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Optimization of Fermentation Conditions for the Biosynthesis of L-Threonine by *Escherichia coli*

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Abstract In this study, the fed-batch fermentation technique was applied to improve the yield of L-threonine produced by *Escherichia coli* TRFC. Various fermentation substrates and conditions were investigated to identify the optimal carbon source, its concentration and C/N ratio in the production of L-threonine. Sucrose was found to be the optimal initial carbon source and its optimal concentration was determined to be 70 g/L based on the results of fermentations conducted in a 5-L jar fermentor using a series of fed-batch cultures of *E. coli* TRFC. The effects of glucose concentration and three different feeding methods on the production of L-threonine were also investigated in this work. Our results showed that the production of L-threonine by *E. coli* was enhanced when glucose concentration varied between 5 and 20 g/L with DO-control pulse fed-batch method. Furthermore, the C/N ratio was a more predominant factor than nitrogen concentration for L-threonine overproduction and the optimal ratio of ammonium sulfate to sucrose (*g*/*g*) was 30. Under the optimal conditions, a final L-threonine concentration of 118 g/L was achieved after 38 h with the productivity of 3.1 g/L/h (46% conversion ratio from glucose to threonine).

Keywords *Escherichia coli* · L-Threonine · Fed-batch fermentation · Condition optimization · Sucrose · C/N ratio

Introduction

L-Threonine, an essential amino acid, has been supplemented with L-lysine to improve the nutritional values of animal feeds and human foods, and it is also used as a precursor of a

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number of commonly used flavoring agents [1]. The production of L-threonine depends mainly on direct fermentation of auxotrophic and regulatory mutants of *Escherichia coli*, *Serratia marcescens*, *Brevibacterium flavum* and *Corynebacterium glutamicum* using carbohydrates as substrates [2], and the *E. coli* mutants generated by mutagenesis or genetic manipulation are commonly used due to their fast growth rate and well-known physiological characteristics [3].

Due to its huge commercial demand, the mass production of L-threonine has been the focus of research and development, especially in the field of microbial production processes development through strain improvement and fermentation process optimization [1, 4]. A mutant *E. coli* HS3 was isolated which was released from repression and inhibition of aspartokinase by the end-products, and the concentrations of the carbon source (glucose) and the nitrogen source (yeast extract) were optimized to improve the production of L-threonine by this strain [5]. To reduce the cost for mass production of L-threonine, yeast extract was replaced by corn steep liquor as the nitrogen source [6]. The increase in L-threonine production by adding biotin as a growth factor was also reported in the literature [3].

It is important to maintain the optimal fermentation conditions throughout the production process, such as those for temperature, aeration rate, pH, moisture, contents for carbon nitrogen sources, as well as the C/N ratio of the media [7]. Fed-batch culture is an efficient technique to control the organism's growth rate and achieve high yields of biomass and metabolites by avoiding the repressive effects of high substrate concentration [8]. The above-mentioned approach has been successfully applied in the productions of erythritol by *Torula* sp. [7], glutathione by recombinant *E. coli* [9], and arachidonic acid by *Mortierella alpine* [10].

Lee et al. [3] applied the fed-batch culture system by adding biotin and oxygen-enriched air to basal minimal salt medium and achieved a high yield of threonine (80.2 g/L). Okamoto [11] established an industrially stable process for L-threonine fermentation with addition of D,L-methionine and iron by a L-methionine autotrophic *E. coli* mutant and achieved a final yield of 101 g/L, which was the highest one ever reported. However, as key factors to the fed-batch culture technique for L-threonine production, analyses of initial carbon source and glucose feeding have not yet been systematically investigated so far. In this study, we applied the fed-batch fermentation technique to investigate the biosynthesis of L-threonine by *E. coli* TRFC. Effects of various influencing factors including different carbon sources, initial sucrose concentrations, feeding media, feeding glucose concentrations and feeding methods, as well as the C/N ratio were investigated and a fed-batch culture method was successfully developed to achieve a higher threonine yield.

Materials and Methods

Microorganism and Medium

E. coli TRFC (ILE^L, AHV^r) used in this study, carrying a pTHR101 plasmid containing a Thr operon, was derived by repeated compound mutagenesis (DES plus UV) from *E. coli* K12 and was capable of use sucrose as the carbon source. The culture was maintained on LB slant agar (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, and 2% agar) and inoculated monthly.

