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Enhancement of L-Threonine Production by Controlling Sequential Carbon-Nitrogen Ratios during Fermentation

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Controlling the residual glucose concentration is important for improving productivity in L-threonine fermentation. In this study, we developed a procedure to automatically control the feeding quantity of glucose solution as a function of ammonia-water consumption rate. The feeding ratio ($R_{C/N}$) of glucose and ammonia water was predetermined via a stoichiometric approach, on the basis of glucose-ammonia water consumption rates. In a 5-L fermenter, 102 g/l L-threonine was obtained using our glucose-ammonia water combined feeding strategy, which was then successfully applied in a 500-L fermenter (89 g/l). Therefore, we conclude that an automatic combination feeding strategy is suitable for improving L-threonine production.

Keywords: L-Threonine, feeding ratio ($R_{C/N}$), feeding strategy, residual glucose concentration, scale-up

During fermentation, the feeding strategy used to control the residual concentration of a key nutrient, typically the carbon source, affects productivity and yield [1, 2]. Several efforts have been made to improve amino-acid production through exponential feeding of glucose [3–5]; particularly, pseudo-exponential and glucose-stat feeding have been used in L-tryptophan production [6]. Feeding strategies may also be based on physiology, such as pH- [7, 8] or DO-stat [9, 10] techniques that depend on acid production or oxygen utilization. However, both strategies require instantly exhausting the carbon source, a process that changes the microbial composition and negatively affects productivity and product yield in the culture broth. Intermittent feeding and continuous feeding strategies are still used in industrial amino acid production through fermentation [11].

In L-threonine fermentation, *Escherichia coli* mutant strains resistant to α-amino-β-hydroxybutyric acid (L-threonine analog) are usually used, and controlling the glucose concentration in the culture broth is more important than controlling other nutrients [12]. Thus, the main parameters in L-threonine production are the nitrogen and carbon sources. In our previous study using this *E. coli* mutant, we optimized the concentrations of the carbon and nitrogen sources to improve L-threonine production [13]. Thus, in this study, we hypothesized that an automatic combination feeding strategy of glucose-ammonia water as substrates would further enhance L-threonine production.

Here, we used *E. coli* MT201 derived from *E. coli* K-12 as AHV^r, Met-, diaminopimelic $acid^L$, and Ile^L, as an L-threonine producer. For the seed culture, one colony was inoculated into a 1-L flask containing 100 ml of seed medium and the following reagents: 40.0 g/l glucose, 10.0 g/l $(NH_4)_2SO_4$, 1.0 g/l KH_2PO_4, 0.5 g/l $MgSO_4{\cdot}7H_2O$, 2.0 g/l yeast extract, 0.1 g/l L-methionine, 0.05 g/l L-lysine, 0.05 g/l L-isoleucine, and 0.6 g/l $CaCO_3$. The colony was cultivated in a shaking incubator (180 rpm) at 37°C for 24 h. The culture medium fermentation contained: 40.0 g/l glucose, 25.0 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 1.0 g/l $MgSO_4{\cdot}7H_2O$, 0.5 g/l $MnSO_4{\cdot}H_2O$, and 0.5 g/l $FeSO_4{\cdot}7H_2O$. Glucose and $MgSO_4{\cdot}7H_2O$ were sterilized separately from

the culture medium. The feeding medium was 800 g/l glucose solution. The L-threonine concentration was determined using the Kase-ninhydrin method [14]. Fermentation studies were conducted on two scales, in 5-L and 500-L fermenters (Korea Fermenter Co., Korea), with pH maintained at 6.3 through automatic addition of 25% NH_4OH. Dissolved oxygen (DO) was maintained at >20% by adjusting the agitation and aeration. Initial working volumes for the 5-L and 500-L fermenters were 2-L and 200-L, respectively.

