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Compound mutation breeding of *Streptococcus equisimilis* ZC-95 for producing high molecular weight hyaluronic acid and optimization of culture conditions

Ronghu Zhang[1], Jiancheng Feng[1,2]*

[1]Key Laboratory of Tropical Biological Resources of Ministry of Education, Hainan University, Haikou 570228 (CHINA)

[2]College of Materials and Chemical Engineering, Hainan University, Haikou 570228 (CHINA)

E-mail : zrh0912@126.com; fjc197228@126.com

ABSTRACT

A strain of mutant *S. equisimilis* ZC-95, which can produce high molecular weight (HMW) hyaluronic acid (HA), was obtained from compound mutation breeding of *S. equisimilis* ZC-0 using 5-Bromouracil, UV rays and nitrosoguanidine as mutagenic agents. After inoculation for 20 generations, *S. equisimilis* ZC-95 showed inheritance stability, but not hemolysis. The culture conditions for producing HMW-HA were optimized in a 5-L fermentor through single-factor and multi-factor orthogonal tests. Results showed that cheap corn steep liquor was an appropriate nitrogen source for *S. equisimilis* ZC-95, and the optimum ratio of fermentation medium comprised 50 g/L sucrose, 80 g/L corn steep liquor, 10 g/L glucose, 2 g/L $MgSO_4$, 2 g/L $NaHCO_3$, 2 g/L Na_2HPO_4, and 0.1 g/L UTP. Under these fermentation conditions, the molecular weight of the produced HA was 2.76×10^6 Da, and HA yield reached 4.12 g/L.

KEYWORDS

Compound mutation; High molecular weight; Hyaluronic acid; Culture conditions; *Streptococcus equisimilis.*

INTRODUCTION

Hyaluronic acid (HA) is a linear polymer mucopolysaccharide formed from the alternative connection of disaccharide units, which are composed of β-1,3-N-acetyl glucosamine and β-1,4-D-glucuronic acid[1]. This substance has unique viscoelasticity, but shows no immunogenicity or toxicity. HA applications are mainly determined by molecular weight. High molecular weight HA (HMW-HA) has good viscoelasticity and can be used in ophthalmology and anaplasty, whereas low molecular weight HA can be used as a moisture factor in cosmetics[2]. HA can be obtained from three main sources, namely, human umbilical cord, cockscomb, and cultures of hemolytic Lancefield group A and C streptococci. The first two sources are inferior because of the low output, the risk of cross-species viral infection, and the high cost of HA purification. In comparison, the third source presents technical, economical, and ethical advantages. Therefore, bacterial fermentation replaced animal tissue extraction as the first HA production model[3].

Lancefield group A and C streptococci are the two major strains used to produce HA. Compared with streptococci-A, which has strong pathogenicity, streptococci-C involves non-human pathogenic bacteria, such as *Streptococcus zooepidemicus*, *S. equi*, and *S. Equisimilis*[4]. Existing research on bacteria selection and optimization of fermentation conditions mainly focuses on *S. zooepidemicus* and *S. equi*[5-7]. Information is lacking on HWM-HA production from *S. equisimilis* fermentation[8]. At present, HA quality rather than quantity is the focus of bacteria selection and process optimization studies. Molecular weight is the most important influencing factor of HA quality[1]. Acquisition of mutant strain for HMW-HA production and optimization of corresponding culture conditions are important solutions to improve HA quality[4,9]. In this paper, mutant strains for HMW-HA production were screened from compound mutation breeding of *S. equisimilis* ZC-0 using 5-Bromouracil (5-BrU), UV rays, and nitrosoguanidine (NTG) as mutagenic agents. The corresponding culture conditions were optimized in a 5-L fermentor through single-factor and multi-factor orthogonal tests.

MATERIALS AND METHODS

Bacteria and culture medium

S. equisimilis ZC-0, which was used as the mutation bacteria, was kept in the Key Laboratory for Tropical Biological Resources, Ministry of Education (Hainan University, Haikou City, China). Martin Broth (MB) medium comprised 30 g/L MB and 10 g/L yeast extract. Blood Agar Plate (BAP) was produced by adding 10% sterilized defibrinated sheep blood (v/v) and 15 g/L agar powder to the MB. Fermentation medium comprised 40 g/L glucose, 70 g/L tryptone, 2 g/L $MgSO_4$, 1.5 g/L $NaHCO_3$, and 2 g/L Na_2HPO_4.

