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Enhanced Production of Gellan by *Sphingomonas paucibilis* NK-2000 with Shifts in Agitation Speed and Aeration Rate after Glucose Feeding into the Medium

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Optimal agitation speed and aeration rate for the production of gellan by *Sphingomnas paucibilis* NK2000 in a 7 l bioreactor were found to be 400 rpm and 1.0 vvm. The best time for glucose feeding into the medium for enhanced production of gellan by *S. paucibilis* NK2000 was 36 hr after cultivation. The concentrations of gellan produced by *S. paucibilis* NK2000 from 1) 20.0 g/l glucose without additional feeding, 2) 20.0 g/l glucose with feeding of 200.0 g/l glucose at 36 hr, in which the final concentration in the medium was 10.0 g/l, 3) 20 g/l glucose with feeding of 200.0 g/l glucose and a shift in an agitation speed from 400 to 600 rpm, 4) 20.0 g/l glucose with feeding of 200.0 g/l glucose at 36 hr and shifts in an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 1.5 vvm, 5) and 20.0 g/l glucose with feeding of 200.0 g/l glucose at 36 hr and shifts in an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 2.0 vvm, were 5.19, 5.74, 6.73, 7.93, and 9.40 g/l, respectively, and their conversion rates from glucose were 26.0, 19.1, 22.4, 26.4, and 31.3%, respectively. Compared to those developed using a normal process, production of gellan by *S. paucibilis* NK2000 from 20.0 g/l glucose was 1.81 times higher, and and its conversion rate was 1.20 times higher when the optimized process developed in this study was used.

Key words : *Sphingomnas paucibilis,* gellan, glucose feeding, agitation speed, aeration rate

Introduction

Gellan, a commercial gelling agent, is an extracellular polysaccharide produced by *Sphingomnas paucibilis* (formerly *Pseudomonas elodea*) [9,17]. Gellan consists of linear repeating tetrasaccharides, which is composed of D-glucose (Glc), D-glucuronic acid (GlcA), and L-rhamnose residues (Rha) [8,9]. Gellan exhibits good stability, which is a distinct advantage in fruit-based products [12]. It has approval in the USA and EU for food use as gelling, stabilizing and suspending agents [19]. Gellan has also been used for immobilization of enzymes and cells as well as gel electrophoresis. Recent reports indicate that the annual production of gelatin is nearly 326,000 tons [13]. One of the possible gelatin alternatives for the food industry is gellan [18].

Factors that affect the production of gellan are carbon and nitrogen sources, temperature, the initial pH of the medium, and oxygen supply [1,4,14]. Effects of agitation and aeration on the production of gellan and its molecular weight have been reported [7]. Agitation speed and aeration rate, which affect the concentration of dissolved oxygen (DO) in culture broths are important in aerobic fermentation processes particularly in polysaccharide production, since the broth becomes highly viscous and limits mass and oxygen transfer which influence the cellular activities and secondary metabolite production [3]. Production of gellan is accompanied by a considerable increase in viscosity as the time of cultivation passes, which leads to a reduction of oxygen supply [6]. Due to viscous and pseudoplastic characteristics of its culture broth, the production of gellan requires high agitation to promote enough concentration of dissolved oxygen in the medium [6,22]. However, an increase in agitation speed means more energy consumed for the production of gellan, and can lead to mechanical damage to the polysaccharide [20]. Studies on strategy for control of agitation speed for enhanced production of gellan by *S. paucimobilis* have been scarce.

Production of gellan is a closely growth-associated process, and thus a factor or process variable that stimulate cell

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growth should enhance it production [21]. The carbon source supporting the maximum cell growth results in the maximum production of gellan [2]. However, higher concentrations of carbon sources, especially glucose, inhibit cell growth as well as the production of gellan due to their catabolite repression [21]. The concentrations of gellan produced by *S. paucimobilis* ranged from 6.0 to 17.7 g/l dependent on kinds of carbon sources and their concentration [2,11,21]. Higher concentration of starch can enhance the production of gellan, however, which interferes with the recovery of gellan from culture broth [2]. Higher production and conversion rate of gellan from glucose were obtained in a fed-batch fermentation, which overcame the catabolite repression and supported the higher production of gellan [11,21].

