Korean J. Chem. Eng., **26**(2), 428-432 (2009)

Fermentation process development for hyaluronic acid production by *Streptococcus zooepidemicus* ATCC 39920

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(*Received 7 July 2008 • accepted 7 September 2008*)

Abstract−The development of a fermentation strategy for hyaluronic acid (HA) production by *Streptococcus zooepidemicus* ATCC 39920 has been explored. The specific HA productivity ($Y_{P/X}$) was affected by the medium carbon-to-nitrogen (C/N) ratio rather than the specific growth rate of cells. Accordingly, HA fermentation should be performed in a balanced medium with an optimum C/N ratio of 2 : 1 in a batch culture. To improve the performance of the batch culture, the operation conditions for the fill-and-draw culture were investigated. It was found that the timing of medium exchange is critical for successfully performing fill-and-draw operations. Since streptococcal cells at the stationary phase might lose the capacity of HA synthesis, the displacement of the medium in a fill-and-draw culture should be started at the late exponential growth phase.

Key words: *Streptococcus zooepidemicus*, Hyaluronic Acid, Carbon-to-Nitrogen Ratio, Specific Growth Rate, Fed-batch, Fill-and-draw Culture

INTRODUCTION

Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan consisting of alternating D-glucuronic acid and N-acetylglucosamine, which are linked alternately by $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ glycosidic linkages [1]. This structural characteristic allows HA to retain large amounts of water and to have a pseudoplastic fluid property. Consequently, HA is endowed with viscoelasticity, demonstrating high viscosity at low shear rates and shows high elasticity at high shear rates [2]. Because of its distinct hydrodynamic properties, HA has been widely applied in the pharmaceutical and cosmetic industries [3-5]. HA is typically found in the connective tissues of animals as well as in the capsules of streptococcal bacteria. Traditionally, HA has been extracted from rooster combs; however, microbial production is receiving increased attention for prevention of viral infection [5]. Furthermore, microbial fermentation of polysaccharides offers additional advantages; for instance, the purification is relatively simple and avoids the use of hazardous solvents during purification processes [6].

Developing an appropriate fermentation strategy for HA production is necessary for an economical fermentation process. Fermentation process development involves the determination of culture conditions and the establishment of a cultivation mode. The influential factors in culture conditions include medium composition, temperature, pH, aeration, and agitation. The design of a cultivation mode is based on the effect of the specific growth rate on metabolite production. If metabolite production is not affected by the specific growth rate, batch culture is preferable. Once metabolite production is affected by the specific growth rate, manipulating the specific growth rate appropriately in a fed-batch or continuous operation can result in the maximum productivity. Therefore, an understanding of the effect of specific growth rate on metabolite production is fundamental for developing a fermentation process.

Several reports have been concerned with the culture conditions of HA production [7-11], the metabolic fluxes for HA production [12], and the optimization of HA production on an industrial scale [13]. However, little information is available regarding the effect of medium carbon-to-nitrogen (C/N) ratios which might affect the metabolic flux. In addition, even though a negative relationship between specific growth rate and specific HA productivity has been reported [5,9], it is possible that the decease in HA productivity can be attributed to other factors because the specific growth rates in the studies were varied by changing cultivation temperature or pH. Therefore, further efforts to examine the effect of specific growth rate on HA production are worth considering.

The current study was undertaken to investigate the effect of C/N ratios on HA fermentation and to develop a balanced medium for producing HA. The effect of specific growth rate on HA production was assessed by using a fed-batch culture. After disclosing that HA production was irrelevant to specific growth rate, a fill-and-draw operation was proposed for improving the performance of a batch culture. The operation strategy for performing fill-and-draw culture was also discussed.

MATERIALS AND METHODS

1. Bacterial Strain

Streptococcus zooepidemicus ATCC 39920 was used as the HA producer in this study. The cells were maintained at −30 °C in 50% (vol/vol) glycerol. Inocula were prepared in a 500-mL Erlenmeyer flask with 100 mL of the TSB medium, which contained (per liter) 17 g of pancreatic digest of casein, 3 g of enzymatic digest of soybean meal, 2.5 g of glucose, 2.5 g of K_2HPO_4, and 5 g of NaCl. After

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inoculum, the flask was incubated at 37 °C in a reciprocal shaker at 150 rpm for 12 h.

