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Effect of Oxygen and Shear Stress on Molecular Weight of Hyaluronic Acid Produced by *Streptococcus zooepidemicus*

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Dissolved oxygen (DO) and shear stress have pronounced effects on hyaluronic acid (HA) production, yet various views persist about their effects on the molecular weight of HA. Accordingly, this study investigated the effects of DO and shear stress during HA fermentation. The results showed that both cell growth and HA synthesis were suppressed under anaerobic conditions, and the HA molecular mass was only (1.22±0.02)×10^6 Da. Under aerobic conditions, although the DO level produced no change in the biomass or HA yield, a high DO level favored the HA molecular mass, which reached a maximum value of (2.19±0.05)× 10^6 Da at 50% DO. Furthermore, a high shear stress delayed the rate of HA synthesis and decreased the HA molecular weight, yet had no clear effect on the HA yield. Therefore, a high DO concentration and mild shear environment would appear to be essential to enhance the HA molecular weight.

Keywords: Oxygen, shear stress, hyaluronic acid, molecular weight, *Streptococcus zooepidemicus*

Hyaluronic acid (HA) is a uniformly repetitive, linear glycosaminoglycan composed of alternating β-1,4-glucuronic acid (GlcA) and β-1,3-*N*-acetylglucosamine (GlcNAc) moieties [31], plus, owing to its variety of biological functions, hyaluronic acid has a number of applications in medicine, cosmetics, and food [11, 19, 23].

The primary production criterion for HA is its molecular weight, which reflects its biological functions, where HA with a high molecular weight has good viscoelasticity and mucoadhesion, allowing it to be used in the areas of ophthalmology, orthopedics, and wound healing, whereas HA with a relatively low molecular weight is used in cosmetics as a moisturizing factor and in eye drops as lubrication [23]. Therefore, it is important to study the factors impacting the molecular weight of HA during its production.

The production of HA *via* aerobic fermentation has already been extensively studied [1, 2, 7, 13, 15, 16, 18, 21, 22], including various factors that affect the biomass, HA yield, and molecular weight, such as the medium composition, pH, temperature, agitation rate, aeration, initial glucose concentration, and mode of operation. Interestingly, different opinions exist on the effect of agitation and the aeration rate during HA fermentation. For example, Gao *et al.* [13] found that agitation enhanced the HA yield, yet the molecular weight decreased when increasing the impeller speed, whereas Kim *et al.* [22] observed that high agitation caused a low biomass and HA production, yet the HA molecular weight increased remarkably. Meanwhile, Armstrong and Johns [1] observed that the HA molecular weight was independent of agitation, yet a high aeration favored the molecular weight, whereas Kim *et al.* [21] observed that the HA concentration decreased at aeration rates exceeding 0.5 vvm.

It is well known that oxygen plays an important role in aerobic fermentation, especially in non-Newtonian systems [20, 27, 29]. Owing to the low solubility of oxygen, providing a sufficient amount to the bioreactor is important so that cell growth is not limited by oxygen availability. However, since cultures are always heterogeneous mixtures, the oxygen transfer can be hindered by a viscous broth, making it essential to focus on the relationship between the oxygen uptake and transfer. Nonetheless, only a few studies have focused on the parameters of oxygen uptake and transfer during HA fermentation.

As with the fermentation of most polysaccharides, an HA culture broth has non-Newtonian fluid properties, and changing the agitation and aeration rates are the two main ways to enhance the DO concentration. However, a low impeller speed is unable to satisfy the oxygen demand of the cells, whereas a high impeller speed brings high shear stress that can damage both the cells and the synthesis of HA [13, 18]. Accordingly, since the effects of agitation

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and aeration clearly always overlap, it is important to investigate the effects of DO and shear stress separately during HA fermentation, as the results can provide guidelines for optimizing and scaling-up the HA fermentation process.

MATERIALS AND METHODS

Organism

S. zooepidemicus G1, a mutant of *S. zooepidemicus* ATCC 39920 induced by exposure to ultraviolet (UV) light and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, was used as the source of HA in this study. The bacterium was maintained as freeze-dried ampoules in the dark at 4°C.

Inoculum and Medium

The isolation of a pure culture was achieved by streaking onto agar plates that contained (g/l) 2 of glucose, 10 of beef extract, 20 of polypeptone, 5 of yeast extract, 2 of NaCl, 1 of Na_2HPO_4, 0.12 of KH_2PO_4, and 20 of agar. The inoculum was prepared in a 250-ml shake flask with 30 ml of the medium and incubated at 37°C for 8 h. The medium consisted of (g/l) 2 of glucose, 10 of beef extract, 20 of polypeptone, 5 of yeast extract, 2 of NaCl, 1 of Na_2HPO_4, and 0.12 of KH_2PO_4.

Cultivation Conditions

The batch culture experiments were performed in a 5-l fermentor (BIOREA-2000; Shanghai, China) with a working volume of 3 l, and the agitation was provided by two six-bladed disk turbines and one anchor. The medium composition was as follows (g/l): 20 of polypeptone, 10 of yeast extract, 2 of NaCl, 1 of $MgSO_4 \cdot 7H_2O$, and 2.5 of K_2HPO_4, and the fermentor was inoculated with 5% (v/v) inoculum. Automatic temperature and pH control were used, where the latter utilized a steam-sterilizable glass pH probe and sterile 5 M NaOH. The standard fermentation conditions were as follows: 40 g/l of glucose (initially), pH 7.0, 37°C, impeller speed increased stepwise from 150 rpm to 600 rpm, which kept the DO concentration above 30%, and aeration rate of 2 l/min, all of which were defined to enable the prevailing culture conditions to be described apart from the parameters used in this study.

