J. Microbiol. Biotechnol. (2006), **16**(12), 1849–1855

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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A Novel Approach to the Production of Hyaluronic Acid by *Streptococcus zooepidemicus*

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Received: March 27, 2006
Accepted: July 21, 2006

Abstract It has been shown that the initial conditions of bacterial cultivation are extremely important for the successful production of hyaluronic acid (HA) by fermentation. We investigated several parameters that affect cell growth rate and the productivity and molecular weight of hyaluronic acid--*i.e.*, agitation speed, aeration rate, culture temperature, pH, and pressure--to determine how to optimize the production of HA by *Streptococcus zooepidemicus* on an industrial scale. Using a 30-l jar fermentor under laboratory conditions, we achieved maximum HA productivity and biomass when the agitation speed and aeration rate were increased simultaneously. By shifting the temperature downward from 35°C to 32°C at key levels of cell growth during the fermentation process, we were able to obtain HA with a molecular weight of 2.8×10^6 at a productivity of 5.3 g/l. Moreover, we reproduced these optimized conditions successfully in three 30-l jar fermentors. By reproducing these conditions in a 3-m^3 fermentor, we were able to produce HA with a molecular weight of 2.9×10^6 at a productivity of 5.4 g/l under large-scale conditions.

Key words: Hyaluronic acid, *Streptococcus zooepidemicus*, fermentation

Hyaluronic acid (HA) is a high molecular-weight polymer with repeating units of D-glucuronic acid and *N*-acetylglucosamine that are bound by alternating β-1,3 and β-1,4 bonds [18, 19, 21]. This structural characteristic results in typical pseudoplastic fluid properties that allow it to retain large amounts of moisture. As a result, HA acts like a gel with a high viscosity at low shear rates, but demonstrates high elasticity and low viscosity at high shear rates [14]. Because of these hydrodynamic properties, HA is used as an additive in high-grade cosmetics and eye drops, as well as medicines for ophthalmic surgery and arthritis [4]. HA was originally extracted from bovine vitreous humor and later from many animal tissues, including the umbilical cord, joints, and combs of fowls [16]. It has also been reported that HA can be produced from Lancefield groups A and C streptococci [15].

Commercially, HA is produced through extraction from rooster combs or by microbial fermentation. Because regulations against the use of animal-derived products are becoming increasingly stringent, it is becoming more attractive to produce HA by fermentation [17]. Although there is much evidence from the literature that HA can be obtained by fermentation using *Streptococcus zooepidemicus* and *S. equi* [2, 3, 5–7, 9, 12, 13, 23], few studies have indicated that this process can be carried out efficiently on an industrial scale.

This study was designed to develop a model for producing HA on an industrial scale by identifying optimal fermentation parameters, such as agitation speed, aeration rate, culture temperature, pH, and operating pressure.

MATERIALS AND METHODS

Bacterial Strain

S. zooepidemicus CKD 117, a mutant of *S. zooepidemicus* ATCC 35246 induced by exposure to ultraviolet (UV) light and *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, was used as the source of HA in this study. This strain has nonhemolytic and hyaluronidase-negative characteristics. The stock culture was prepared in the form of working cell bank by using 20% skim milk as a cryoprotectant, and it was preserved in a deep freezer at −75±5°C until the fermentation study began.

Culture Medium

The medium used for the germination culture was composed of 25 g/l trypticase soy broth, 18 g/l yeast extract, 4.5 g/l

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KH_2PO_4, 11.8 g/l $Na_2HPO_4 \cdot 12H_2O$, and 10 g/l glucose. The prefermentation medium contained 15 g/l yeast peptone, 0.8 g/l L(+)-glutamine, 10 g/l yeast extract, 4.18 g/l KH_2PO_4, 5.22 g/l K_2HPO_4, 0.055 g/l NaCl, 1.39 g/l $MgSO_4 \cdot 7H_2O$, 40 g/l glucose, and 0.5 g/l antifoaming agent. The main fermentation medium was prepared by adding 100 g/l glucose to the prefermentation medium.