Previous study was to develop cheap nitrogen sources instead of yeast extract or peptone for the production of gellan by *S. paucimobilis* NK-2000 [10]. Soybean pomace was substituted for yeast extract as a nitrogen source for production of gellan, which could reduce the cost. Soybean pomace is a by-product from the soy sauce industry and about 20,000 tons are generated each year in the Republic of Korea. In this study, the effect of agitation speed and aeration rate as well as shifts in agitation speed and aeration rate after glucose feeding into the medium on the production of gellan was investigated in order to solve the problem regarding mixing and oxygen supply, which is believed to enhance its productivity.

Materials and Methods

Bacterial strain and medium

Sphingomnas paucibilis NK2000 (formerly *Pseudomonas elodea*) is a UV-induced mutant of *S. paucibilis* ATCC 31461, which was purchased from American Type Culture Collection (ATCC). The medium used for cell growth and production of gellan contained 20.0 g/l glucose, 10.0 g/l soybean pomace, 0.5 g/l K_2HPO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$, and 0.1 ml mineral salt solution [10]. The mineral salt solution contained 1.8 g/l $MnCl_2 \cdot 4H_2O$, 2.49 g/l $FeSO_4 \cdot 7H_2O$, 0.29 g/l H_3BO_3, 27.0 g/l $CuCl_2$, 21.0 g/l $ZnCl_2$, 74 g/l $CoCl_2 \cdot 6H_2O$, 23.0 g/l $MgMoO_4$, and 2.1 g/l sodium tartrate (dihydrate). The pH of the medium was adjusted to 6.5-6.8 before sterilization. The carbon source was autoclaved separately for 15 min at 121°C and added to the medium under aseptic conditions. The soybean pomace used in this study was a by-product from a domestic factory (O-Bok Food Co., Korea), which produces traditional Korean foods.

Production of gellan

Starter cultures were prepared by transferring cells from agar slants to 100 ml of the medium with 20.0 g/l glucose and 2.5 g/l yeast extract in 500 ml Erlenmeyer flasks. These cultures were incubated at 30°C for 1 day with an agitation speed of 200 rpm. These starter cultures were used as inoculum for a 5 l medium with 20.0 g/l glucose and 10.0 g/l soybean pomace in a 7 l bioreactor (Ko-Biotech Co., Korea). Working volumes of the 7 l bioreactors were 5 l and inoculum size of batch fermentations for production of gellan by *S. paucibilis* NK2000 was 5.0% (v/v). Agitation speeds ranged from 200 to 400 rpm and aeration rates ranged from 0.5 to 2.0 vvm. Samples were periodically withdrawn from the culture to determine cell growth and production of gellan.

To isolate gellan, the culture broth was heated at 95°C for 15 min in a boiling water bath. The pH of the heated culture broth was adjusted to 10.0 by 2.0 N NaOH and neutralized with 2.0 N H_2SO_4. The pretreated broth was centrifuged at 12,000× *g* for 20 min to separate the cells. The supernatant was mixed with 2 vol of isopropanol and then kept overnight at 4°C. This precipitated gellan was centrifuged at 8,000× *g* for 30 min and dried at 100-105°C until constant weight [16].

Analytical methods

Cell growth was determined by directly weighing biomass after drying to a constant weight at 100-105°C. Production of gellan and reducing sugar were determined by a method described in a previous report [10].