Cell Growth

The cell concentration was measured at 660 nm with a spectrophotometer (Model 752N; Shanghai, China). Culture samples were diluted in water to give an optical density (OD) of less than 0.5, and the OD was then multiplied by the dilution factor. The biomass (g/l) was determined from the OD using a calibration curve.

Chemical Analysis

The glucose concentration was determined using the Miller method [25], the HA concentration determined using the Bitter-Muir method [6], and the weight-average HA molecular weight measured using the Laurent *et al.* method [24], where single-point measurements were performed on diluted samples containing 200 to 500 mg/ml in 0.2 M NaCl at pH 7.0. The Mark-Houwink constants were k= 3.6×10^{-4} and a=0.78.

The oxygen uptake rate (OUR), specific oxygen uptake rate (Q_{O2}), and volumetric mass transfer coefficient (k_La) were measured using the dynamic gas-out/gas-in method reported by Bandyopadhyay and Numphrey [5], plus the gassing out/in data were modified by taking account of the electrode response time [3].

The broth viscosity, in the presence of cells, was measured with TA rheometrics (ARES) at 37°C.

The HA capsules were observed using electron microscopy according to Jacques and Graham [17], and the quantification of HA capsules was carried out according to Fong Chong and Nielsen [12]. Briefly, the cell suspension was treated with an equal volume of 0.1% (w/v) sodium dodecyl sulfate for 1 h, and then the cells were removed by centrifugation at 9,500 ×*g* for 30 min and the supernatant was assayed for the HA content.

RESULTS AND DISCUSSION

Profile of Oxygen Uptake and Transfer During HA Fermentation

Batch fermentations of *S. zooepidemicus* G1 were performed under the standard conditions mentioned in Materials and Methods (Fig. 1). During the HA fermentation, the impeller speed ranged from 150 rpm to 600 rpm, and the DO concentration was kept above 30%.

Fig. 2 shows the biomass, HA concentration, and molecular weight as a function of time. The time course of the fermentation could be divided into three phases: lag phase (0–1 h), exponential phase (1–10 h), and stationary phase (10–14 h). The biomass grew rapidly (μ_{max}=0.67 h^{-1}) after 4 h during the early exponential phase, with the maximum biomass (OD_{660}) and HA yield attained after 9 h and 10 h, respectively. The HA production reached a value of 3.65 g/l at the end of the fermentation, with a molecular weight of 2.05×10^6 Da. Thus, the HA production appeared to be closely associated with the biomass; *i.e.*, the HA was produced in a growth-associated manner, as reported in previous literature [22].

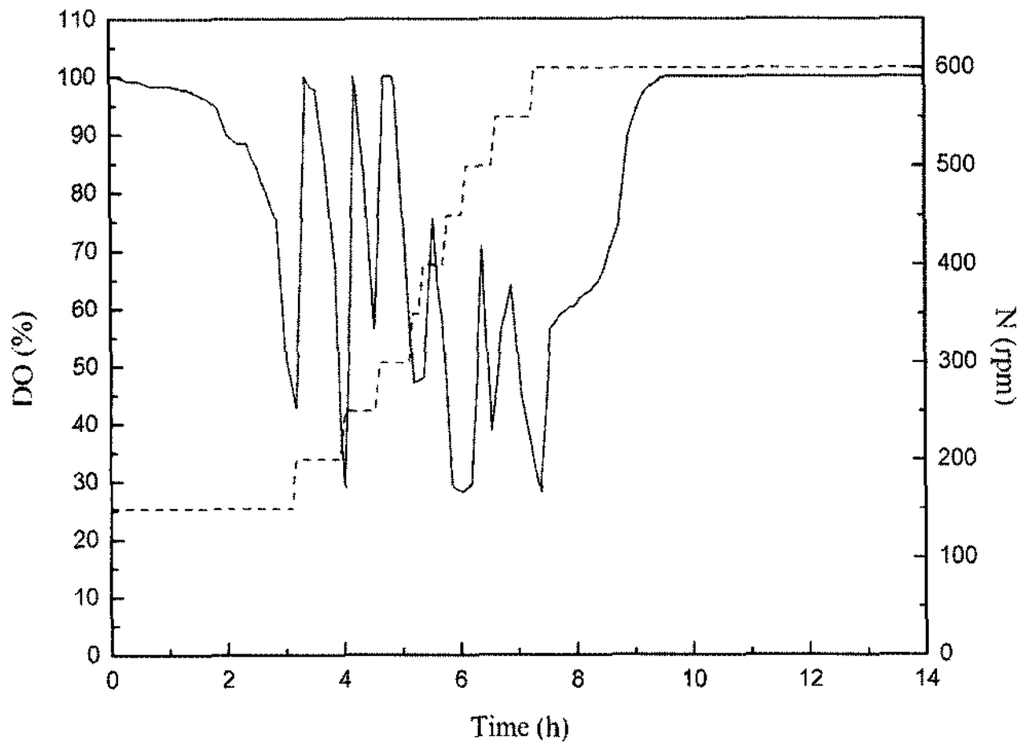


Fig. 1. Typical profiles of impeller speed and DO in time course of HA fermentation.
Symbols: (-------), impeller speed; (——), DO.