The medium used for cell growth and L-threonine production contained the following components (g/L): The carbon source, 90; KH_2PO_4, 2; $(NH_4)_2SO_4$, 10; $MgSO_4$, 1; $FeSO_4 \cdot 7H_2O$, 0.01, and $MnSO_4 \cdot 4H_2O$, 0.01.

Culture Conditions

A single colony of *E. coli* was inoculated to a 500-mL baffled flask containing 25 mL growth media and was cultivated at 37°C, 250 rpm for 11 h. Five percent (*v*/*v*) inocula were added aseptically to baffled flasks (500 mL) containing 20 mL media or to a fermentor according to different purposes for the experiment.

The initial pH of the media was adjusted to 6.7 with 1 M sodium hydroxide, and then the inoculated 500-mL baffled flasks, each containing 20 mL of production media with 90 g/L carbon source were incubated at 37°C on a rotary shaker at 250 rpm for 48 h.

Fed-batch fermentation was performed with 5-L jar fermentors (Biotech-2002 Bioprocess Controller, Baoxing, Shanghai, China), each containing 3 L media. The temperature was maintained at 37°C and the pH was adjusted to 7.0 with addition of 25% (*m*/*m*) ammonia. Dissolved oxygen level was maintained above 20% by adjusting agitation rate between 500 and 800 rpm, and maintaining aeration at 2 vvm.

When initial sucrose was depleted, 800 g/L glucose solution was used as feeding medium and was intermittently fed into the fermentor. The tubing pump for feeding medium was controlled manually to keep glucose concentration approximately 5–20 g/L with DO-control pulse fed-batch methods. When glucose concentration is lower than 5 g/L, the level of dissolved oxygen will increase rapidly to 100%, indicating the time to add feeding medium.

Analytical Methods

All experiments were carried out in triplicate, and the experimental data were analyzed using the Data Processing System (version 2.0).

Dry cell weight (DCW) was gravimetrically determined using the pellet fraction from 20 mL samples. After centrifugation at 12,000 rpm for 10 min and washed twice with distilled water, the biomass was poured into preweighed aluminum cups and placed in a ventilating oven at 80°C overnight until constant weights were obtained [12].

Dissolved oxygen, pH, and temperature were measured automatically with electrodes attached to the fermentors. The concentration for the carbon source was determined with HPLC (Hitachi L-2000, Hitachi, Tokyo, Japan). For determination of L-threonine concentration, the supernatant fractions were diluted 100 times with doubly-distilled water, and derivatized with equal amount of 1% (*m*/*m*) Fluoro-2,4-dinitrobenzene (FDNB) dissolved in acetonitrile at 60°C for 1 h. A sample of 25 μL was loaded and analyzed by a high speed amino acid analyzer (Elite-AAA, Elite, Dalian, China). L-Threonine concentrations were measured by integrating the absorbance at 360 nm and then compared to that of an authentic sample.

Results and Discussion

Effects of Initial Carbon Sources on L-Threonine Production

In order to find the optimal carbon source for L-threonine production by *E. coli* TRFC, different types of carbohydrates, including glucose, galactose, sorbose, sucrose, maltose, and lactose were added to the fermentation media at 90 g/L using baffled flasks. The yields of L-threonine produced from various carbon sources were determined after 48 h of cultivation in baffled flasks. The dry cell weight and the yield of L-threonine in each case are shown in Fig. 1.