Cultivation Conditions

The germination culture for the lab-scale experiments (30-l jar fermentor; KF-30L, Korea Fermentor Company, Ltd., Korea) and the large-scale experiments (3-m^3 fermentor; Altieri Asrl, Italy) was inoculated with 7 ml of working cells into a 7-l round flask containing 700 ml of the germination medium. The flask was cultivated at 35°C for 6 to 8 h under static conditions, and its contents were injected into the prefermentation medium when it achieved an optical density of 1 to 2 at 600 nm (OD_{600}) and a pH of 5.2 to 5.4. Preculturing was carried out in a 30-l jar fermentor with a working volume of 15 l for use in the lab-scale experiment and in a 200-l pilot fermentor with a working volume of 120 l for use in the large-scale experiment. Both cultures were incubated at 35°C for 8 to 10 h and then transferred to the main fermentor when an OD_{600} of 5 to 7 was achieved and the pH fell in the range of 5.5 to 5.8.

The lab-scale experiment for optimizing the culture conditions was carried out in a 30-l jar fermentor with a 15-l working volume. Fermentation parameters (*e.g.*, agitation speed, aeration rate, culture temperature, pH, and pressure) were varied according to the study design. The main fermentation was carried out with 6% of the inoculum, and the pH of the culture broth was maintained by adding 10 N NaOH. The fermentor used for the large-scale experiment was a regular stirred tank with 3 Rushton turbines, 4 baffles, blades measuring 170 mm wide and 110 mm tall (which is larger than the standard size), and a total volume of 3 m^3.

Analytical Methods

Cell growth was monitored by the broth OD using a UV-Vis spectrophotometer (Hewlett Packard, Waldbronn, Germany) at 600 nm. The glucose concentration was monitored using a glucose analyzer (YSI model 2700, Yellow Springs Instrument Incorporated, Yellow Springs, OH, U.S.A.). HA concentrations in the culture broth were measured using the carbazol method [10]. The molecular weight of HA was determined by high-performance liquid chromatography using a column equipped with a refractive index detector (Waters, Milford, MA, U.S.A.) and a gel permeation chromatography program. A column of ultrahydrogel 2000 was used, with polyethylene oxide (Sigma Chemical Company, St. Louis, MO, U.S.A.) serving as a reference standard.

Experimental Design and Statistical Analysis

The central composite design [22] was performed to assess the effect of agitation speed and aeration rate on HA production and cell growth. Statistical analysis system (SAS) [20] was used for the statistical analysis.

RESULTS AND DISCUSSION

Effect of Agitation Speed, Aeration Rate, and Operating Pressure

HA fermentation is as strongly influenced by mass transfer of the fermentation broth as it is by other aspects of microbial biopolymer production. Therefore, the effects of the agitation speed, aeration rate, and operating pressure--which can play an important role in mass transfer--were investigated. The preliminary results of these experiments indicated that the cell growth for *S. zooepidemicus* was

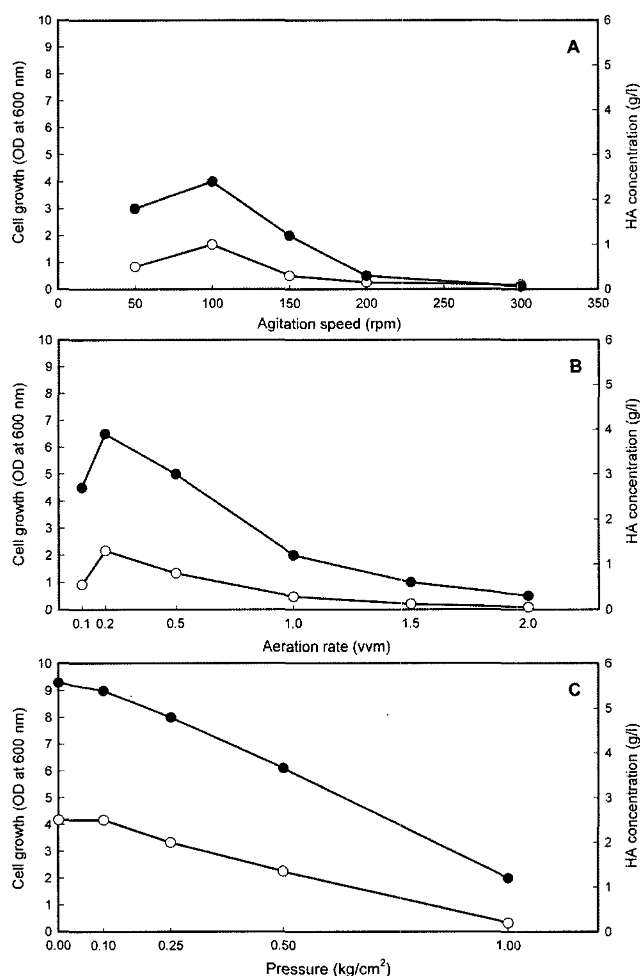

Fig. 1. Effects of the initial agitation speed, aeration rate, and operating pressure on the HA production and cell growth for *S. zooepidemicus*.

