polysaccharides that play a broad range of essential biological roles. They are widely distributed in the extracellular matrix and on the cell surfaces of animal tissues. They interact with signaling molecules and play a role in regulating cell proliferation and differentiation [1, 2]. GAGs can be classified into four main classes based on their structures: hyaluronic acid (HA), heparin sulfate, chondroitin sulfate, and keratin sulfate. HA is the only non-sulfated linear polysaccharide in the GAG family that is not bound to proteins. The basic structure of HA is composed of dglucuronic acid (GlcUA) and N-acetyl-D-glucosamine (GlcNAc), which are alternately linked by β-1,3 and β-1,4 glycosidic bonds. HA is widely used in medicine, cosmetics, and food products because of its unique viscoelasticity, hygroscopicity, non-immunogenicity, and biocompatibility properties [3, 4, 5]. In medical applications, HA acts as a lubricant to protect the ends of bones [6]. In cosmetics, an aqueous solution of HA is primarily used to form a viscoelastic gel that can be applied to the skin to moisturize, rejuvenate, and improve wound healing [7]. Studies on the use of HA in food have focused on increasing the amount of HA in the body via oral administration. Schwartz *et al*. reported that oral dietary supplements containing HA reduced facial wrinkles and increased skin elasticity and collagen content [8]. In addition, oral HA supplementation prevents symptoms such as arthritis, arteriosclerosis, and an irregular pulse.

HA is primarily derived from animal tissues and microbial fermentation processes. Due to the limited quality and quantity of raw materials, HA yield costs are high. Coupled with the frequent occurrence of animal epidemics, source cross-infection events lead to increased health and safety concerns, thus limiting the application of HA in biomedicine and clinical practice [9, 10]. In recent years, microbial fermentation has gradually replaced tissue extraction as the main source of HA, mainly using the fermentation of *Streptococcus zooepidemicus* [11, 12]. The mechanism for HA synthesis has been continuously analyzed due to the continuous developments in synthetic biology [13, 14]. The use of microorganisms with well-defined genetic backgrounds and high biosecurity to synthesize HA has become a trend in developing microbial fermentation to synthesize HA. The HA synthesis pathway has been successfully developed for efficient recombinant HA yield in safe microbial hosts, such as Bacillus subtilis [15], *Corynebacterium glutamicum* [16], and *Bacillus amyloliquefaciens* [17]. As shown in our previous studies, compared to the traditional *B. subtilis chassis*, B. *amyloliquefaciens* is an important, safe microbial host that has been developed for the production of biopolymers such as HA [17], poly (γ-glutamic acid) [18, 19], and other highvalue-added chemicals such as ornithine [20]. Although Ma *et al*. confirmed that the HA synthesis pathway constructed in *B. amyloliquefaciens* could synthesize HA, its yield was 2.89 g/L, which was still low [17].

In the present study we have used *Streptococcus mutans* obtained from dental caries as alternative organism to *Streptococcus zooepidemicus*. The yield was considerable eventhough it is a wild strain.

Materials & Methods

Isolation of bacteria

Swabs from dental caries of a five-year child were obtained from a local dental hospital and streaked across M17 agar medium in Petri dishes. The plates were incubated at 42° C for 48 h under aerobic conditions [10]. Round mucoid colonies representing *S. mutans* were selected and the organism confirmed by IMVIC tests.

Three culture media were used to produce MRS broth, Skim milk, and M17 broth, adding 1% lactose [11]. The diagnosed bacterial strains were grown in 100 ml of culture media, placed in volumetric flasks of 250 ml capacity, and incubated at 42˚C for 24 hours, pH 6.8, inoculum volume 1%, and a vibrating incubator at 150 rpm [12]. Biomass was estimated according Izawa, Serata [13].

Extraction and purification of hyaluronic acid

After the end of the fermentation period, the isolation and purification of HA were performed by the procedure described previously [14]. Briefly, proteins were removed using 1% trichloroacetic acid (TCA), and HA in the supernatant was collected by isopropanol precipitation. The collected HA was dialyzed against ultrapure water and lyophilized. The amount of acid produced was estimated according to the method of Sciabica [15].

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 H2SO4, the test detects the glucuronic acid that is liberated.

Optimal conditions for HA production

Several carbon sources were used to replace it with lactose in the medium of optimal production. The prepared alternatives included date juice according to what was mentioned in Al-Roomi and Al-Sahlany [16], grape juice [17, 18], and whey [19]. The total lactose of these substitutes was estimated by the Lane-Eynon method mentioned in Ranganna [20]. Lactose constitutes 20 g/L in the medium of optimal production, with equivalents consisting of date juice, grape juice, and whey with the ratios of 58, 210.5, and 321.15 ml/L ml of medium, respectively.

The remaining ingredients were added, 1% inoculated from the activated bacterial culture, and incubated at 42˚C in a vibrating incubator at a speed of 150 rpm for 24 hours. The hyaluronic acid production was estimated to select the best concentration of whey to be used as a substitute for the carbon source for acid production.

Effect of various physical parameters on HA production and biomass like incubation temperature (35-42°C), inoculum volume (0.5-10%), pH (5.5-7.8), fermentation period (6-48 hours), and different agitation speed (100-400 rpm) were studied in alternative production medium under shake flask using *S. mutans.*

Results

As previously documented (Armstrong and Johns, 1997) [4], 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 Acid Molecules 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. These circumstances favour a slower growth rate and more hyaluronic acid production, resulting in a yield greater than 5 g/l.

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	100	3.5	0.57	57	342	16.6
Silica gel	90	3.3	0.16	290	13.8	4.8
Carbon	90	3.2	0.03	280	1.9	0.7
Diafiltration	128	1.8	0.002	216.5	0.07	0.15
0.22 µ M Filtration	128	1.8	0.002	216.5	0.07	0.15
Hyaluronic acid was precipitated from clarified broth with 1:1 2-propanol and resuspended in 3% sodium acetate. 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 40C), the hyaluronic acid solution was clarified. At a flow rate of 14 ml/min, hyaluronic acid solution was filtered via a 0.45 µm charcoal filter assembly. 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. A 0.22 µm filter was used to sterilise the hyaluronic acid solution resulting from the diafiltration procedure.						

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

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.

Conclusion

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%. Consequently, the procedure described here is straightforward, cost-effective, and repeatable, yielding a high output of hyaluronic acid.

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