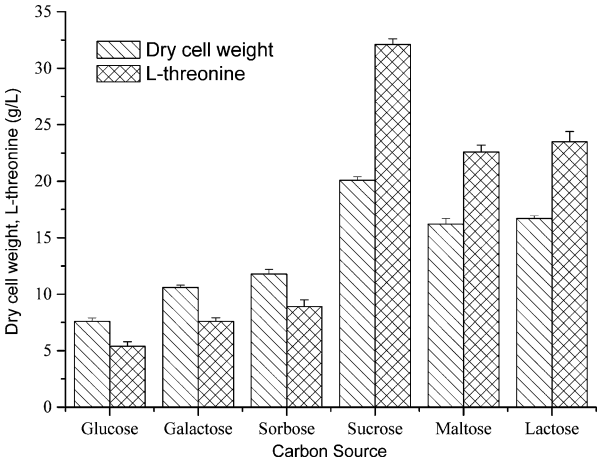

Fig. 1 Effects of carbon sources on L-threonine production in baffled flasks

It is generally accepted that bacteria can grow to various extents in a wide range of carbon sources [13]. With carbohydrates, such as sucrose, maltose, and lactose, *E. coli* TRFC exhibited a relatively high cell growth, and the weight of L-threonine produced overpassed that of the dry cell, whereas with maltose and lactose, the yield of L-threonine is relatively lower comparing to sucrose. The dry cell weight and yield for L-threonine were both the highest when sucrose was involved, indicating that sucrose is the optimal initial carbon source for L-threonine production by *E. coli* TRFC.

When glucose, usually an excellent carbon source for growth, was used as the initial carbon source, the lowest yields for biomass and L-threonine were found, possibly due to the fact that *E. coli* was able to uptake sugar and turned on glycolysis immediately after sugar was transported into the cell. The oxidative phosphorylation process and the tricarboxylic acid cycle (TCA cycle) led to accumulation of acetic acid that would restrain the cell growth and reduce the production of L-threonine. However, when sucrose was used as the initial carbon source, it was gradually hydrolyzed into glucose and fructose, making it relatively tolerant to glycolysis. In addition, the relative lower osmotic pressure of sucrose would be more favorable for the growth of *E. coli*.

Effects of Initial Sucrose Concentrations on L-Threonine Production

In order to determine the optimal initial sucrose concentration for L-threonine production, four fed-batch cultures were evaluated with media containing different concentrations of sucrose. After sucrose depletion, an 800 g/L sucrose solution was fed into the jar fermentor until the total sucrose in the media reached approximately 10 g/L. Sucrose consumption by *E. coli* at different initial concentrations is shown in Fig. 2. With high initial sucrose concentration, sucrose-uptaken was slow in the lag phase of cultivation, but became fast after 13 h. When initial sucrose concentrations were 30 and 50 g/L, the sucrose was depleted at 9 and 11 h, respectively. When initial sucrose concentration was increased from 70 to 90 g/L, the depletion time was increased from 14.5 to 18 h accordingly.

The dry cell yields of *E. coli* TRFC at different initial sucrose concentrations are shown in Fig. 3. In the early phase of cultivation, *E. coli* TRFC grew faster in media with sucrose concentration of 30 and 50 g/L comparing to that of 70 and 90 g/L. Due to the presence of enough carbon source in the media, the dry cell weight increased gradually during the entire period of cultivation, but with a higher rate in the early phase and a lower rate in the late

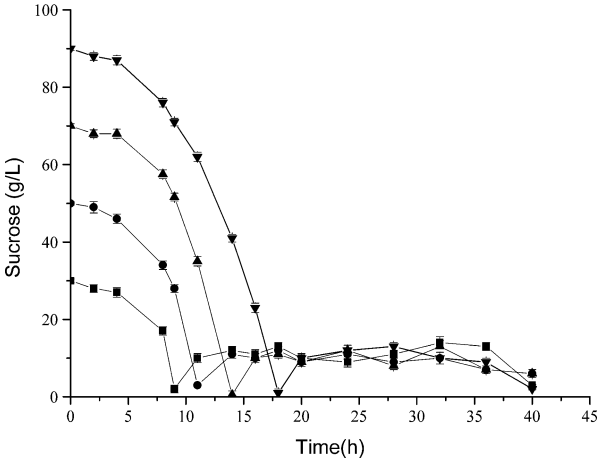

Fig. 2 Plots of sucrose consumptions by *E. coli* at different initial sucrose concentrations. The initial sucrose concentrations were: (*filled square*) 30 g/L, (*filled circle*) 50 g/L, (*filled upright triangle*) 70 g/L, (*filled inverse triangle*) 90 g/L

phase. Although the *E. coli* strain grew faster at low initial sucrose concentrations, higher dry cell weights were achieved when initial concentrations of sucrose were 70 and 90 g/L, respectively.