Symbols: ●, cell growth; ○, HA concentration.

highly influenced by the initial operating conditions for bacterial cultivation.

Fermentation was carried out at a constant temperature of 35°C, an aeration rate of 0.5 vvm, an operating pressure of 0.5 kg/cm^2, and a pH of 7.0, while the agitation speed varied (50, 100, 150, 200, and 300 rpm). Maximum cell growth was observed at an agitation speed of 100 rpm. Cell growth was poor at agitation speeds exceeding 150 rpm and much worse at an agitation speed of 300 rpm (Fig. 1A). This finding suggests that the initial agitation speed is a critical factor in the optimization of cell growth and could be a key factor to consider for a scaled-up production of HA.

The temperature was maintained at 35°C, the agitation speed at 100 rpm, the operating pressure at 0.5 kg/cm^2, and the pH at 7.0 during the actual fermentation process. The effect of the initial aeration rate was investigated by increasing it from 0.1 vvm to 2.0 vvm (Fig. 1B). The aeration rate and the agitation speed were both found to have significant effects on the initial cell growth. At aeration rates exceeding 0.5 vvm, the rate of cell growth decreased. This suggests that a low aeration rate is favorable for cell growth during the early stages of fermentation. An aeration rate of 0.2 vvm appeared to be optimal for the initial cell growth, resulting in a broth OD_{600} of 6.5 and an HA productivity of 1.3 g/l.

The effect of the operating pressure on HA production and cell growth was investigated under the conditions of a constant temperature of 35°C, an agitation speed of 100 rpm, an aeration rate of 0.2 vvm, and a pH of 7.0, while various operating pressures were used: 0, 0.1, 0.25, 0.5, and 1.0 kg/cm^2 (Fig. 1C). As the operating pressure increased, the HA productivity and cell growth rate decreased dramatically. This result indicates that cell growth is adversely affected by the elevated partial pressure of oxygen (pO_2) or the elevated partial pressure of carbon dioxide (pCO_2), as reported by Hasegawa *et al.* [11]. Therefore, HA fermentation should be carried out under a low pCO_2 with sufficient ventilation. To prevent contamination by adventitious agents, an operating pressure of 0.1 kg/cm^2 is recommended.

To obtain the highest HA productivity and cell growth, we investigated the effects of various agitation speeds and aeration rates on cell growth after achieving optimal initial operating conditions. The central composite design was carried out at various agitation speeds from 200 to 500 rpm and aeration rates from 0.5 to 2.0 vvm. The experiment was performed initially under the optimized initial operating conditions. When the OD_{600} of the culture broth reached 4, the agitation speed and the aeration rate were changed according to experimental design. Table 1 shows the HA productivity and cell growth with various agitation speeds and aeration rates determined using the central composite design. Analysis with SAS software showed an R^2 value of 0.881 and 0.930 on cell growth and HA productivity, respectively. Therefore, these values suggested that the proposed experimental design was suitable to simulate the HA production of *S. zooepidemicus*. The estimated parameters from simulation with the model equation are as follows:

$$\text{Cell growth } (OD_{600}) = 12.0834 - 0.017521X + 3.03335Y - 1.17464e^{-0.005}X^2 - 1.77434Y^2 \ (P<0.1)$$

$$\text{Productivity (g/l)} = 2.4804 - 0.000684335X + 0.547762Y - 6.62712e^{-0.006}X^2 - 0.306153Y^2 \ (P<0.1)$$

where X and Y represent the agitation speed and aeration rate, respectively. Hence, the predicted values of cell growth and productivity based on the range of X and Y in response surface methodology were estimated, as illustrated in Fig. 2.

Table 1. Matrix of the experimental design (coded and values) with responses in terms of cell growth and HA production.