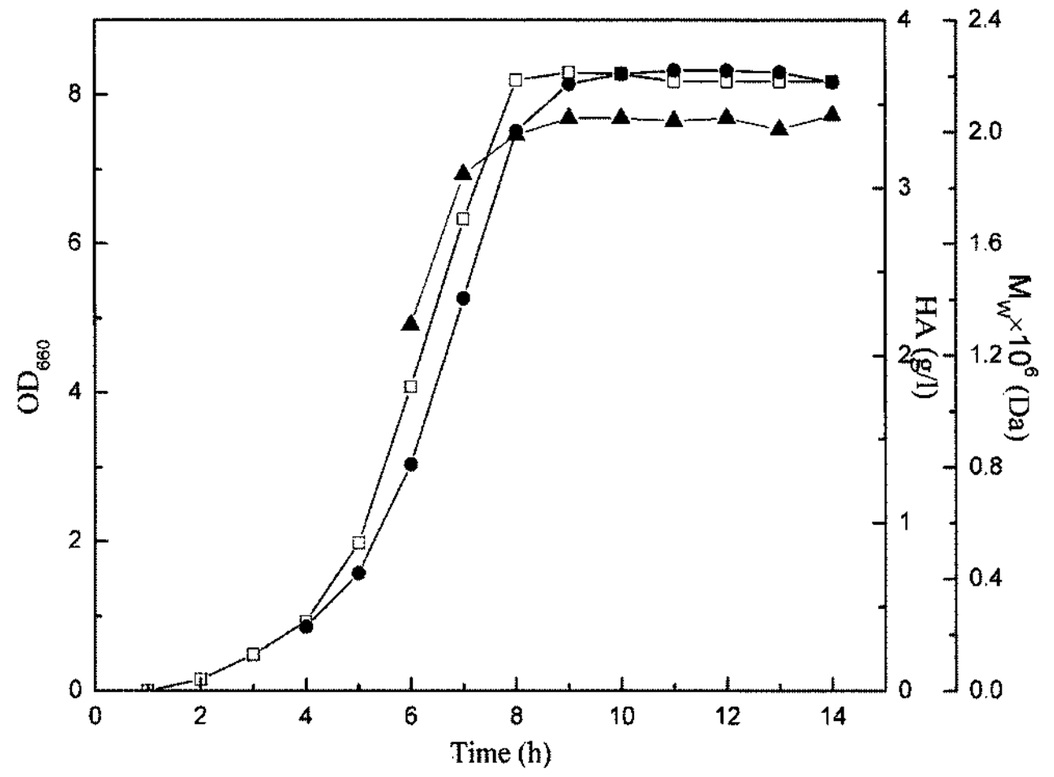


Fig. 2. Batch culture of *S. zooepidemicus* G1 under standard conditions during HA fermentation.
Symbols: (●), HA concentration; (□), Biomass; (▲), HA molecular weight.

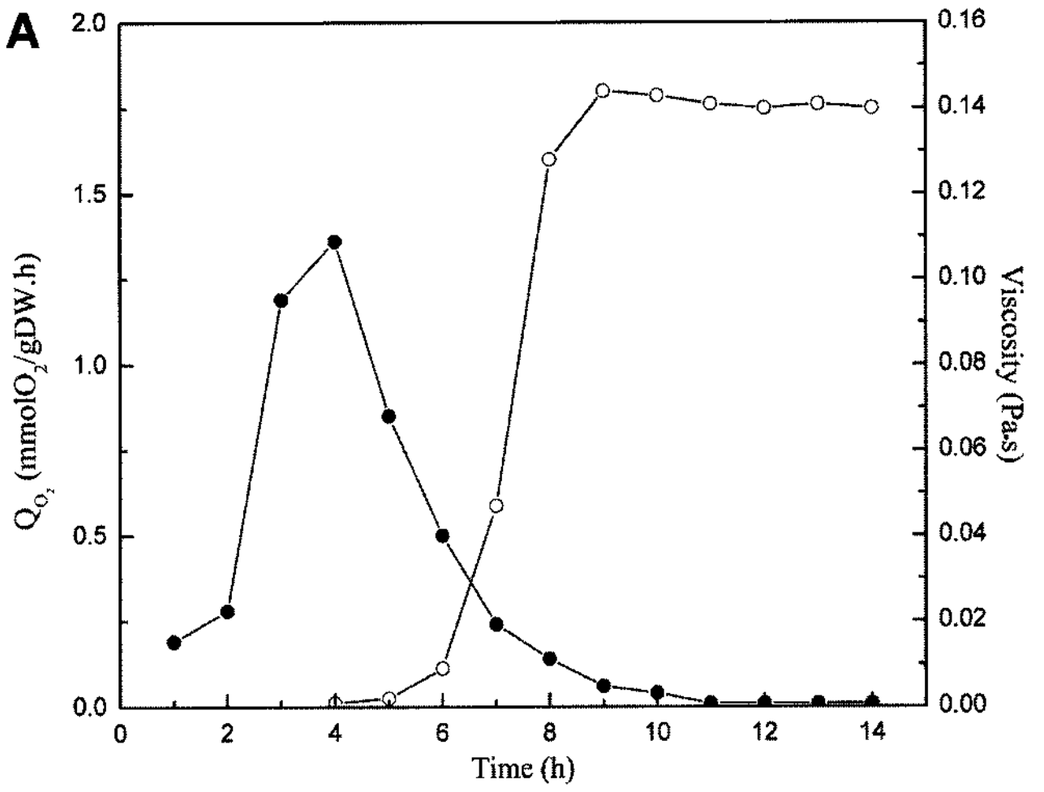
Figs. 3A and 3B show the tendencies of the oxygen uptake rate (OUR), specific oxygen transfer rate (Q_{O2}), volumetric mass transfer coefficient (k_La), and broth viscosity (η) during the HA fermentation. The maximum OUR (0.76 mmolO_2/L·h) and Q_{O2} (1.36 mmol O_2/gDW·h) were observed after 6 h and 4 h, respectively, during the early exponential phase when the broth viscosity was very low (0.002 Pa·s). As the HA production increased rapidly, the broth viscosity also increased and reached a maximum of 0.143 Pa·s at the beginning of the stationary phase (9 h). In Fig. 3B, the oxygen mass transfer coefficient k_La increased at the beginning of the exponential phase when increasing the impeller speed, and reached a maximum of 180 h^{-1} after 7 h. During the stationary phase, k_La decreased to around 100 h^{-1}, due to the high viscosity of the broth.