2. Batch Fermentation

Batch production of HA was carried out in a 2.5-L fermentor (M-100, Tokyo Rikakikai, Japan) with a working volume of 1.5 L. The fermentation medium was comprised of (per liter) 20 g of glucose, 10 g of yeast extract, 2.5 g of K_2HPO_4, 2 g of NaCl, and 1.5 g of $MgSO_4 \cdot 7H_2O$. The conditions during fermentation were the following: a temperature of 37 °C, a pH of 7.0 (with 5 N NaOH). The dissolved oxygen level was maintained at 20% of air saturation by increasing the aeration rate and/or supplementing pure oxygen. A gate impeller, similar to that of Hiruta et al. [14], with a speed of 300 rpm was used for agitation.

3. Fed-Batch Operation

Fed-batch operation was carried out under the same conditions as that in batch fermentation. The operation was initiated in batch mode, and contained 1,000 mL of the fermentation broth with 5 g of glucose, 2.5 g of yeast extract, 2.5 g of K_2HPO_4, 2 g of NaCl, and 1.5 g of $MgSO_4 \cdot 7H_2O$. When the initial glucose was depleted, the operation was changed to fed-batch mode by introducing 500 mL of the feeding medium containing 25 g of glucose, 12.5 g of yeast extract, 1.25 g of K_2HPO_4, 1 g of NaCl, and 0.75 g of $MgSO_4 \cdot 7H_2O$. The specific growth rate (μ) in the fed-batch phase was maintained at a constant value by exponentially feeding the feeding medium [15-17]. The feeding rate (F) was determined by using a mass balance equation of cells and the glucose, represented by:

$$F = \frac{\mu X_0 V_0}{Y_{X/S} S_0} e^{\mu t} \quad (1)$$

where $Y_{X/S}$ is the growth yield, S_0 is the feeding concentration of glucose, and X_0 and V_0 are the cell concentration and the culture volume at the beginning of the fed-batch phase, respectively. From preliminary batch cultivation, it was found that $Y_{X/S}$=0.15 g cell/g glucose, X_0=0.9 g/L, and V_0=1,000 mL.

4. Analytical Methods

Cell concentration was measured from the optical density (OD) of the broth at 660 nm with a spectrophotometer (model UV-1201, Shimadzu, Japan); the OD obtained (after adequate dilution) was then correlated with dry cell weight (DCW). Because of a change in cell morphology after entry into the stationary phase, cell concentrations in the exponential growth phase and the stationary phase were correlated separately; the former was correlated by using DCW (g/L)=0.399×OD−0.003, while the latter used DCW (g/L)=0.456×OD−0.012 [18].

In the analysis of HA concentration, the sample was first incubated with an equal volume of 0.1% (w/v) sodium dodecyl sulfate for 10 min to liberate the capsular HA and to facilitate the separation of the cells [19]. After the cells were removed by centrifugation at 10,000×*g* for 10 min, the supernatant was then subjected to HA precipitation by mixing it with four volumes of ethanol. The precipitate was collected by centrifugation at 3,000×*g* for 10 min, and redissolved in water. Finally, the HA concentration was determined by the carbazole method [20]. The optical density was measured at 525 nm with D-glucuronic acid used as the standard.

Residual glucose was assayed to be a reducing sugar by the DNS method [21]. The concentration of organic acids was determined by an HPLC system (L-7000, Hitachi, Tokyo, Japan) equipped with an ionic exchange column (CARBO Sep COREGEL 87H3, Transgenomic, USA), which was maintained at 55 °C. 0.01 N H_2SO_4 was used as the mobile phase at a flow rate of 0.5 mL/min, and the effluent was monitored by a Hitachi L-7455 diode array detector with a detection wavelength of 210 nm.

RESULTS AND DISCUSSION

1. Effect of Carbon-to-Nitrogen (C/N) Ratio

The effect of the carbon-to-nitrogen (C/N) ratio of the culture medium on cell growth, HA production, and lactic acid formation is shown in Fig. 1. Using a fixed glucose concentration (20 g/L), the supplemented yeast extracts were 5, 10, and 15 g/L, respectively. As illustrated in Fig. 1, the increase of yeast extract improves the growth of streptococcal cells. Although the yield of cell mass increased with decreasing C/N ratio, a C/N ratio of 2 : 1 was observed to produce the maximum yield of HA. In addition to HA, lactic acid is a major metabolite of streptococcal cells, which accumulates as glucose is consumed. At C/N ratios of 2 : 1 and 1.3 : 1, the carbon source was depleted at the onset of the stationary phase. However, at a C/N ratio of 4 : 1, the consumed glucose in the cell growth stage was only 13 g/L, and the remaining glucose was not converted to biomass or HA. The cessation of cell growth can be viewed as the ex-