Results and Discussion

Effect of agitation speed on production of gellan

The effect of agitation speed on cell growth and the production of gellan by *S. paucibilis* NK2000 was investigated in a 7 l bioreactor (Ko-Biotech Co., Korea). Agitation speed ranged from 200 to 500 rpm and aeration rate was 1.0 vvm. The temperature and initial pH of the medium for the production of gellan by *S. paucibilis* NK2000 were 30°C and 6.8, respectively. A higher agitation speed, which resulted in an increase of dissolved oxygen in the medium, enhanced cell growth, as shown in Fig. 1A. The optimal agitation speed

for cell growth of *S. paucibilis* NK2000 was found to be 400 rpm. Production of gellan by *S. paucibilis* NK2000 also increased with elevated agitation speed, as shown in Fig. 1B. The optimal agitation speed for production of gellan was also 400 rpm. The highest production of gellan by *S. paucibilis* NK2000 was 5.55 g/l from 20.0 g/l glucose and 10.0 g/l soybean pomace as carbon and nitrogen sources. Production of gellan was increased according to cell growth of *S. paucibilis* NK2000 [10]. It seems that higher concentrations of dissolved oxygen in the medium promote cell growth of *S. paucibilis* NK2000, which results in higher production of gellan.

Effect of aeration rate on production of gellan

The effect of aeration rate on cell growth and the production of gellan by *S. paucibilis* NK2000 was also investigated. Aeration rate ranged from 0.5 to 2.0 vvm and agitation speed was 400 rpm. Optimal aeration rates for cell growth of *S. paucibilis* NK2000 as well as for production of gellan were 1.0 vvm, as shown in Fig. 2. The highest production of gellan by *S. paucibilis* NK2000 was 5.65 g/l with an agitation speed of 400 rpm and an aeration rate of 1.0 vvm. The concentration of dissolved oxygen in the medium can be influenced by agitation speed, aeration rate, and the inner pressure of bioreactors [6,7]. It seems that higher dissolved oxygen in the medium due to higher aeration rates and agitation speeds, leads to enhance cell growth, which resulted in improved production of gellan.

Effect of glucose feeding time on production of gellan

The effect of glucose feeding into the culture broth at different time on cell growth and the production of gellan by *S. paucibilis* NK2000 was investigated. Agitation speed and aeration rate for the production of gellan by *S. paucibilis*

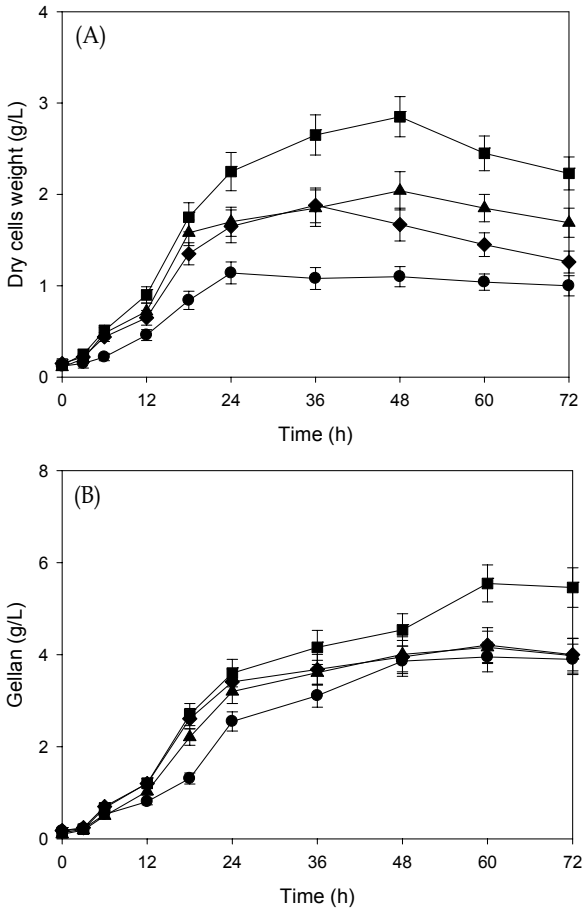

Fig. 1. Effect of agitation speed on cell growth (A) and the production of gellan (B) by *S. paucibilis* NK2000 (●, 200 rpm; ▲, 300 rpm; ■, 400 rpm, and ◆, 500 rpm).