Compound mutation and mutant strain screening

A single colony of *S. equisimilis* ZC-0 was inoculated in 10 mL MB at 37 °C for 16 h. Grown cells were collected by centrifugation at 2,500 *g* for 10 min and washed with 0.02 M NaCl solution. The cells were starved, and 5-BrU was added at a concentration of 40 μg/mL. The mixture was processed for 30 min, and 5 mL of the processed solution was injected into a 60 mm culture dish for UV mutagenesis (power, 20 W; wavelength, 254 nm; exposure time, 90 s; and exposure distance, 20 cm). The bacterial solution was transferred into 25 mL MB, which was incubated in the dark at 37 °C for 6 h. Grown cells were collected by centrifugation at 2,500 *g* for 10 min and resuspended in 50 mM pH 7.0 phosphate-buffered solution containing 300 μg NTG/mL for chemical mutation. After NTG treatment, the cells were incubated at 37 °C for 3 h in MB and plated on BAP. Strains without hemolytic zone on BAP were selected and fermented in the shake flask. HA outputs and molecular weights were tested. Experiments were performed in triplicate, and the results are presented as mean values. Strains with high HA outputs and molecular weights were selected and stored on a divided slope.

Inheritance stability test of the mutant strain

The screened mutant strain was inoculated on the agar slope (recorded as Generation 1). After growth was achieved, the strain was inoculated onto another fresh agar slope (recorded as Generation 2). This procedure was repeated until Generation 20 was obtained. *S. equisimilis* ZC-0 was considered as the control group. The diameters of the hemolytic zone of the control group, Generation 1, and Generation 20 on BAP were measured. Cell growth, molecular weight and HA output were tested after fermentation in a shake flask. Experiments were performed in triplicate, and the results were given as mean values.

Multi-factor orthogonal test

A multi-factor cross experiment was carried out for investigating the effects of medium component on hyaluronic production by the strain *S. equisimilis* ZC-95. Three different levels and seven medium factors (sucrose, corn steep liquor, glucose, $MgSO_4$, $NaHCO_3$, Na_2HPO_4, and UTP) were used to design an orthogonal experiment of $L_{18}(3^7)$ form. Sucrose and glucose were taken as carbon source factors, and corn steep liquor were used as nitrogen source factor in the medium. $MgSO_4$, Na_2HPO_4, and $NaHCO_3$ were used as mineral salts, and UTP was used as nucleotide adding factor. Batch culture experiments were performed in a 5-L fermentor with a working volume of 1 L for 44 h at 37 °C. Other conditions were as follows: aeration, 0.5 vvm; agitation, 400 rpm. Experiments were performed in triplicate, and the results are reported as mean values.

Analytical method

To prevent interference in the assay by media components, the HA in the cell-free broth was precipitated with 2-propanol (1:1 v/v) and re-dissolved in 3% sodium acetate (w/v). HA output was determined by carbazole method[5]. The intrinsic viscosity of HA was measured using an Ubbelohde viscometer to evaluate HA MW[9]. Cell growth was monitored by measuring the optical density of the culture broth at 620 nm.

RESULTS

Strain screening for HMW-HA production

A total of 101 mutant strains without hemolysis were obtained from compound mutation breeding. Among these strains, three could produce HMW-HA (Figure 1). After compound mutation, molecular weights and HA outputs produced by these 3 mutant strains were significantly higher than those of *S. equisimilis* ZC-0. In particular, *S. equisimilis* ZC-95 had the highest molecular weight (2.05×10^6 Da) and HA output (3.53 g/L) after fermentation in the shake flask. Therefore, *S. equisimilis* ZC-95 was selected for inheritance stability testing.