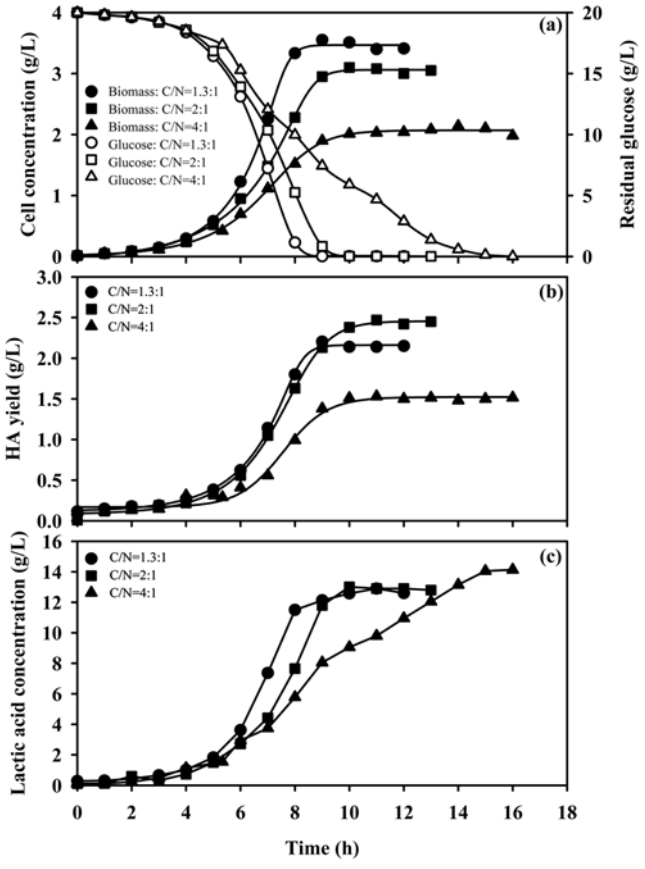

Fig. 1. Effect of carbon-to-nitrogen (C/N) ratio in culture medium with a fixed glucose concentration (20 g/L) on cell growth, HA production and lactic acid formation. Conditions: C/N=1.3 : 1 (15 g/L yeast extract); C/N=2 : 1 (10 g/L yeast extract); C/N=4 : 1 (5 g/L yeast extract).

Table 1. Effect of carbon-to-nitrogen (C/N) ratio on the specific HA productivity, yield coefficients of biomass, HA, and lactic acid (LA)

	Carbon-to-nitrogen ratio		
	1.3 : 1	2 : 1	4 : 1
HA yield (g HA/L)	2.20	2.45	1.52
Specific HA productivity $Y_{P/X}$ (g HA/g cell)	0.59	0.76	0.72
Yield coefficient of biomass $Y_{X/S}$ (g cell/g glucose)	0.18	0.15	0.15* 0.10#
Yield coefficient of HA $Y_{P/S}$ (g HA/g glucose)	0.10	0.12	0.11* 0.07#
Yield coefficient of LA $Y_{LA/S}$ (g LA/g glucose)	0.64	0.64	0.64* 0.70#

*The yield coefficients estimated from the cell growth phase.
#The overall yield coefficients calculated from the whole fermentation process.

haustion of the nitrogen source. In the absence of a nitrogen source, almost all of the residual glucose was converted to lactic acid.

The specific HA productivity ($Y_{P/X}$), defined as the ratio of total increment of HA to total increment of biomass, is used as an indicator for assessing the efficiency of HA fermentation. As shown in Table 1, fermentation of *S. zooepidemicus* ATCC 39920 with a C/N ratio of 2 : 1 gave the maximum $Y_{P/X}$ and the highest yield coefficient of HA ($Y_{P/S}$). A much higher growth yield ($Y_{X/S}$) and a lower $Y_{P/S}$ were observed when the C/N ratio was lower than 2 : 1. This suggests that a relatively larger amount of nitrogen source in the medium shifted the flux of glucose from HA synthesis to cell growth. The fermentation parameters ($Y_{P/X}$, $Y_{X/S}$ and $Y_{P/S}$) calculated from the cell growth phase at the C/N ratios of 2 : 1 and 4 : 1 were comparable. However, at a C/N ratio of 4 : 1, the excess glucose was converted to organic acids rather than biomass or HA, leading to an obvious decrease in overall $Y_{X/S}$ and $Y_{P/S}$. Apparently, the streptococcal cells are capable of transforming glucose to organic acids even if the nitrogen is insufficient.