Exp. No.	**X** agitation speed (rpm)	**Y** aeration rate (vvm)	Cell growth (OD_{600})	Productivity (g/l)
1	300(0)	2.0(2)	4.70	1.50
2	400(1)	0.5(−1)	2.70	1.10
3	200(−1)	1.5(1)	8.80	2.10
4	200(−1)	2.0(2)	4.50	1.60
5	300(0)	0.5(−1)	7.90	1.93
6	400(1)	1.5(1)	3.60	1.32
7	300(0)	1.5(1)	6.50	1.84
8	200(−1)	0.5(−1)	10.50	2.60
9	200(−1)	1.0(0)	9.70	2.40
10	400(1)	1.0(0)	3.10	1.27
11	300(0)	1.0(0)	8.20	2.05
12	400(1)	2.0(2)	2.90	1.18
13	500(2)	0.5(−1)	0.78	0.54
14	500(2)	1.0(0)	1.10	0.64
15	500(2)	1.5(1)	1.20	0.65
16	500(2)	2.0(2)	1.10	0.59

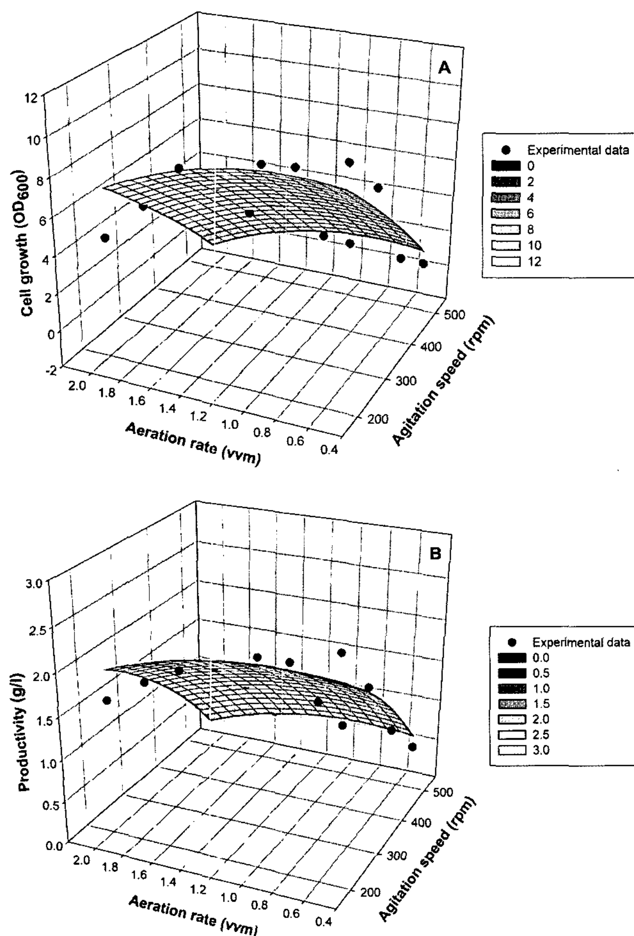

Fig. 2. The response surface curve for the effect of the agitation speed and aeration rate on the HA production and cell growth for *S. zooepidemicus*.
Symbols: curves, predicted value; points, experimental data.