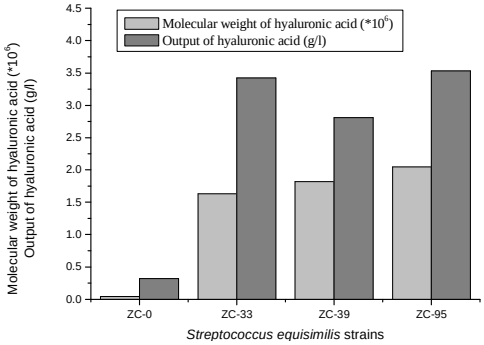

Figure 1 : HA fermentation of wild-type and mutant strains. *S. equisimilis* ZC-0 was the wild-type strain, whereas *S. equisimilis* ZC-33, ZC-39, and ZC-95 were the mutant strains screened from compound mutation. All strains were submersion-cultured in fermentation medium by batch culture in a 1 L shake flask for 44 h at 37 °C and at a speed of 350 rpm.

Inheritance stability of *S. equisimilis* ZC-95

S. equisimilis ZC-0, as the control group, presented evident hemolysis on BAP. However, neither Generation 1 nor Generation 20 showed hemolysis, indicating that *S. equisimilis* ZC-95 remained non-hemolytic after inoculation for 20 generations (Figure 2A). Biomass grew rapidly to a maximum level at 16 h, and there was no significant difference between Generation 1 and Generation 20 (Figure 2B). The molecular weight and HA output produced by Generation 1 of *S. equisimilis* ZC-95 were 2.02×10^6 Da and 3.48 g/L, respectively, whereas Generation 20 produced 1.95×10^6 Da and 3.32 g/L, respectively (Figure 2C). These results reflected the stable HA productivity of *S. equisimilis* ZC-95. *S. equisimilis* ZC-95 had high inheritance stability.

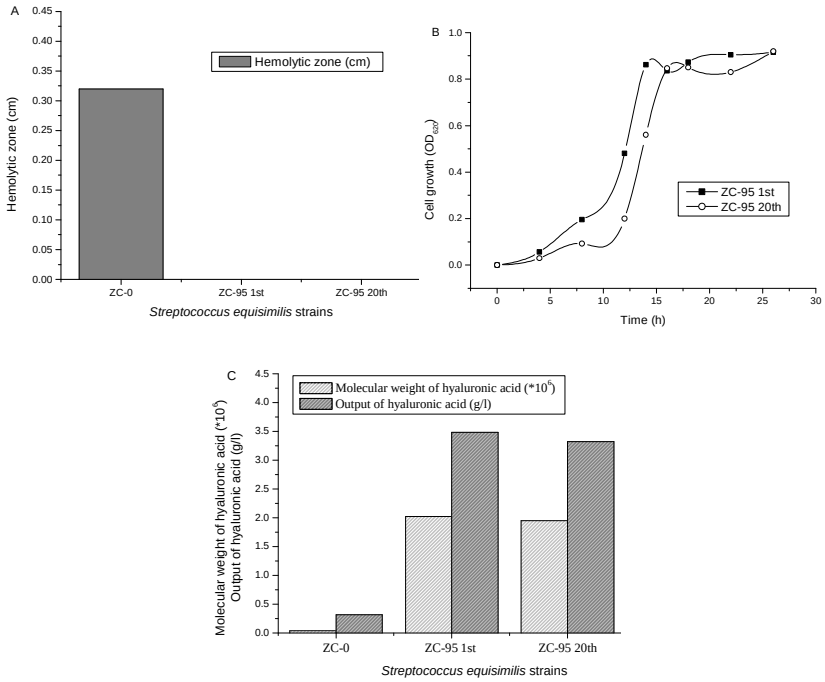

Figure 2 : Comparison of growth and HA production in Generation 1 (ZC-95 1st) and Generation 20 (ZC-95 20th) by *S. equisimilis* ZC-95. *S. equisimilis* ZC-0 was designated as the control group. A. All strains were incubated on Blood Agar Plate (BAP) at 37 °C for 48 h. B. Both strains were submersion-cultured in fermentation medium by batch culture in a 1 L shake flask at 37 °C for 44 h and at a speed of 350 rpm. C. All strains were submersion-cultured in fermentation medium by batch culture in a 1 L shake flask at 37 °C for 44 h and at a speed of 350 rpm.