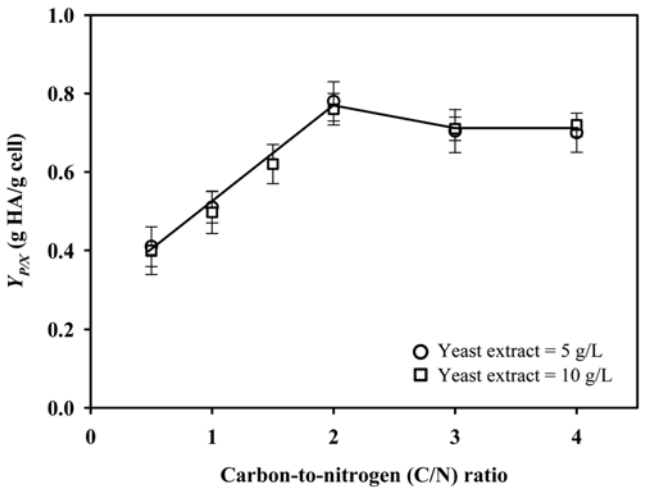

Fig. 2. Effect of carbon-to-nitrogen ratio in culture medium with equal concentrations of yeast extract on the specific HA productivity ($Y_{P/X}$).

The optimum C/N ratio for producing HA was further confirmed by using equal concentrations of yeast extract, where various concentrations of glucose were supplemented. As illustrated in Fig. 2, the highest $Y_{P/X}$ was produced with a C/N ratio of 2 : 1 for concentration of yeast extract of 5 and 10 g/L. This implies that a balanced medium is necessary for conducting HA fermentation, and, therefore, the fed-batch culture should be performed by feeding a medium with an optimum C/N ratio.

2. Effect of Specific Growth Rate on HA Production

Microbial specific growth rate can be manipulated by fed-batch or continuous operation. However, HA production in a continuous culture is hard to achieve at a high dilution rate [21], where the occurrence of non-HA-producing variants is observed. In addition, continuous fermentation is subject to a low efficiency of substrate utilization, which inherently results from the residence time distribution of the substrate. The specific growth rate of streptococcal cells was therefore controlled at a desired value by a fed-batch operation in this study.

The effect of specific growth rate on HA production is shown in Fig. 3. In the first stage of batch culture, the cells grew to a cell concentration of 0.9 g/L until the initial glucose was depleted; then, after the first stage of cultivation, the feeding medium was introduced by using a pulse or an exponential feeding strategy. As can be seen in Fig. 3, the pulse feeding resulted in a specific growth rate of 0.58 h^{-1} at 37 °C; the other specific growth rates of cells were obtained with different exponential feeding rates. Therefore, the in-

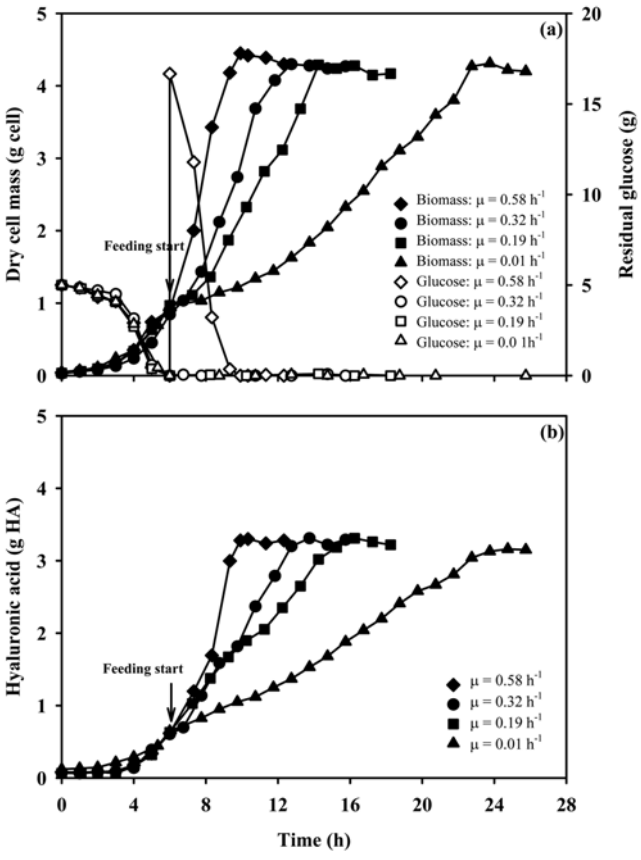

Fig. 3. Effect of specific growth rate on HA fermentation.