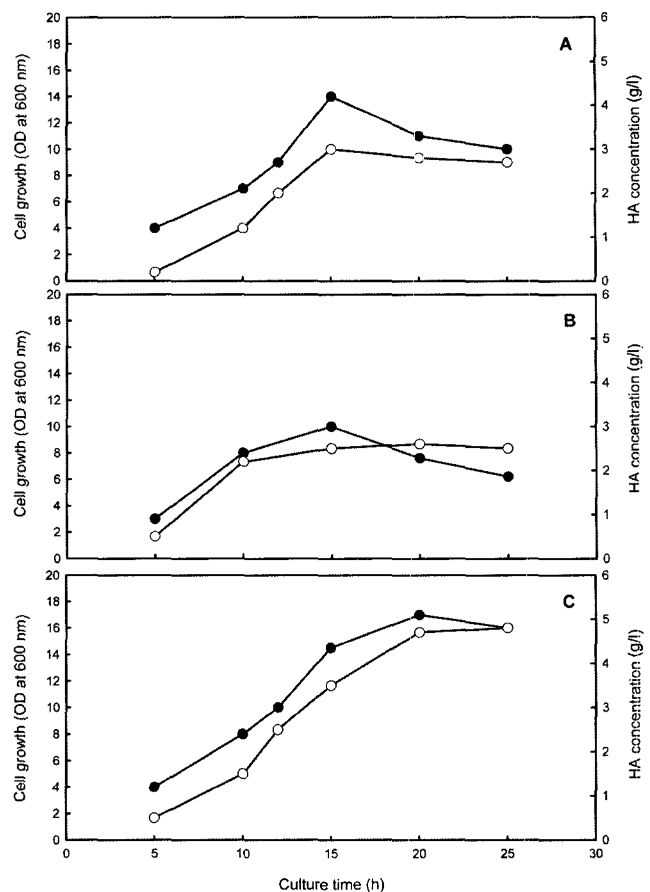

Fig. 3. Comparison of HA production and cell growth for *S. zooepidemicus* at different agitation speeds and aeration rates.
A, Maintain 100 rpm and 0.2 vvm until OD_{600}=4, then increase agitation speeds according to cell growth (200 rpm at OD_{600}=4; 300 rpm at OD_{600}=8; 400 rpm at OD_{600}=12); **B**. Maintain 100 rpm and 0.2 vvm until OD_{600}=4, then increase aeration rates according to cell growth (0.5 vvm at OD_{600}=4; 1.0 vvm at OD_{600}=8; 1.5 vvm at OD_{600}=12); **C**. Maintain 100 rpm and 0.2 vvm until OD_{600}=4, then increase agitation speeds and aeration rates according to cell growth (200 rpm and 0.5 vvm at OD_{600}=4; 300 rpm and 1.0 vvm at OD_{600}=8; 400 rpm and 1.5 vvm at OD_{600}=12). Symbols: ●, cell growth; ○, HA concentration.

It shows that the predicted values matched the experimental data quite well and the predicted maximum value (cell growth: OD_{600}=10.5, productivity: 2.6 g/l) under the operating condition of 200 rpm and 0.5 vvm. It has been reported in the literature that the HA production by *S. zooepidemicus* is growth dependent [13]. Indeed, the same behavior was observed in our fermentation, as the transient profiles of cell growth and HA productivity were quite consistent (Table 1), indicating that the HA production by *S. zooepidemicus* occurred in a growth-associated fashion. In the present study, different agitation speeds and aeration rates were found to have a significant effect on cell growth and HA production. A relatively high agitation speed and aeration rate at specific stages of bacterial growth were favorable for cell growth and HA production. Therefore, a new set of experiments was performed, where the agitation speeds and aeration rates were increased according to cell growth (Fig. 3). An HA productivity of 3 g/l and cell growth yielding a broth OD_{600} of 14 were achieved at 15 h with a fixed aeration rate of 0.2 vvm (Fig. 3A). An increase in the aeration rate from 0.2 to 1.5 vvm resulted in an increase in the cell biomass and HA productivity, but was problematic in terms of poor mixing and limiting mass transfer (Fig. 3B). The highest HA productivity (4.7 g/l) and cell growth (OD_{600}= 17) were obtained at 20 h when the agitation speed and the aeration rate were increased simultaneously (Fig. 3C). The increase in cell growth and the HA productivity may be the result of facultative anaerobes producing more HA to protect themselves from their aerobic surroundings [8]. These results indicate that the production of HA is most likely to be stable when the initial culture broth contains a limited amount of oxygen, and the oxygen supply is then increased to create an aerobic condition after the desired cell biomass has been achieved.