Effect of nitrogen source on HA production of *S. equisimilis* ZC-95

Tryptone, yeast extract powder and meat extracts (beef) for laboratory use, as well as corn steep liquor, soy peptone and malt extract for industrial use were used as nitrogen source of fermentation medium. Strains were submersion-cultured in fermentation medium by batch culture in a 5-L fermentor for 44 h at 37 °C. Other conditions were as follows: aeration, 0.5 vvm; agitation, 400 rpm. Results are presented in TABLE 1.

Among the three common nitrogen sources used in the laboratory, tryptone was the best source for *S. equisimilis* ZC-95 for HA production. Among the three plant-derived nitrogen sources for industrial use, corn steep liquor was the best. HA output and molecular weight of *S. equisimilis* ZC-95 were 3.22 g/L and 1.92×10^6 Da, respectively, when corn steep liquor was the nitrogen source. HA output and molecular weight were 3.45 g/L and 2.01×10^6 Da, respectively, when tryptone was the nitrogen source. Corn steep liquor and tryptone contributed similar outputs and molecular weights of HA. Corn steep liquor, a processing by-product of agriculture products, is used for industrial production because of its abundance and low cost.

TABLE 1 : Effect of nitrogen source on HA production of *S. equisimilis* ZC-95.

Nitrogen source	Output of Hyaluronic acid (g/L)	Molecular weight of Hyaluronic acid ($\times 10^6$ Da)
Tryptone	3.45	2.01
Yeast extract powder	3.25	1.88
Meat extracts (beef)	3.11	1.65
Corn steep liquor	3.22	1.92
Soy peptone	2.35	1.82
Malt extract	2.65	1.47

Experiments were performed in triplicate, and the results are reported as mean values

Effect of medium components on HMW-HA production

To determine the optimum culture conditions of *S. equisimilis* ZC-95 during fermentation, a multi-factor orthogonal test was implemented. In the table, K_{jm} is sum of the test results of factors in the j column (j = A, B...G) and level m (m = 1, 2, 3); X_{jm} is the mean of K_{jm} ($X_{jm} = K_{jm}/6$) and $R_j = (X_{jm})\max - (X_{jm})\min$. The values of R_j were consistent with the effects of factors on various levels. A higher R_j value indicates that greater effect was obtained in the j factor. Results are presented in TABLES 2 and 3.

The significance values of different medium components are in the following order: sucrose > corn steep liquor > $MgSO_4$ > glucose > UTP > Na_2HPO_4 > $NaHCO_3$. The optimum medium comprised the following: 50 g /L sucrose, 80 g/L corn steep liquor, 10 g/L glucose, 2 g/L $MgSO_4$, 2 g/L $NaHCO_3$, 2 g/L Na_2HPO_4, and 0.1 g/L UTP. Under such cultureconditions, the HA output reached 4.12 g/L, and the molecular weight was 2.76×10^6 Da. These were significantly higher values than those obtained prior to optimization.

TABLE 2. Effects of the medium composition on HA production by *S. equisimilis* ZC-95.

$L_{18}(3^7)$	A (g/L)	B (g/L)	C (g/L)	D (g/L)	E (g/L)	F (g/L)	G (g/L)	Molecular weight of hyaluronic acid ($\times 10^6$ Da)
	Sucrose	Corn steep liquor	Glucose	$MgSO_4$	$NaHCO_3$	Na_2HPO_4	UTP	
1	30	70	15	2	1	2	0.2	0.75
2	40	70	5	1	1.5	1	0.1	1.237
3	50	70	10	3	2	3	0.3	2.063
4	30	80	10	1	2	1	0.2	0.989
5	40	80	15	3	1	3	0.1	1.724
6	50	80	5	2	1.5	2	0.3	2.48
7	30	90	5	2	2	3	0.1	0.881
8	40	90	10	1	1	2	0.3	1.524
9	50	90	15	3	1.5	1	0.2	2.251
10	30	70	5	3	1	1	0.3	0.481
11	40	70	10	2	1.5	3	0.2	1.527
12	50	70	15	1	2	2	0.1	2.032
13	30	80	15	1	1.5	3	0.3	0.812
14	40	80	5	3	2	2	0.2	1.682
15	50	80	10	2	1	1	0.1	2.683
16	30	90	10	3	1.5	2	0.1	0.968
17	40	90	15	2	2	1	0.3	1.718
18	50	90	5	1	1	3	0.2	2.11

TABLE 3 : Analysis of variance of medium composition on HA production by *S. equisimilis* ZC-95.