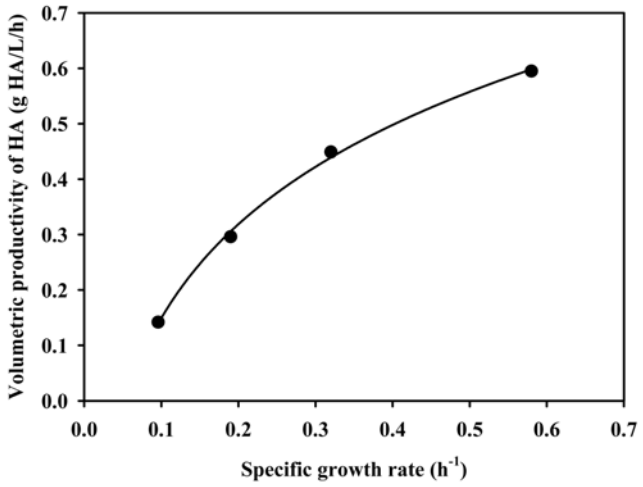

Fig. 4. Effect of specific growth rate on the volumetric productivity of HA at a fermentation temperature of 37 °C.

fluence of specific growth rate on HA fermentation can be examined from the results of the fed-batch stage. The increased biomass and the produced HA obtained at various specific growth rates were quite similar. This means that $Y_{P/X}$ is irrelevant to specific growth rate; additionally, $Y_{P/X}$ is a constant during the whole fermentation process. The specific production rate of HA (q_p) is thus expected to increase with a higher specific growth rate. In addition, the volumetric productivity, calculated by dividing the increased HA yield at the fed-batch phase with the corresponding culture time, shows a positive correlation to specific growth rate, as illustrated in Fig. 4. All together, these results suggest that batch culture is the optimum mode for HA production.

3. HA Production in a Batch Fill-and-Draw Culture

Since the highest volumetric productivity of HA is obtained from the maximum specific growth rate, one would expect that HA production should be performed in a batch culture. However, the batch process requires cleaning, sterilization, and inoculation procedures after each fermentation cycle. To reduce the time spent on fermenter turnover, the operation of batch fill-and-draw culture is worth investigating.

In the operation of a fill-and-draw culture, two critical factors are the timing of medium exchange and the amount of medium displacement. To maintain an adequate cell density, our test exchanged one-third of the medium (500 mL). The results of the fill-and-draw operation, starting at 9 h corresponding to the onset of the stationary phase, are shown in Fig. 5. Although cell density could be recovered within four cycles, the HA yield decreased from 2.0 g/L to 1.3 g/L, indicating that the cells gradually lost the ability to synthesize HA during the repeated culture. van de Rijn [22] indicated that the membrane derived from the streptococcal cells at the stationary phase lacked the capacity to synthesize HA. The stationary phase was considered an inappropriate time for starting a fill-and-draw operation.

To prevent the loss of HA synthesis capacity, the fill-and-draw operation was advanced to the fermentation time of 8 h corresponding to the late exponential growth phase. As can be seen in Fig. 6, both cell density and HA yield could be recovered and performed repeatedly for six cycles when one-third of the medium was exchanged. The volumetric productivity of HA, defined as the total HA yield obtained in one replacement divided by the total working volume and the time interval, was 0.105 g HA L^{-1} h^{-1}. When the amount of medium displacement increased to two-thirds of the medium (1,000 mL), the operation could still be performed repeatedly for six cycles, which resulted in a volumetric productivity of