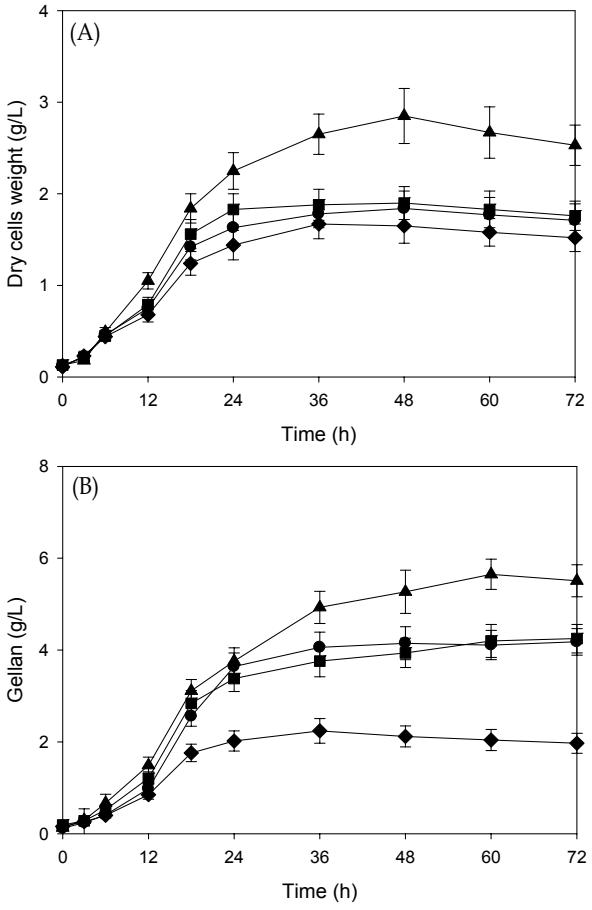

Fig. 2. Effect of aeration rate on cell growth (A) and the production of gellan (B) by *S. paucibilis* NK2000 (●, 0.5 vvm; ▲, 1.0 vvm; ■, 1.5 vvm, and ◆, 2.0 vvm).

NK2000 were 400 rpm and 1.0 vvm. Concentration of glucose in the feeding solution was 200.0 g/l and its final concentration of glucose fed into the medium was 10.0 g/l. Reducing sugars in culture broths increased from 18.9 to 29.2 g/l, from 8.4 to 18.2 g/l, from 4.4 to 14.1, and from 2.4 to 12.1 g/l with glucose feeding after 0, 24, 36, and 48 hr cultivation, respectively, as shown in Fig. 3A. Production of gellan from 20.0 g/l glucose without glucose feeding was 5.07 g/l, whereas those from 20.0 g/l glucose with feeding of glucose feeding at a different time ranged from 5.45 to 6.16 g/l, as shown in Table 1. Significance of each value was analyzed by DPS software version 3.01 (DPS Co., Middlesex,