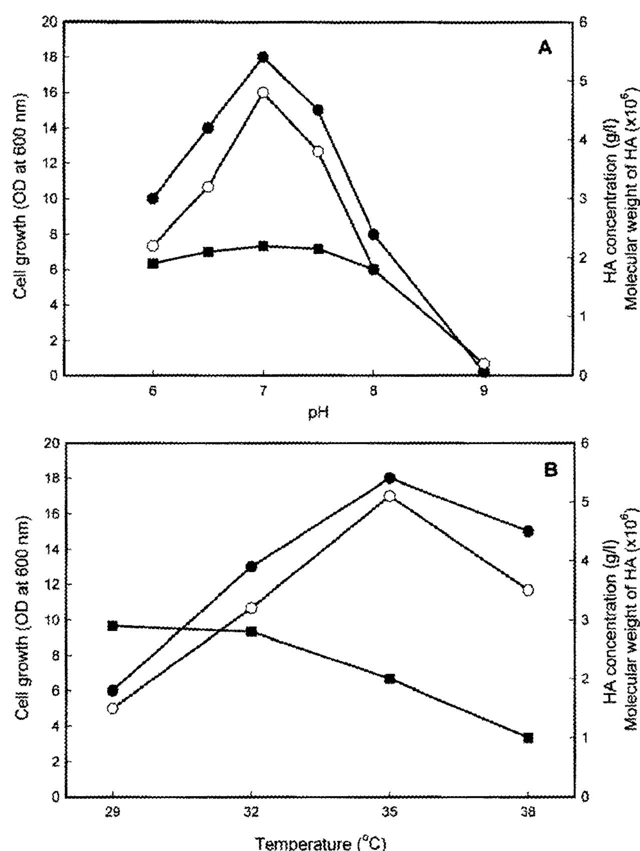

Fig. 4. Effects of pH and temperature on the production of HA and the cell growth in *S. zooepidemicus*.
Symbols: ●, cell growth; ○, HA concentration; ■, molecular weight of HA.

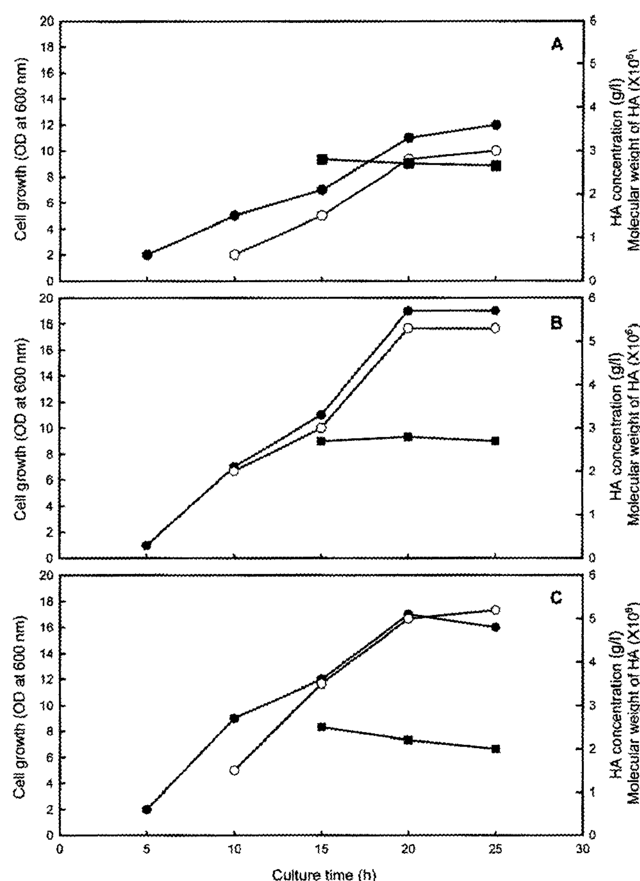

Fig. 5. Effects of the temperature downshift on the production of HA and the cell growth in *S. zooepidemicus*.
A. Maintain 35°C until OD_{600}=4, then decrease temperature to 32°C; **B.** Maintain 35°C until OD_{600}=8, then decrease temperature to 32°C; **C.** Maintain 35°C until OD_{600}=12, then decrease temperature to 32°C. Symbols: ●, cell growth; ○, HA concentration; ■, molecular weight of HA.

Effect of pH and Temperature

The effects of pH and temperature on cell growth and the production and molecular weight of HA were investigated under the optimized fermentation conditions (Fig. 3C). The maximum cell growth rate, HA productivity (4.8 g/l), and HA molecular weight (2.2×10^6) were obtained at a pH of 7.0 (Fig. 4A). The HA productivity and molecular weight decreased at other pHs, especially when the pH exceeded 8.0. However, there was no change in molecular weight in a pH range of 6.0 to 8.0. This result indicates that the culture pH is a critical factor in cell growth and HA production.