The residual glucose concentration was controlled by connecting glucose and ammonia reservoirs to the fermenter via pH-regulator-controlled peristaltic pumps (Miniplus 3; Gilson, France). Glucose-ammonia water was added at predetermined pump speeds and intervals. Additionally, the culture broth pH was adjusted whenever it decreased to below the threshold, triggering peristaltic pumps connected to the ammonia container and set to a pre-fixed speed. Simultaneously, peristaltic pumps connected to the glucose reservoir operated at $R_{C/N}$ of the pre-fixed speed. Here, $R_{C/N}$ is the ratio of carbon and nitrogen consumption or the molar ratio of the carbon and nitrogen content in the biomass. To calculate $R_{C/N}$, the consumption rates of glucose-ammonia water were determined using an electronic balance and monitoring system that measured bottle-weight variation. In the 5-L and 500-L fermentations using intermittent and continuous feeding methods, the average of $R_{C/N}$ in the growth and production phases was calculated.

For L-threonine production, a 5-L fed-batch cultivation was performed using continuous and intermittent glucose feeding methods (Fig. 1). Plotting glucose consumption as a function of ammonia water requirements revealed that $R_{C/N}$ was 1.35 in the growth phase and 1.84 in the L-threonine production phase (Fig. 1A). The average $R_{C/N}$ was calculated as 2.12 in the growth phase and 3.37 in the L-threonine production phase (Fig. 1B). Next, a 500-L fed-batch cultivation was also performed to determine $R_{C/N}$ based on continuous (Fig. 2A) or intermittent feeding (Fig. 2B). The two methods respectively resulted in $R_{C/N}$ of 1.47 and 1.92 in the growth phase, as well as 2.23 and 2.87 in the production phase respectively.

The $R_{C/N}$ of maximum L-threonine production with the intermittent method at 5-L was applied to 5-L and 500-L fermentations (Fig. 3). To control the fed-batch cultures, the