It should be noted that the broth viscosity was still very low when the OUR reached its maximum value. The broth viscosity then increased to its maximum value during the stationary phase, during which time the OUR decreased to its minimum value. Thus, it is suggested that the effect of the DO on the biomass and HA synthesis was much less because of the low demand for oxygen.

Effect of DO During HA Fermentation

Table 1 shows the biomass and HA production with different DO levels, while the impeller speed control remained the same along with the standard fermentation conditions mentioned above.

Both the biomass and the HA production increased very slowly under anaerobic conditions, with final measurements of only 0.60 g/l and 0.73 g/l, respectively. However, under aerobic conditions, the final biomass and HA production were not affected by various DO levels, even at 2% DO,



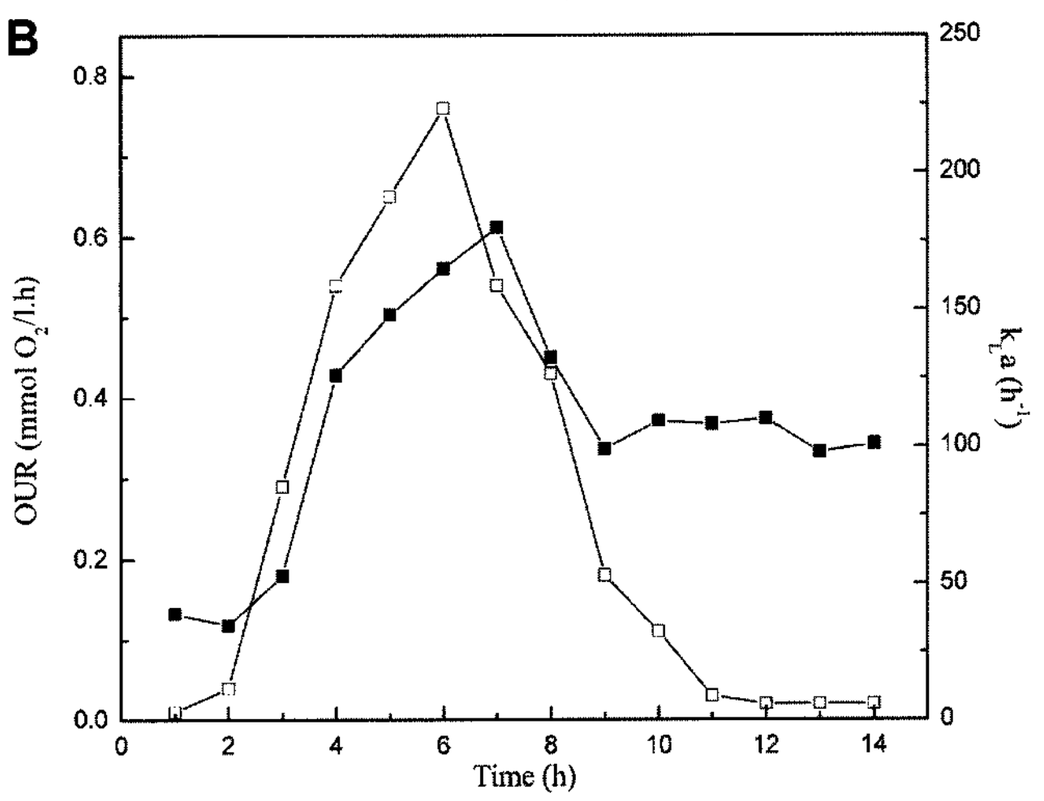


Fig. 3. Characteristics of oxygen uptake and transfer during HA production by *S. zooepidemicus* G1.
Symbols: (●), Specific oxygen transfer rate (Q_{O_2}); (○), broth viscosity (η); (□), oxygen uptake rate (OUR); (■), volumetric mass transfer coefficient (k_La).

indicating that the biomass and HA synthesis decreased under anaerobic conditions, yet were unaffected in the presence of oxygen, regardless of the DO level. In contrast, Huang *et al.* [16] found a critical DO level of 5% during HA fermentation, meaning the capacity of HA synthesis was affected below this level. It should be noted that the critical DO for *S. zooepidemicus* G1 may be very low (less than 2%), although this was not established in the present study.

Furthermore, the yield coefficient $Y_{X/HA}$ was investigated under anaerobic and aerobic conditions, and the results are summarized in Table 1. There were no distinct differences in the values of $Y_{X/HA}$, indicating that the efficiency of the HA synthesis was unaffected with various DO levels. However, both the cell growth and the HA synthesis were hindered under anaerobic conditions owing to a lack of oxygen. Once cells grew in the presence of oxygen, the

Table 1. Biomass, HA yield, and the yield coefficient $Y_{X/HA}$ under different DO levels.

DO level (%)	Biomass (gDW/l)	HA (g/l)	$Y_{X/HA}$ (gDW/gHA)
Anaerobic	0.60±0.05	0.73±0.04	0.82±0.08
2	2.80±0.08	3.61±0.12	0.78±0.03
10	2.72±0.10	3.55±0.08	0.77±0.03
30	2.88±0.11	3.48±0.09	0.83±0.04
50	2.75±0.12	3.51±0.08	0.78±0.04
80	2.79±0.09	3.50±0.10	0.80±0.03

biomass and HA production increased rapidly without any change in $Y_{X/HA}$.