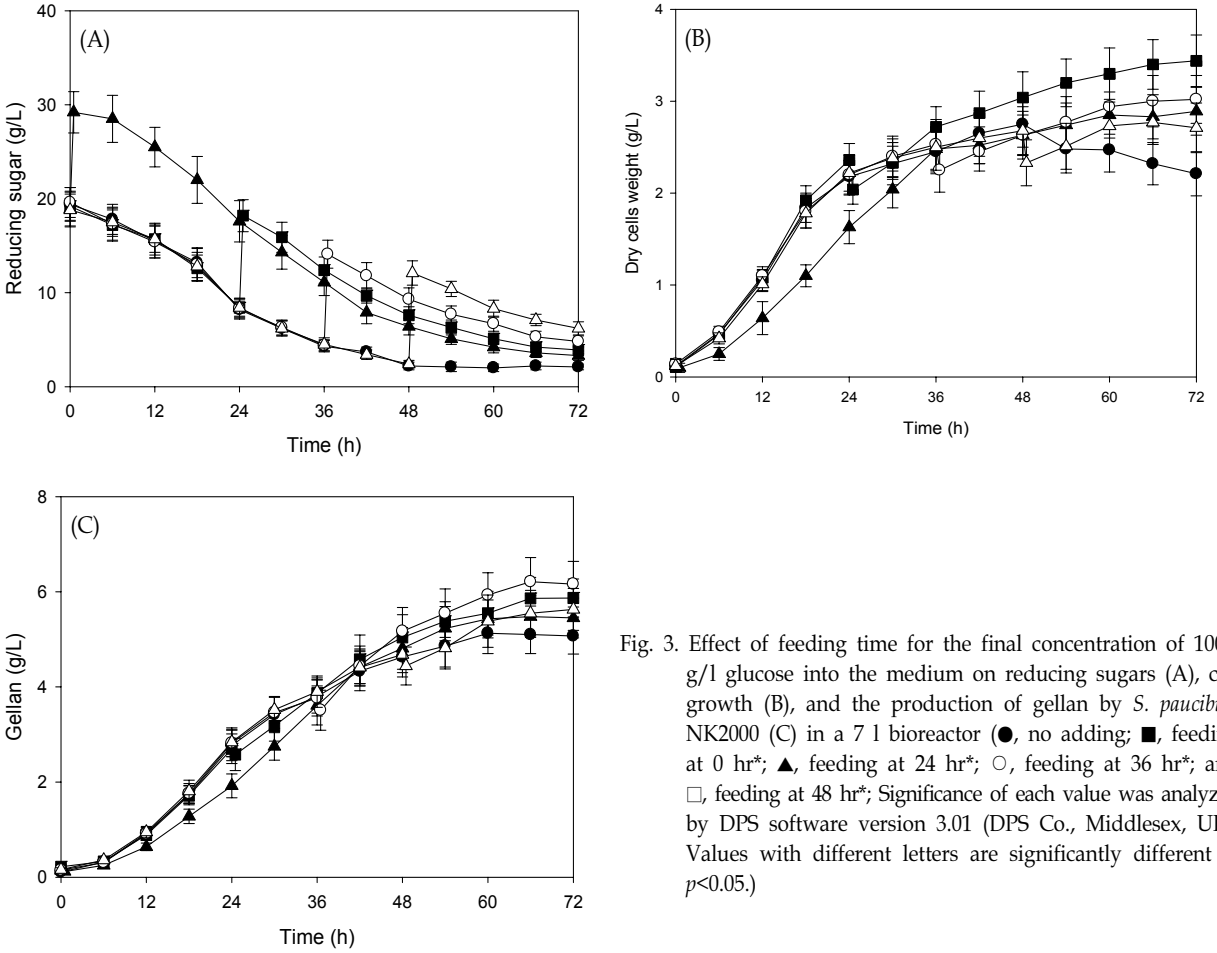

Fig. 3. Effect of feeding time for the final concentration of 100.0 g/l glucose into the medium on reducing sugars (A), cell growth (B), and the production of gellan by *S. paucibilis* NK2000 (C) in a 7 l bioreactor (●, no adding; ■, feeding at 0 hr*; ▲, feeding at 24 hr*; ○, feeding at 36 hr*; and □, feeding at 48 hr*; Significance of each value was analyzed by DPS software version 3.01 (DPS Co., Middlesex, UK). Values with different letters are significantly different at $p<0.05$.)

Table 1. Effect of feeding time for the final concentration of 10.0 g/l glucose in the medium on cell growth and the production of gellan by *S. paucibilis* NK2000 for 72 hr cultivation

Feeding time (hr)	Final pH	DCW (g/l)	Gellan (g/l)	Reducing sugars (g/l)	$Y_{x/s}$[1]	$Y_{p/s}$[2]	$Y_{p/x}$[3]
No feeding	5.6	2.21±0.22^{c}[4]	5.07±0.32^{b}	2.11±0.25^{d}	0.11	0.25	2.29
0	5.5	2.89±0.24^{b}	5.45±0.34^{ab*}[4]	3.32±0.28^{c*}	0.10	0.18	1.89
24	5.6	3.44±0.22^{a}	5.87±0.35^{ab*}	3.91±0.36^{c*}	0.11	0.20	1.71
36	5.8	3.02±0.23^{b}	6.16±0.42^{a*}	4.84±0.42^{b*}	0.10	0.21	2.04
48	6.0	2.71±0.18^{b}	5.63±0.33^{ab*}	6.37±0.49^{a*}	0.09	0.19	2.00