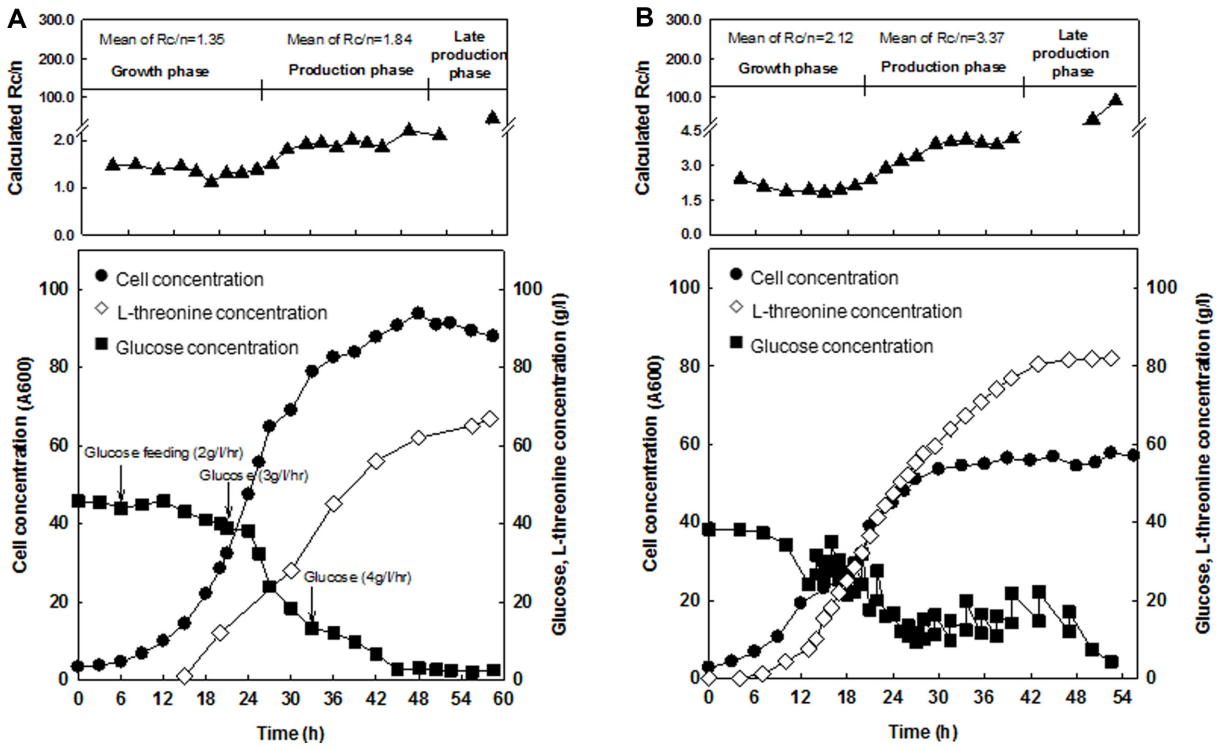

Fig. 1. Time-course profiles of 5-L fed-batch cultivation for L-threonine production using (**A**) continuous and (**B**) intermittent glucose feeding strategies.

Optical density, closed circles; glucose concentration, closed squares; L-threonine concentration, open diamonds.

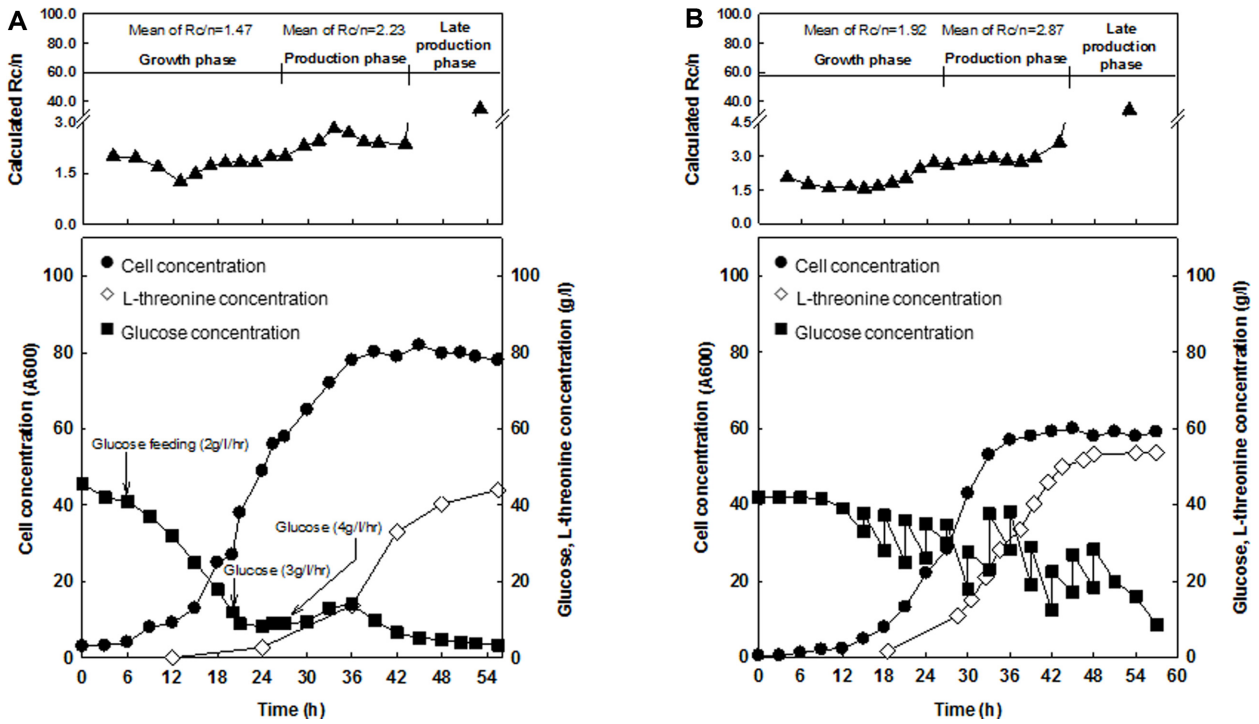

Fig. 2. Time-course profiles of 500-L fed-batch cultivation for L-threonine production using (**A**) continuous and (**B**) intermittent glucose feeding strategies.