Electron micrographs of *S. zooepidemicus* G1 are shown in Fig. 4. Under anaerobic conditions, no capsules were found around the cells (Fig. 4A). In contrast, the thickness of the capsules was 0.05 μm compared with the cell diameter of 0.5 μm (Fig. 4B). Thus, the thickness of

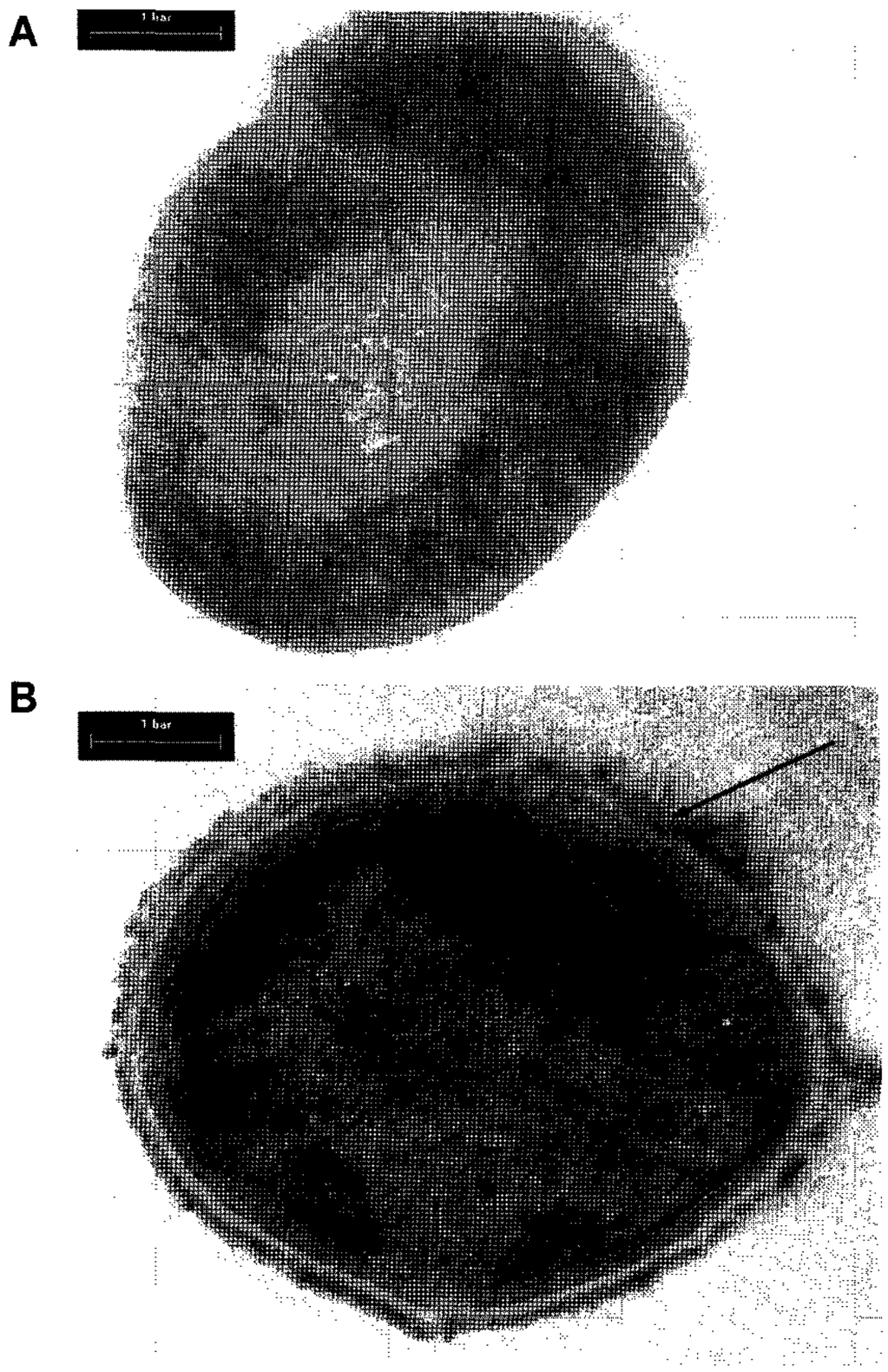


Fig. 4. Electron micrograph sections of *S. zooepidemicus* G1 cells obtained from the exponential phase (7 h). 1 bar=100 nm.
A. Anaerobic condition; **B**. Aerobic condition at 50% DO level.

Table 2. Effect of DO level on the HA molecular weight.

DO level (%)	HA molecular weight (×10^6 Da)
Anaerobic	1.22±0.02
2	1.54±0.07
10	1.75±0.06
30	1.90±0.04
50	2.19±0.05
80	2.06±0.02

capsules was thin under aerobic conditions. However, there was no substantial variation in the thickness of the capsules with different DO levels. Cleary and Larkin [8] previously proposed a defense mechanism where HA capsules protect cells from oxygen in Group A, and the protective mechanism is activated in the presence of