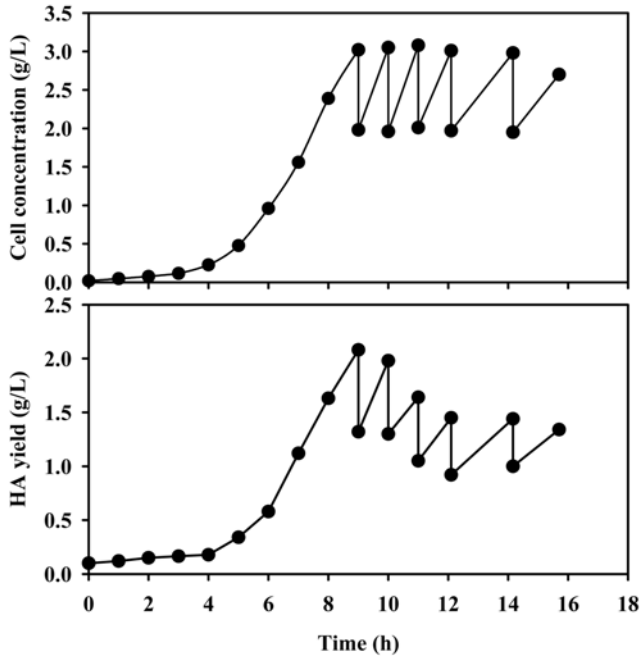

Fig. 5. Fill-and-draw culture of *S. zooepidemicus* with an exchange of 500 mL of medium starting at the fermentation time of 9 h.

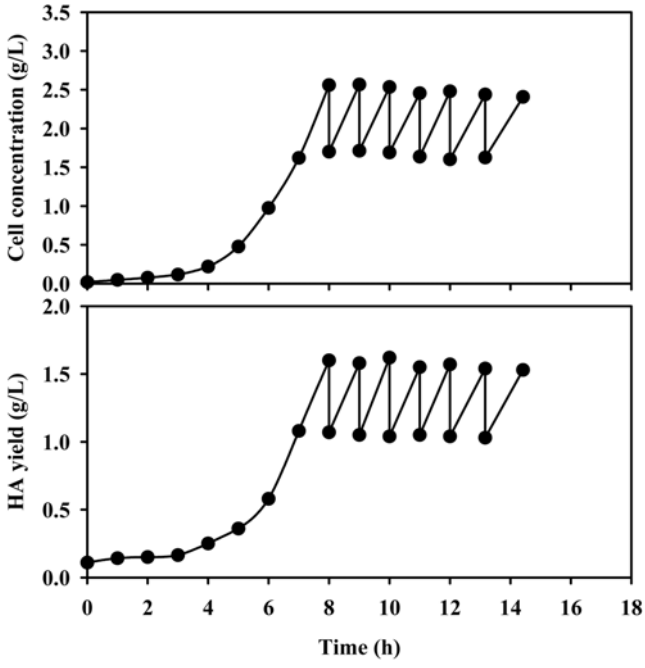

Fig. 6. Fill-and-draw culture of *S. zooepidemicus* with an exchange of 500 mL of medium starting at the fermentation time of 8 h.

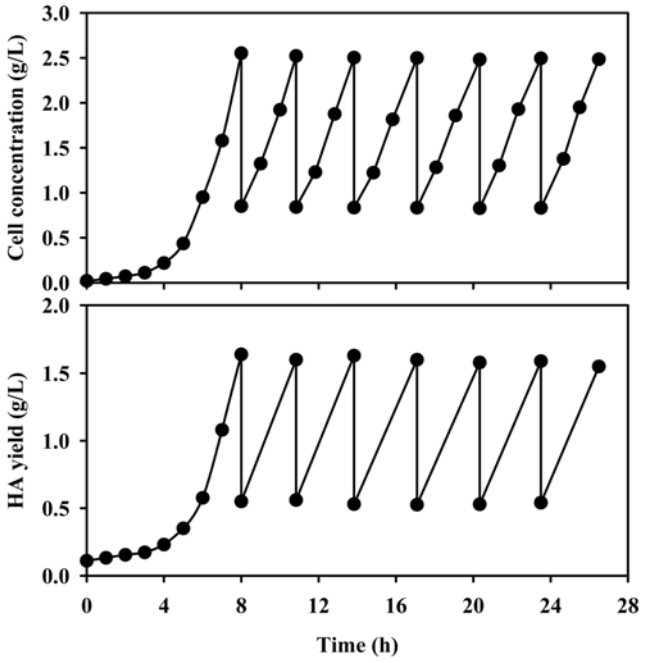

Fig. 7. Fill-and-draw culture of *S. zooepidemicus* with an exchange of 1,000 mL of medium starting at the fermentation time of 8 h.