Fig. 4B shows the results of fermentation at different culture temperatures (29, 32, 35, and 38°C). The culture temperature was also found to be a critical factor in cell growth and HA production, and especially on the molecular weight of HA. The maximum HA productivity of 5.1 g/l and cell growth with a broth OD_{600} of 18 were obtained in a culture grown at 35°C. When the temperature was increased to 38°C, the molecular weight of HA decreased significantly. When the temperatures were below 35°C, the highest molecular weight (2.9×10^6) was obtained, even though HA productivity and cell growth rate had decreased considerably. This finding is in accord with those of Armstrong and Johns [1], who also found that the molecular weight of HA increases at lower temperatures.

We attempted to cultivate these organisms while lowering the culture temperature from 35°C to 32°C at various stages of cell growth to determine the effect of a temperature downshift on cell growth and HA productivity and molecular weight (Fig. 5). When the culture was maintained at 32°C during the early stages of cell growth (*i.e.*, when OD_{600}=4), an adequate molecular weight was achieved (2.8×10^6), but the cell growth rate and HA productivity were very poor (Fig. 5A). When a downshift in temperature occurred at a broth OD_{600} of 8, the maximum HA productivity was 5.3 g/l, the maximum cell growth produced a broth OD_{600} of 19, and the highest molecular weight was 2.8×10^6 (Fig. 5B). When the temperature downshift occurred at the cell growth broth OD_{600} of 12, the cell mass and the HA productivity were very good, but the molecular weight fell

to 2.1×10^6, which was similar to the molecular weight observed with a constant temperature of 35°C (Fig. 5C). These results indicate that a downshift in the culture temperature at specific stages of bacterial growth is a very effective way to maximize productivity and achieve a higher molecular weight for HA.

Reproducibility of the Fermentation Process

To confirm the reproducibility of the fermentation process, we attempted to establish a standard process model based on the optimal conditions found thus far (Figs. 5B and 6). This was accomplished by focusing on the cell growth pattern, HA production, and molecular weight of HA using three 30-l jar fermentors with a similar geometry. In spite of intrinsic variability in biological processes, we had very similar findings (Fig. 7). Cell growth increased linearly during cultivation, and an HA productivity of 5.1±0.2 g/l and a molecular weight of $2.8\pm0.1\times10^6$ were obtained.