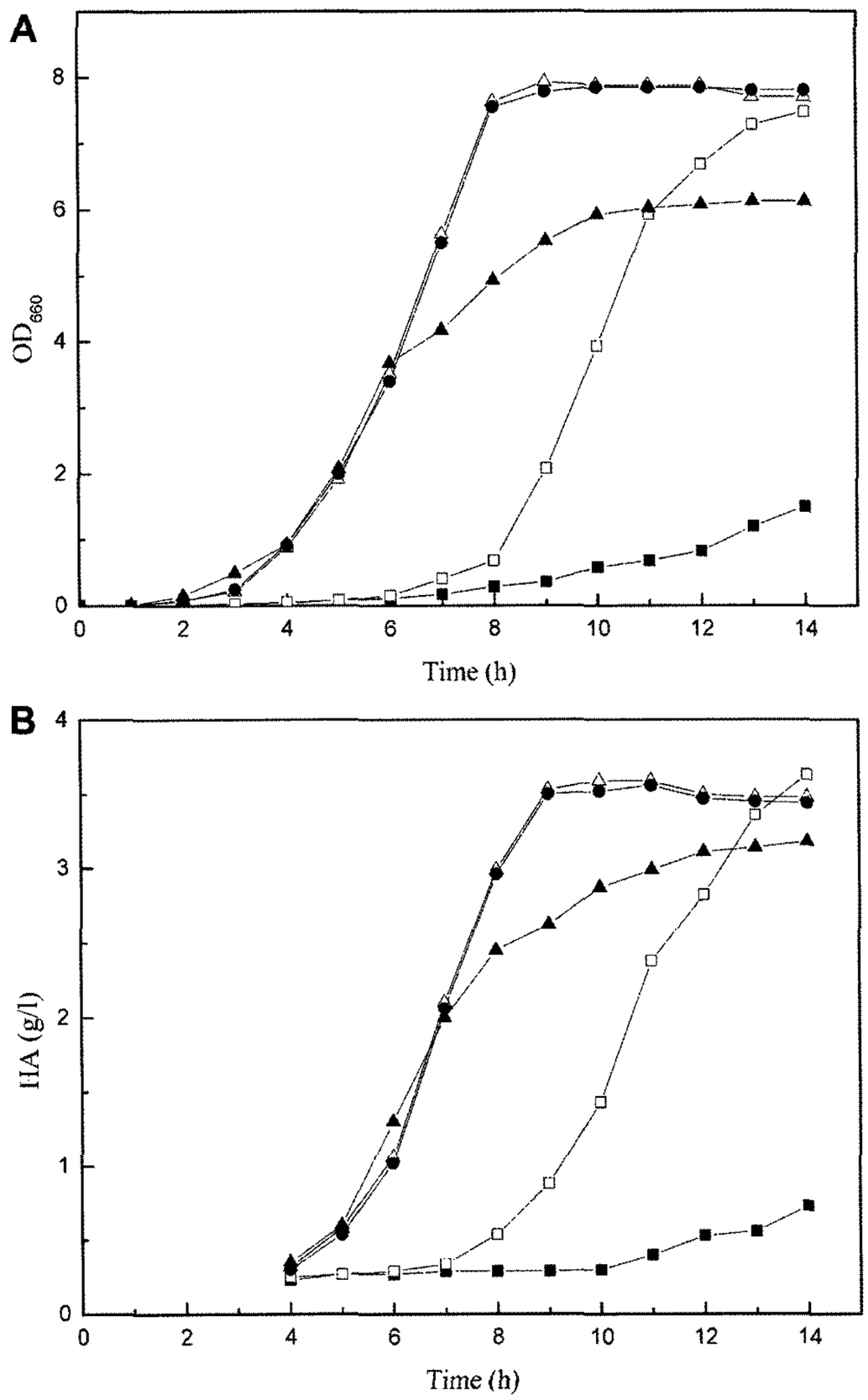


Fig. 5. Effect of phase oxygen control strategy on biomass (OD_{660}) and HA produced by *S. zooepidemicus* G1.
Symbols: (■), anaerobic condition; (□), procedure (a); (▲), procedure (b); (●), procedure (c); (△), aerobic condition at 50% DO level.

Table 3. Effect of phase oxygen control strategy on the HA molecular weight.

Procedure	HA molecular weight ($\times 10^6$ Da)
a	1.85±0.05
b	1.94±0.02
c	2.16±0.04
Control*	2.19±0.05

*50% DO level shown in Table 2.

oxygen. Moreover, capsular HA with a high molecular weight can effectively protect cells from damage [28], yet this was not evident based on the capsule thickness.

In this study, the HA molecular weight was found to be sensitive to the DO level, and the results are shown in Table 2. Under anaerobic conditions, the HA molecular weight was only $(1.22\pm0.02)\times10^6$ Da, yet it reached $(2.19\pm0.05)\times10^6$ Da at a DO level of 50%, and then decreased slightly to $(2.06\pm0.02)\times10^6$ Da at 80%. This phenomenon was caused by the balance between HA synthesis [1] and oxygen-mediated degradation [14] with various DO levels. As such, the HA molecular weight increased owing to the effect of the DO on the HA synthesis, and then decreased because of oxygen radical degradation. Thus, the HA molecular weight at 80% DO was less than that at 50% DO owing to oxygen radical degradation [14].

To investigate the role of oxygen in the HA synthesis process, a phase oxygen control strategy was adopted (Fig. 5) based on the following three procedures: (a) anaerobic conditions for the first 5 h, followed by a 50% DO level for the next 9 h; (b) a 50% DO level for the first 5 h, followed by anaerobic fermentation; (c) a 50% DO level for the first 10 h, followed by anaerobic fermentation.

The cells grew slowly under the anaerobic conditions for the first 5 h under procedure (a), and then grew fast with a DO level of 50%, resulting in a final biomass and HA production that were almost equal to those with a constant 50% DO level. In contrast, owing to lack of oxygen under procedure (b), the biomass and HA synthesis were hindered during the exponential phase, meaning the final biomass and HA yield were much lower. However, the DO level did not affect the HA production during the stationary phase under procedure (c), possibly due to lost capacity for HA synthesis [9, 30].