[1]yield of g dry cells weight/g glucose
[2]yield of g gellan/g glucose
[3]yield of g gellan/g dry cells weight
[4]*Values with different letters are significantly different at $p<0.05$.

UK). Values with different letters are significantly different at $p<0.05$.

The best feeding time of glucose for cell growth was not the same as that for the production of gellan by *S. paucibilis* NK2000, as shown Fig. 3B and C. The highest cell growth of *S. paucibilis* NK2000 was obtained when 200.0 g/l glucose was fed into the culture broth after 24 hr cultivation, whereas the highest production of gellan was obtained when the same concentration of glucose was fed into the culture broth after 36 hr cultivation. Glucose, as a carbon source, seems to be first utilized for cell growth of *S. paucibilis* NK2000 and then biosynthesis of precursors for production of gellan. The highest production of gellan from 20.0 g/l glucose with feeding of 200.0 g/l glucose after 36 hr of cultivation was 6.16 g/l, which conversion rate was 20.5%. Fed-batch culture is a batch culture fed continuously or sequentially with substrate without the removal of fermentation broth, which is generally superior to batch and continuous cultures [5].

Effect of shifts in agitation speed and aeration rate on production of gellan

Cell growth and production of gellan by five different processes were compared to investigate the effect of shifts in agitation speed and aeration rate after feeding of glucose into the medium. Its final concentration of glucose fed into the medium was 10.0 g/l. Concentrations of dissolved oxygen in culture broths rapidly decreased and reached around 0% after 36 hr of cultivation. Concentrations of dissolved oxygen after a shift in an agitation speed from 400 to 600 rpm, shifts in an agitation speed from 400 to 600 rpm and