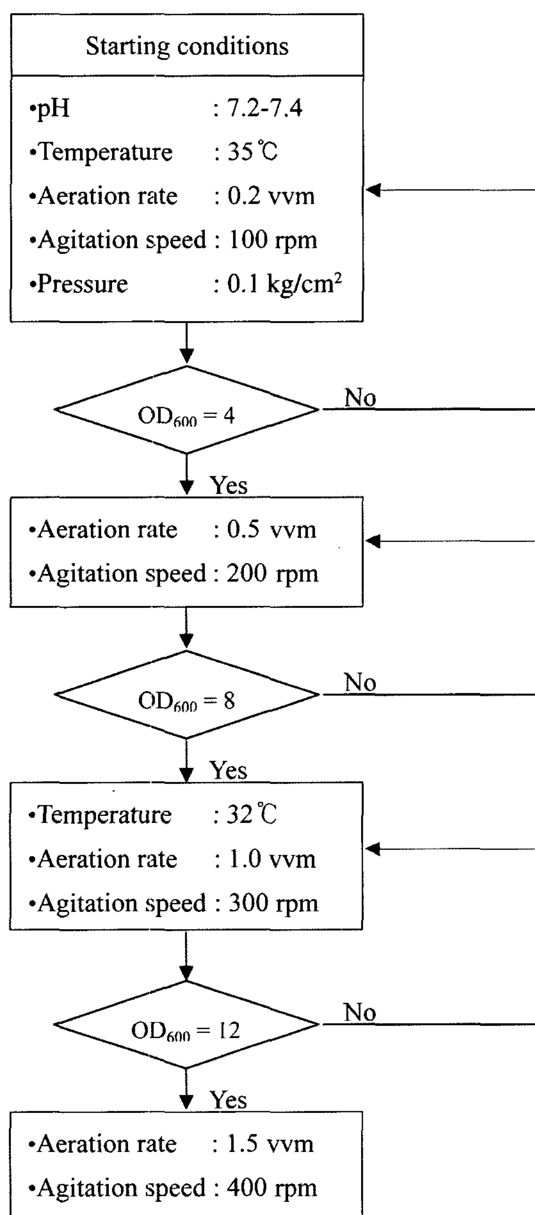

Fig. 6. The flow chart for the production of HA in *S. zooepidemicus.*

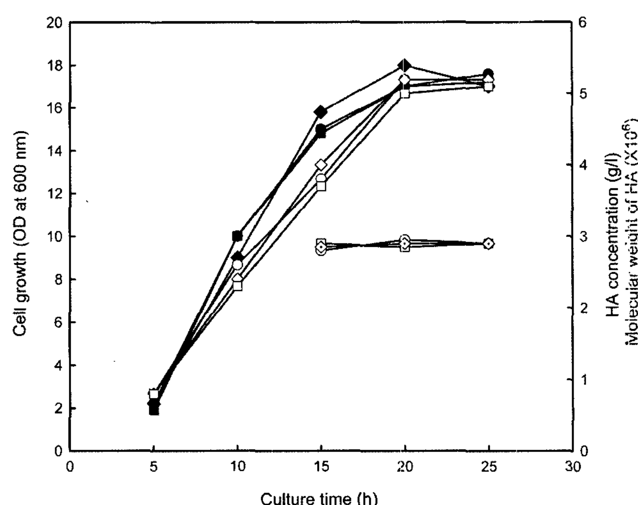

Fig. 7. Reproducibility of the fermentation process shown with 3 similar 30-l jar fermentors.
Symbols: ●, ◆, ■, cell growth; ○, ◇, □, HA concentration; ⊙, ◈, ▣, molecular weight of HA in the 3 cultivations, respectively.

Large-Scale Cultivation

The large-scale production of HA was carried out in a 3-m^3 fermentor with 2 m^3 of working volume, based on the standard process model shown in Fig. 6. The fermentation process was carried out initially at a temperature of 35°C, an agitation speed of 25 rpm, an aeration rate of 0.2 vvm, an operating pressure of 0.1 kg/cm^2, and a pH of 7.0. When the OD_{600} of the culture broth reached 4, the agitation speed was increased to 50 rpm and the aeration rate to 0.5 vvm. The fermentation process was continued until the cell growth resulted in a broth OD_{600} of 8. The agitation speed and the aeration rate were increased again and the temperature was downshifted to 32°C. When the broth OD_{600} reached 12, the agitation speed was increased to 100 rpm and the aeration rate to 1.5 vvm, and they remained at these rates until the end of the experiment. This control method allowed us to achieve a normal cell growth pattern, with a broth OD_{600} of 18 achieved at 18 h (Fig. 8). The HA productivity appeared to be closely related to the cell growth rate, and a maximum productivity of 5.4 g/l was achieved at 22 h. The highest molecular weight (2.9×10^6) was obtained at 18 h.

Based on these findings, we can conclude that the operating parameters (*e.g.*, agitation speed, aeration rate, culture temperature, pH, and pressure) were major factors in the production of HA, cell growth rate, and the

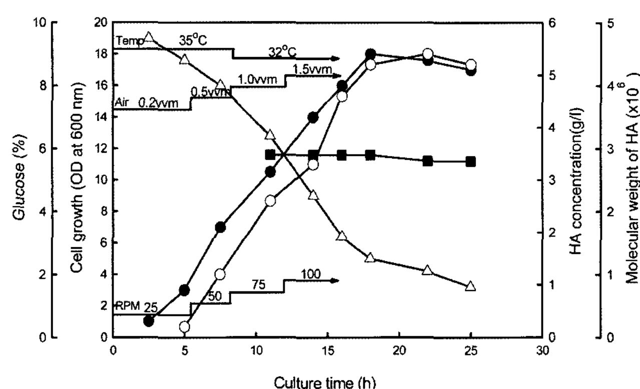

Fig. 8. Typical time course for HA production by *S. zooepidemicus* in a 3-m^3 fermentor.

Symbols: ●, cell growth; ○, HA concentration; ■, molecular weight of HA; △, glucose.

molecular weight of HA. In particular, HA fermentation was found to be highly influenced by the initial cultivation conditions. The downshift in temperature proved to be a very effective method for maximizing productivity and obtaining the highest molecular weight for HA.

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