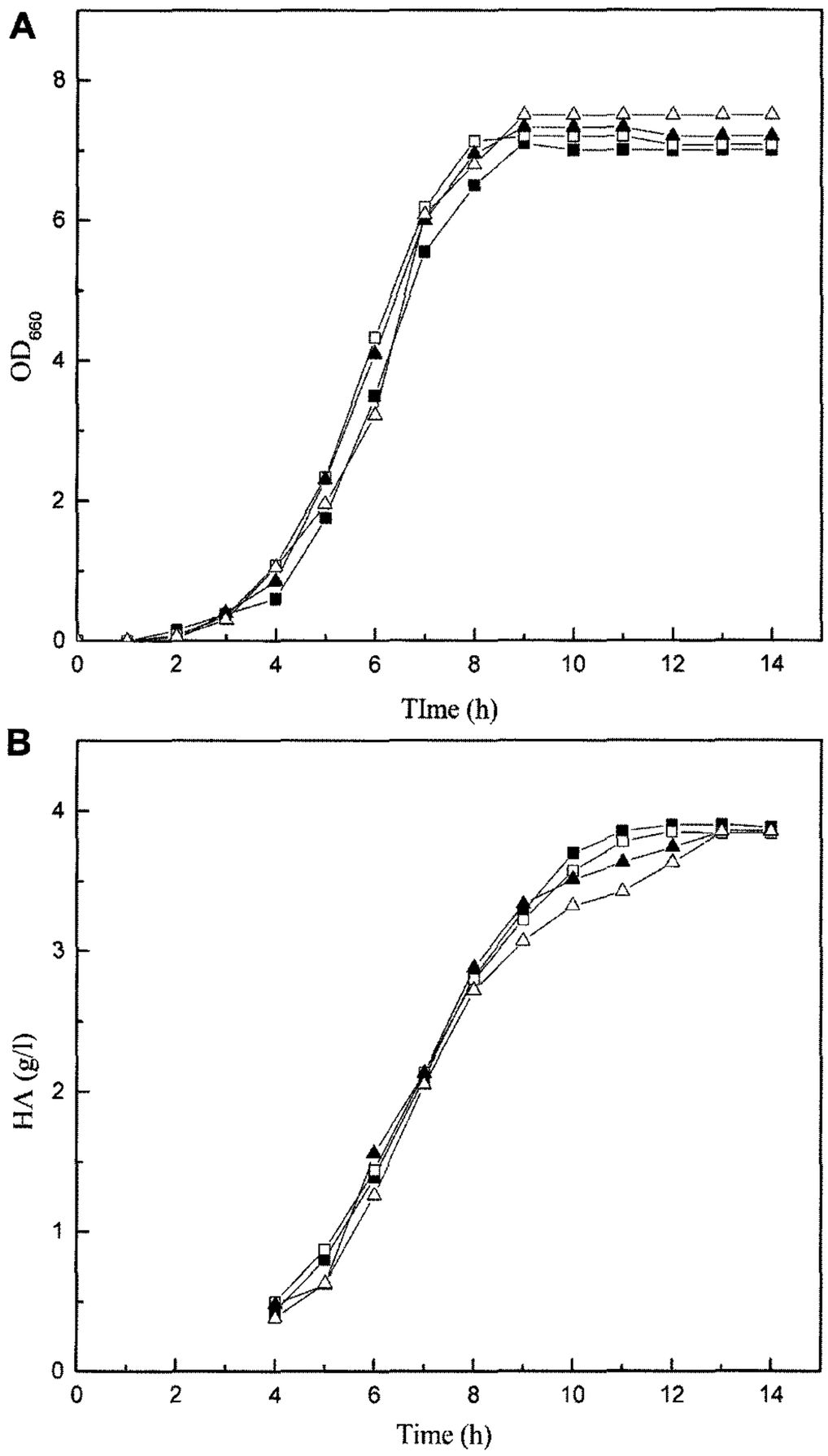


Fig. 6. Effect of impeller speed on biomass (OD_{660}) and HA produced by *S. zooepidemicus* G1.
Symbols: (■), 150 rpm; (□), 450 rpm; (▲), 700 rpm; (△), 1,000 rpm.

The HA molecular weight decreased under both procedures (a) and (b) (Table 3), due to the absence of oxygen during the early or late exponential phase. There was no difference in the HA molecular weight between

Table 4. Distribution of HA at different impeller speeds during different growth phases.

Impeller speed (rpm)	Exponential phase (6 h)			Stationary phase (12 h)		
	Free HA (g/l)	Total HA (g/l)	Proportion of free HA to total HA	Free HA (g/l)	Total HA (g/l)	Proportion of free HA to total HA
150	0.86±0.04	1.02±0.05	0.80±0.05	3.75±0.09	3.85±0.10	0.93±0.03
450	1.08±0.06	1.32±0.07	0.78±0.06	3.65±0.10	3.78±0.12	0.92±0.04
700	1.10±0.08	1.35±0.07	0.77±0.07	3.74±0.09	3.86±0.11	0.92±0.03
1,000	0.89±0.04	1.02±0.06	0.81±0.06	3.65±0.11	3.76±0.08	0.92±0.03

procedure (c) and the control. Thus, since the content of oxygen with procedure (c) was much more than that with the control process, it would seem that the effect of oxygen radicals on the HA molecular weight with 50% DO was negligible.

Effect of Shear Stress During HA Fermentation

Fig. 6 shows the effect of the impeller speed on the biomass (OD_{660}), HA yield, and molecular weight, while maintaining a DO level of 50%. Shifting the speed from 150 rpm to 1,000 rpm had a minimal effect on the biomass, as the HA synthesis was slightly delayed under a high impeller speed, yet the final HA yield was unaffected. Nonetheless, Nickel *et al.* [26] found that chain initiation or elongation was stimulated if the nascent hyaluronan was dissociated from the synthase into the medium, implying that high shear stress may favor HA production by releasing HA capsules.

The total amount of HA consisted of two parts: the capsular HA surrounding the cells and the free HA in the medium. Thus, the total HA and capsular HA were determined under different impeller speeds, and the results are summarized in Table 4. The proportion of free HA to total HA was hardly affected under different impeller speeds. During the exponential phase, the ratio of free HA to total HA was about 79%, and then increased slightly to 92%. Thus, most of the HA was released into the medium, especially during the stationary phase [9, 30].