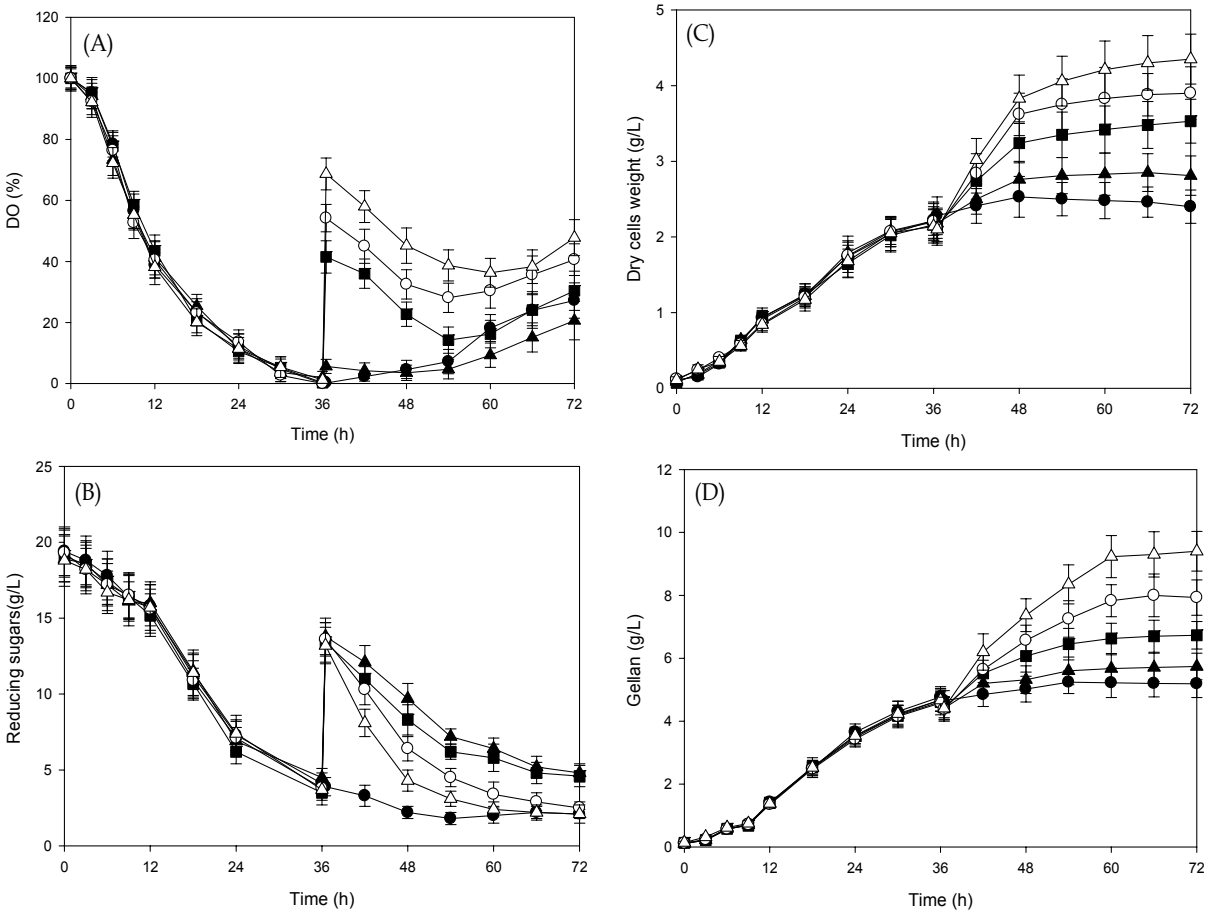

Fig. 4. Effect of feeding of the final concentration of 10.0 g/l glucose and shifts of agitation speed and aeration rate on dissolved oxygen in the medium (A), reducing sugars (B), cell growth (C), and the production of gellan (D) by *S. paucibilis* NK2000 (●, without feeding; ▲, feeding at 36 hr; ■, feeding at 36 hr and a shift of a agitation speed from 400 to 600 rpm*; ○, feeding at 36 hr and shifts of an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 1.5 vvm*, and △, feeding at 36 hr and shifts of an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 2.0 vvm*).

Table 2. Effect of shifts in agitation speed and aeration rate after glucose feeding on cell growth and the production of gellan by *S. paucibilis* NK2000

Process	Glucose[1] (g/l)	Agitation speed (rpm)		Aeration rate (vvm)		DCW (g/l)	Gellan (g/l)
		Before[2]	After[3]	Before	After		
1	20	400	400	1.0	1.0	2.40±0.32[c4]	5.19±0.42[d]
2	30	400	400	1.0	1.0	2.81±0.24[c]	5.74±0.36[cd*4]
3	30	400	600	1.0	1.0	3.53±0.31[b*]	6.73±0.58[c*4]
4	30	400	600	1.0	1.5	3.90±0.36[ab*]	7.93±0.61[b*]
5	30	400	600	1.0	2.0	4.35±0.42[a*]	9.40±0.85[a*]

[1]final concentration of glucose as a carbon source in the medium after feeding of 200.0 g/l glucose
[2]agitation speed before 36 hr cultivation
[3]agitation speed after 36 hr cultivation
[4]* Values with different letters are significantly different at $p<0.05$.

an aeration rate from 1.0 to 1.5 vvm, and shifts in an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 2.0 vvm were increased to 41.5, 54.2, and 68.7%, respectively, as shown in Fig. 4A. Reducing sugars in culture broths were about 3.0 g/l after 36 hr cultivation and those after glucose feeding increased to about 13.0 g/l. Consumption rates of reducing sugars after glucose feeding increased with elevated agitation speeds and aeration rates, as shown in Fig. 4B. Elevated concentrations of dissolved oxygen (DO) in the culture broth by shifts in agitation speeds were reported to enhance productivity of microbial metabolites [14,22].