Optical density, closed circles; glucose concentration, closed squares; L-threonine concentration, open diamonds.

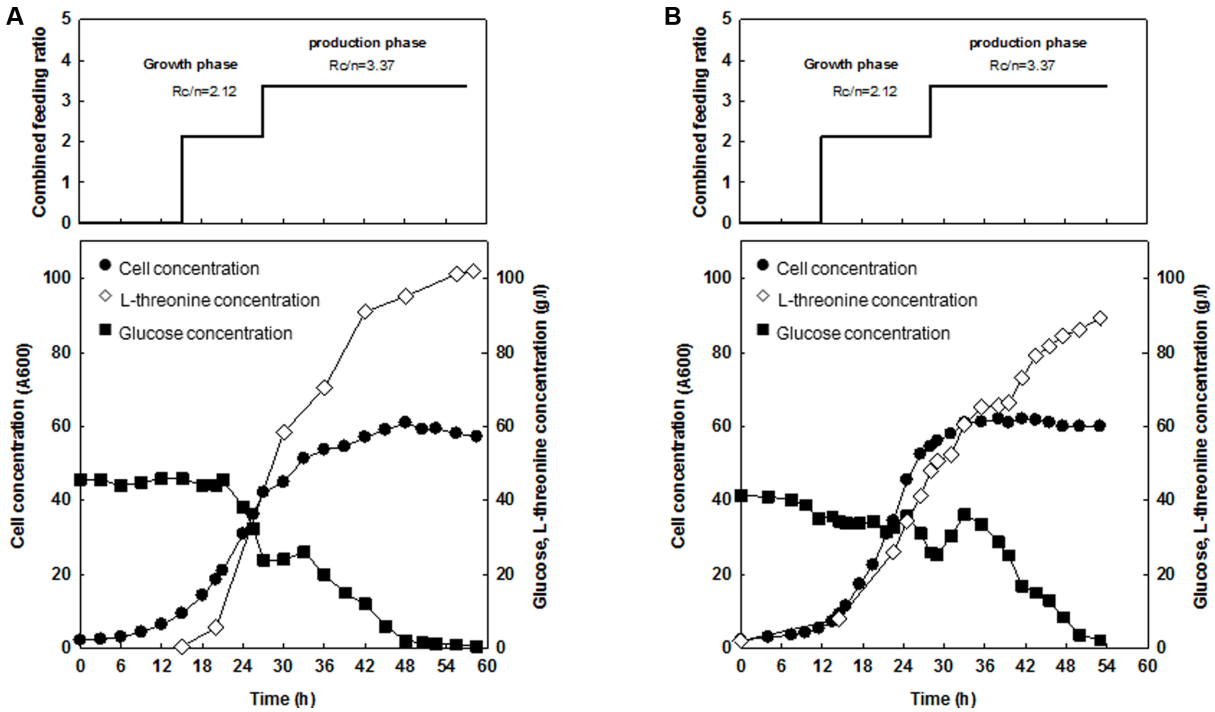

Fig. 3. Time-course profiles of (**A**) 5-L and (**B**) 500-L fed-batch cultivations for L-threonine production using the automatic combination (of glucose and ammonia water) feeding strategy.

Optical density, closed circles; glucose concentration, closed squares; L-threonine concentration, open diamonds.

Table 1. Summary of the cultivation results under different glucose feeding strategies.