The yields of L-threonine versus different initial sucrose concentrations are plotted in Fig. 4. When the initial concentration of sucrose was 30 g/L, the yield of L-threonine reached its peak at 36 h. However, this peak value was obviously lower than those of other initial sucrose concentrations, indicating that the yield of L-threonine was determined by the cell growth rate and the final dry cell weight. When the initial concentrations of sucrose were 70 and 90 g/L, the yields of L-threonine increased continuously throughout the process, and the highest yield and productivity of L-threonine were achieved at an initial sucrose concentration of 70 g/L. Likely due to the fact that in the early stage of cultivation, the growth of *E. coli* TRFC was inhibited by the higher osmotic pressure of the media with 70 and 90 g/L sucrose. With the cultivation going on, the consumption of sucrose led to the decrease of the osmotic pressure and resulted in accumulation of certain biomass. Within a certain range of the osmotic pressures, higher sucrose concentration promoted the growth of the *E. coli* strains, which is influential to L-threonine production. Therefore, the optimal initial sucrose concentration is 70 g/L.

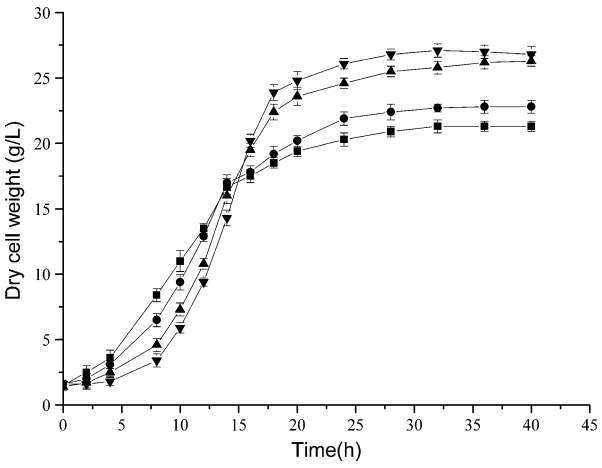

Fig. 3 Plots of the dry cell weights of *E. coli* at different initial sucrose concentrations. The initial sucrose concentrations were: (*filled square*) 30 g/L, (*filled circle*) 50 g/L, (*filled upright triangle*) 70 g/L, (*filled inverse triangle*) 90 g/L

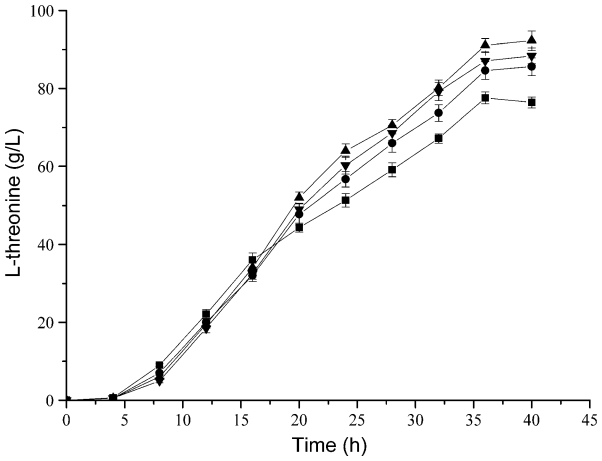

Fig. 4 Plots of L-threonine productions by *E. coli* at different initial sucrose concentrations. The initial sucrose concentrations were: (*filled square*) 30 g/L, (*filled circle*) 50 g/L, (*filled upright triangle*) 70 g/L, (*filled inverse triangle*) 90 g/L

Effects of Feeding Media on L-Threonine Production

The feeding medium is important for the production of L-threonine, and over 60% of carbon flux derived from it according to Figs. 2 and 4. Four fed-batch cultures were performed to investigate the effect of the feeding medium for L-threonine production. When the initial 70 g/L sucrose was depleted, the feeding media containing different carbon sources were added into the fermentors at intervals to maintain carbon source concentration at about 10 g/L. As shown in Fig. 5, no remarkable differences in DCW and the yield of L-