0.059 g HA L^{-1} h^{-1}, as shown in Fig. 7. The above results indicate that the fill-and-draw operation should be started before the onset of the stationary phase. An increase in the amount of medium exchanged might decrease the volumetric productivity of HA.

CONCLUSIONS

In HA production by *Streptococcus zooepidemicus* ATCC 39920, the principle of developing a fermentation strategy was illustrated. A balanced medium with an optimum carbon-to-nitrogen ratio of 2 : 1 should be prepared to maximize the capacity of HA synthesis. The specific productivity of HA was irrelevant to the specific growth rate of streptococcal cells, indicating that batch culture is the optimum mode for producing HA. To reduce the turnover time of batch culture, the fill-and-draw operation is a promising fermentation mode. The medium should be exchanged at the late exponential growth phase when the fill-and-draw culture is performed.

ACKNOWLEDGMENTS

The authors would like to thank the National Science Council of Taiwan for their financial support under grants NSC 95-2622-E-151-024-CC3 and NSC 96-2221-E-151-043-MY2.

REFERENCES

1. B. Weissmann and K. Meyer, *J. Am. Chem. Soc.*, **76**, 1753 (1954).
2. T. J. Liesegang, *Survey Ophthalmol.*, **34**, 268 (1990).
3. I. F. Radaeva, G. A. Kostina and A. V. Zmievskii, *Appl. Biochem. Microbiol.*, **33**, 111 (1997).
4. L. Lapčik, Jr., L. Lapčik, S. De Smedt, J. Demeester and P. Chabreček, *Chem. Rev.*, **8**, 2663 (1998).
5. B. Fong Chong, L. M. Blank, R. Mclaughlin and L. K. Nielsen, *Appl. Microbiol. Biotechnol.*, **66**, 341 (2005).
6. T. Khan, J. K. Park and J.-H. Kwon, *Korean J. Chem. Eng.*, **24**, 816 (2007).
7. M. R. Johns, L. T. Goh and A. Oeggerli, *Biotechnol. Lett.*, **16**, 507 (1994).
8. J.-H. Kim, S.-J. Yoo, D.-K. Oh, Y.-G. Kweon, D.-W. Park, C.-H. Lee and G.-H. Gil, *Enzyme Microb. Technol.*, **19**, 440 (1996).
9. D. C. Armstrong, M. J. Cooney and M. R. Johns, *Appl. Microbiol. Biotechnol.*, **47**, 309 (1997).
10. D. C. Armstrong and M. R. Johns, *Appl. Environ. Microbiol.*, **63**, 2759 (1997).
11. S. Hasegawa, M. Nagatsuru, M. Shibutani, S. Yamamoto and S. Hasebe, *J. Biosci. Bioeng.*, **88**, 68 (1999).
12. H.-J. Gao, G.-C. Du and J. Chen, *World J. Microbiol. Biotechnol.*, **22**, 399 (2006).
13. S.-J. Kim, S.-Y. Park and C.-W. Kim, *J. Microbiol. Biotchnol.*, **16**, 1849 (2006).
14. O. Hiruta, K. Yamamura, H. Takebe, T. Futamura, K. Iinuma and H. Tanaka, *J. Ferment. Bioeng.*, **83**, 79 (1997).
15. A. Patkar, D. H. Lee and J.-H. Seo, *Korean J. Chem. Eng.*, **10**, 146 (1993).
16. L.-C. Cheng, J.-Y. Wu and T.-L. Chen, *Biochem. Eng. J.*, **10**, 227 (2002).
17. A. Arpornwichanop and N. Shomchoam, *Korean J. Chem. Eng.*, **24**, 11 (2007).
18. W.-C. Huang, S.-J. Chen and T.-L. Chen, *Biochem. Eng. J.*, **32**, 239 (2006).
19. B. Fong Chong and L. K. Nielsen, *J. Biotechnol.*, **100**, 33 (2003).
20. T. Bitter and H. M. Muir, *Anal. Biochem.*, **4**, 330 (1962).
21. G. L. Miller, *Anal. Chem.*, **31**, 426 (1959).
22. L. M. Blank, R. L. McLaughlin and L. K. Nielsen, *Biotechnol. Bioeng.*, **90**, 686 (2005).
23. I. van de Rijn, *J. Bacteriol.*, **156**, 1059 (1983).