Cell growths of *S. paucibilis* NK2000, measured as dry cells weight (DCW), from 20.0 g/l glucose without feeding, 20.0 g/l with feeding of 200.0 g/l glucose at 36 h, 20.0 g/l glucose with feeding of 200.0 g/l glucose at 36 hr and a shift in an agitation speed from 400 to 600 rpm, 20.0 g/l glucose with feeding of 200.0 g/l glucose at 36 hr and shifts in an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 1.5 vvm, and 20.0 g/l glucose with feeding of 200.0 g/l glucose at 36 hr and shifts in an agitation speed from 400 to 600 rpm and an aeration rate from 1.0 to 2.0 vvm, were 2.40, 2.81, 3.53, 3.90, and 4.35 g/l, respectively, as shown in Table 2 and Fig. 4C. Productions of gellan by *S. paucibilis* NK2000 from those of five different processes were 5.19, 5.74, 6.73, 7.93, and 9.40 g/l, respectively, and their conversion rates from glucose as a carbon source were 26.0, 19.1, 22.4, 26.4, and 31.3%, as shown in Table 2 and Fig. 4D. Production of gellan and its conversion rate by *S. paucibilis* NK2000 from 20.0 g/l glucose as a carbon source with feeding of 200.0 g/l glucose at 36 hr and shifts in an agitation speed from 400 to 600 rpm and an aeration rate of 1.0 to 2.0 vvm were 9.40 g/l and 31.3%, which were 1.81 and 1.20 times higher than those without feeding as well as shifts in an agitation speed and an aeration rate. The investigation of intermediate two-step addition of glucose under identical conditions of fermentation showed an enhanced production of gellan with a concentration of 8.1 g/l as compared with the control of 6.0 g/l [11]. There are several reports with respect to the application of shifts in an agitation speed, an aeration rate, temperature, and/or pH of culture medium during cultivation to enhance productivity of microbial metabolites [15,23-25]. In this study, a simple process was developed to enhance production of gellan with shifts in an agitation speed and an aeration rate after glucose feeding at an optimal time.

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초록 : *Sphingomonas paucibilis* NK-2000 균주가 생산하는 젤란의 생산 농도 향상을 위한 포도당 첨가 및 교반속도와 통기량 변화 방법의 최적화

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Sphingomnas paucibilis NK2000 균주를 사용한 젤란의 최적 교반속도 및 통기량은 각각 400 rpm 및 1.0 vvm이었다. 이 균주를 사용하여 젤란의 생산성을 향상시키기 위한 포도당의 최적 첨가시기는 배양을 시작한 36시간이었다. 젤란의 생산성을 향상시키기 위한 5가지 방법, 1) 포도당을 첨가하지 않는 방법, 2) 배양 36시간 후에 포도당을 첨가하지만 교반속도 및 통기량을 변화시키지 않는 방법, 3) 배양 36 시간 후에 포도당을 첨가하고 교반속도를 400 rpm에서 600 rpm으로 변화시키는 방법, 4) 배양 36 시간 후에 포도당을 첨가하고 교반속도를 400 rpm에서 600 rpm으로 증가시키고 통기량을 1.0 vvm에서 1.5 vvm으로 증가시키는 방법 및 5) 배양 36 시간 후에 포도당을 첨가하고 교반속도를 400 rpm에서 600 rpm으로 증가시키고 통기량을 1.0 vvm에서 2.0 vvm으로 증가시키는 방법 등을 실험한 결과, 젤란의 생산성은 각각 5.19, 5.74, 6.73, 7.93, 및 9.40 g/l이었으며, 변환률은 각각 26.0, 19.1, 22.4, 26.4, and 31.3%이었다. 최적의 방법으로 생산한 젤란의 생산농도 및 포도당 전환율은 포도당을 첨가하지 않은 방법으로 생산한 젤란의 생산농도 및 포도당 전환율에 비하여 각각 1.81 및 1.20 배 증가하였다.