	Sucrose	Corn steep liquor	Glucose	$MgSO_4$	$NaHCO_3$	Na_2HPO_4	UTP
K_{j1}	4.884	8.09	8.874	8.706	9.27	9.36	9.528
K_{j2}	9.414	10.37	9.75	10.04	9.276	9.438	9.312
K_{j3}	13.62	9.45	9.29	9.168	9.366	9.12	9.078
X_{j1}	0.814	1.348	1.479	1.451	1.545	1.56	1.588
X_{j2}	1.569	1.728	1.625	1.673	1.546	1.573	1.552
X_{j3}	2.27	1.575	1.548	1.528	1.561	1.52	1.513
R_j	1.456	0.38	0.146	0.222	0.015	0.053	0.075

DISCUSSIONS

The use of mutant strains for HMW-HA production is one of the key methods for improving HA quality. Research on the culture of non-hemolytic mutant strains for HMW-HA production based on chemical mutation and a series of screenings has been conducted[3,7]. However, in previous studies, *S. zooepidemicus* and *S. equi* have been used. Few studies used *S. equisimilis* for mutation. Y.H.Chen[8] induced physical mutation of *S. equisimilis* CVCC55116 using UV and $_{60}$Co-γ rays. He reported that the mutant strain showed high HA output. However, he did not analyze HA molecular weight changes. To date, no study on integrated physical and chemical mutation, especially UV and 5-BrU successive mutations, of *S. equisimilis* is available. In this paper, the non-hemolytic mutant strain screened from the compound mutation of *S. equisimilis* ZC-0 using 5-BrU, UV rays, and NTG as mutagenic agents showed high inheritance stability and could be used to produce HMW-HA. 5-BrU mutation is used to induce A-T to G-C transitions, thereby changing the molecular structure of the bacterial DNA. 5-BrU can increase the mutation rate of strains in combination with UV mutation. 5-BrU is viewed as a sensitizer of irradiation mutation[10]. NTG, a "super mutagenic agent", is an effective chemical mutagen that is used to induce mutation[7]. Consequently, the compound mutagen of 5-BrU, UV rays, and NTG easily caused mutation and resulted in strains that could produce HMW-HA.

Several reports have stated that the optimization of cultureconditions can increase HA output[6,11]. Because of increasing demand for HA, researchers are focusing on how to lower fermentation production cost and overcome the growing waste pollution problems. Fermentation production cost is mainly caused by carbon and nitrogen sources in the culture medium. Therefore, seeking alternative cheaper carbon and nitrogen sources from organic wastes or processing by-products of agriculture products is an important method to lower the cost of HA production. Several scholars have attempted to investigate this topic, but more in-depth studies are required. J.A.Vázquez[12] used mussel processing wastewater as carbon source and tuna peptone from viscera residue as nitrogen source for *S. zooepidemicus* fermentation. He concluded that the molecular weight of the produced HA reached 2.5×10^6 Da, and the production cost decreased by 50%. This strategy also lowered organic waste pollution. A.M.Pires[13] stated that cashew apple juice can replace common glucose and yeast powder as a reliable culture medium for *S. zooepidemicus* fermentation for HA production. In this paper, when cheaper sucrose was used as carbon source and corn steep liquor (processing by-products of corn starch) was used as nitrogen source for *S. equisimilis* ZC-95 fermentation, the relative molecular weight and output of HA reached 2.76×10^6 Da and 4.12 g/L, respectively. This result reveals that cheaper raw materials can be used to produce HMW-HA from *S. equisimilis* ZC-95 culture.

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