Mode of operation	Feeding strategy	Cell concentration (A_{600})	Consumed glucose (g/l)	Consumed ammonia water (ml/l)	Feeding ratio of carbon and nitrogen source ($R_{C/N}$)		Produced L-threonine (g/l)
					Growth phase	Production phase	
5-L fed-batch	Continuous feeding of glucose	90.8 ± 1.2	324 ± 3.7	158 ± 3.5	1.35	1.84	67.2 ± 0.6
5-L fed-batch	Intermittent feeding of glucose	58.6 ± 2.1	252 ± 4.2	110 ± 2.8	2.12	3.37	82 ± 0.8
500-L fed-batch	Continuous feeding of glucose	68.1 ± 0.3	282 ± 1.8	128 ± 2.6	1.47	2.23	44.6 ± 0.5
500-L fed-batch	Intermittent feeding of glucose	59.1 ± 2.1	241.3 ± 2.8	107.6 ± 3.3	1.92	2.87	58.6 ± 0.4
5-L fed-batch	Combined feeding of glucose and ammonia water	61.2 ± 0.8	272 ± 2.2	110 ± 3.2	2.12	3.37	102 ± 1.2
500-L fed-batch	Combined feeding of glucose and ammonia water	63.4 ± 1.9	250 ± 2.0	98 ± 1.2	2.12	3.37	89 ± 0.6

Data are shown as the mean ± standard error of triplicate experiments.

culture phase was divided into three sections with different $R_{C/N}$ ratios: initial, exponential, and L-threonine production. The initial phase corresponded to the log phase of cell growth and had an $R_{C/N}$ near zero, implying the lack of glucose feeding despite continuous ammonia-water consumption for pH control. When cell growth entered the exponential growth after culturing for 14 h (5-L) and 9 h (500-L), glucose-ammonia water was automatically fed with a predetermined $R_{C/N}$ of 2.12. After 28 h (5-L) and 29.5 h (500-L) of culture, $R_{C/N}$ was increased to 3.37. The amount of L-threonine obtained in the 5-L fermentation was 102 g/l (automatic combination feeding strategy), 1.5- and 1.24-fold higher than the amount from continuous (67.2 g/l) and intermittent (82 g/l) feeding strategies, respectively (Fig. 3A). In 500-L fermentation, L-threonine concentration (89 g/l) increased over the concentrations from continuous (44.6 g/l) and intermittent (58.6 g/l) feeding (Fig. 3B). Table 1 summarizes the L-threonine productivity and the glucose to ammonia ratio under various feeding strategies and bioreactor scales. We note that L-threonine yield in a lab-scale fermenter could not be fully reproduced in a large-scale fermenter, even with appropriately scaled-up parameters (*e.g.*, K_La and power number). The variation in production rate may depend on fermenter geometry. Therefore, poor mixing (or nonhomogeneous mixing) in the large-scale fermenter is likely the main reason for the observed differences. Our previous study also showed that the production of L-ornithine was abnormally decreased by poorer mixing during scale-up in a 500-L fermenter [15].

A number of previous studies have attempted to improve L-threonine productivity, for instance through testing different concentrations of glucose and yeast extract as carbon and nitrogen sources, respectively [16]. The concentration of ammonium sulfate as a nitrogen source was also considered important for increasing cell density and L-threonine production [12]. In contrast, we have performed an automatic combination feeding strategy using peristaltic pumps connected to reservoirs containing carbon (glucose) and nitrogen (ammonia water) sources. We performed a two-phase fed-batch culture to determine $R_{C/N}$. To maximize growth during the growth phase, $R_{C/N}$ was set to 2.12. The ratio was then raised to 3.37 to avoid a decrease in cell activity, which occurs in response to excessive base addition during the production phase. $R_{C/N}$ determined from the 5-L intermittent feeding of glucose solution was then scaled up for application in 500-L fermentation. We also showed that the feeding ratio of carbon to nitrogen was a useful control method, given that consumption rates of the carbon and nitrogen sources are correlated. The feeding ratio of carbon to nitrogen is shown to be one of the useful control methods in our study.

Currently, no reports besides this one are available that describe L-threonine production using an automatic combination feeding strategy of glucose-ammonia water, with an online monitoring system to measure product weight variation. In conclusion, this feeding strategy allowed us to determine $R_{C/N}$ for L-threonine production in 5-L fermentation and to successfully implement it in 500-L fermentation. The $R_{C/N}$ value should be useful for efficient control of the fermentation, thereby enhancing L-threonine production.

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