Table 5 shows the effect of the impeller speed on the HA molecular weight. The HA molecular weight was only $(1.69\pm0.03)\times10^6$ Da at a speed of 150 rpm, as the low impeller speed probably caused poor mixing and a weak oxygen mass transfer, plus the value of k_La was less than 50 h^{-1}. The HA molecular weight reached its maximum value of $(2.01\pm0.05)\times10^6$ Da at a speed of 450 rpm, and then decreased with an increasing impeller speed. The NMR spectra for sheared HA solutions have shown that tertiary structures persist even at quite high shear rates [10], indicating that the effect of shear stress on the HA structure can be ignored. In this study, the broth viscosity was measured during the different growth phases under a shear rate of 1,400 s^{-1} for an hour, and the results are shown in Fig. 7. The viscosity did not decrease even at 1,400 s^{-1}, and the HA molecular weight remained the same (Table 6). Therefore, the decrease in the HA molecular weight was likely caused by damage to the HA synthase

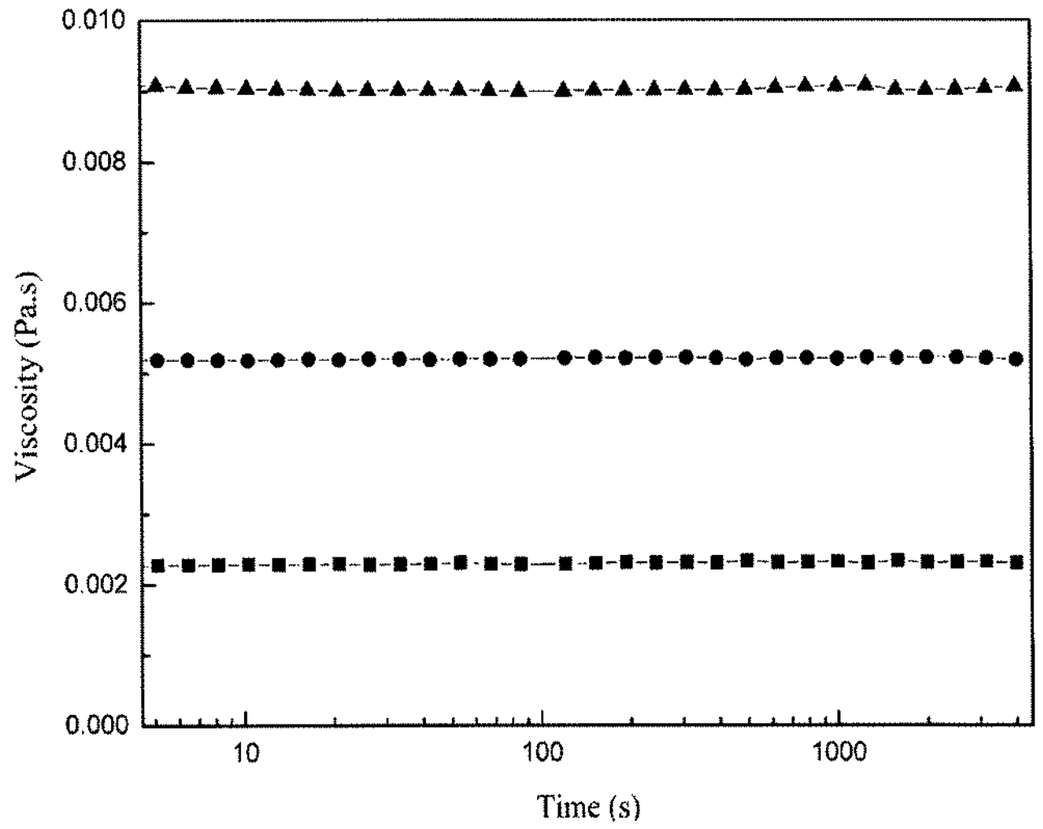


Fig. 7. The broth viscosity in different growth phases under 1,400 s^{-1} shear rate (shear time=4,000 s).
Symbols: (■), exponential phase (6 h); (●), exponential phase (9 h); (▲), stationary phase (12 h).

activity [13], where the HA chains were forced to release into the medium prematurely owing to high shear stress. However, whether and how shear stress is responsible for the activity of HA synthases remains to be further investigated [4].

The characteristics of oxygen uptake and transfer were investigated during HA fermentation by *S. zooepidemicus* G1. As a result, the OUR was found to decrease when the broth became viscous, and reached its minimum value when the viscosity reached its maximum value. Moreover, the role of oxygen lost it importance during the stationary phase, even when a high viscosity hindered oxygen transfer. Nonetheless, the synthesis of HA was stimulated in the presence of oxygen. Although the DO level was found to have little effect on the cell growth or HA yield under aerobic conditions, a high DO concentration favored the molecular weight. Shear stress exhibited no effect on the final biomass and HA production. However, the HA synthesis was slightly delayed with a high impeller speed. The decrease in the HA molecular weight with a high impeller speed may have been due to an abnormal synthesis of HA. Therefore, the effects of oxygen and shear stress on the HA molecular weight should be applied to optimize HA fermentation, as well as the bioreactor design and scaling-up.

Table 5. Effect of impeller speed on HA molecular weight.

Impeller speed (rpm)	HA molecular weight ($\times10^6$ Da)
150	1.69±0.03
450	2.01±0.05
700	1.84±0.03
1,000	1.54±0.06

Table 6. Effect of 1,400 s^{-1} shear rate on HA molecular weight.

Time (h)	HA molecular weight unaffected ($\times10^6$ Da)	HA molecular weight affected ($\times10^6$ Da)
6	1.30±0.06	1.26±0.05
9	2.18±0.07	2.16±0.05
12	2.21±0.10	2.17±0.04

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