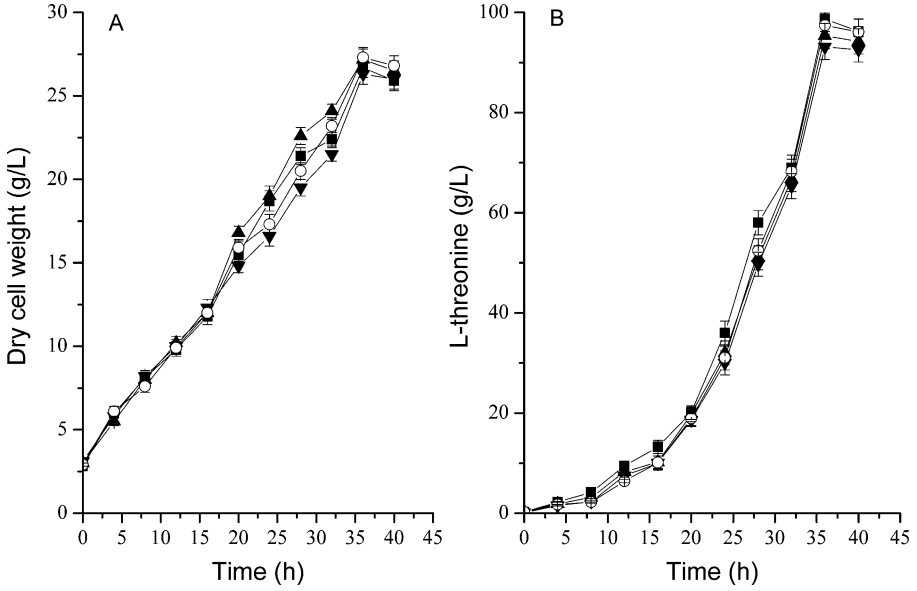

Fig. 5 Plots of the dry cell weights and L-threonine productions by *E. coli* with different carbon sources as the feeding media. The carbon sources were: (*filled square*) sucrose, (*empty circle*) glucose, (*filled upright triangle*) sorbose, (*filled inverse triangle*) maltose

threonine were observed with different carbon sources as the feeding media. Considering its low cost, glucose was selected as the final feeding medium.

Zhu et al. [10] reported that higher initial glucose concentration impaired the growth of microorganism at the early phase of cultivation and lower one was able to shorten the lag phase in microorganism growth after inoculation. Similarly, glucose could be used as the feeding medium at a lower concentration, but was not suitable as the initial carbon source at a higher concentration in the process of L-threonine fermentation.

Effects of Glucose Concentrations and Feeding Methods During L-Threonine Production

Although the highest yield of L-threonine was achieved after 40 h of cultivation when 70 g/L sucrose solution was used, the *E. coli* strain grew slowly at the metaphase of cultivation and the dry cell weight was low at the end of fermentation. In order to obtain higher yield of L-threonine, different ranges of glucose concentration were evaluated when initial sucrose was depleted at about 14 h. The rates of glucose consumption in fed-batch cultures using three different feeding methods were shown in Fig. 6. Glucose was consumed quickly between 14 and 22 h during cultivation, but became slower after 24 h. The maximum glucose consumption rate (13.5 g/L/h) was observed at 22 h and then gradually declined to a constant value (approximately 4.2 g/L/h) when the fed medium (800 g/L glucose solution) was continuously supplied to maintain the residual glucose concentration constant at about 10 g/L. For the remaining two feeding methods, the medium containing 800 g/L glucose was intermittently supplied to maintain the concentration of residual glucose to oscillate in the ranges of 5 to 20 g/L and 0 to 35 g/L, respectively. The maximum glucose consumption rates were 13.9 at 20 h, and 12.8 g/L/h at 21 h, respectively. During the whole cultivation process, the *E. coli* strain displayed its highest assimilating ability for glucose in the second feeding method.

As shown in Fig. 7, the time when the highest rates for L-threonine production (5.6, 6.0, and 5.3 g/L/h, respectively) were achieved with different feeding methods was around 21 h, which corresponded to the time for maximum glucose consumption. While the maximum L-threonine productions (103, 109.2, and 94.1 g/L) were achieved at 38 h with different feeding methods. Compared with continuously feeding method, both the production and

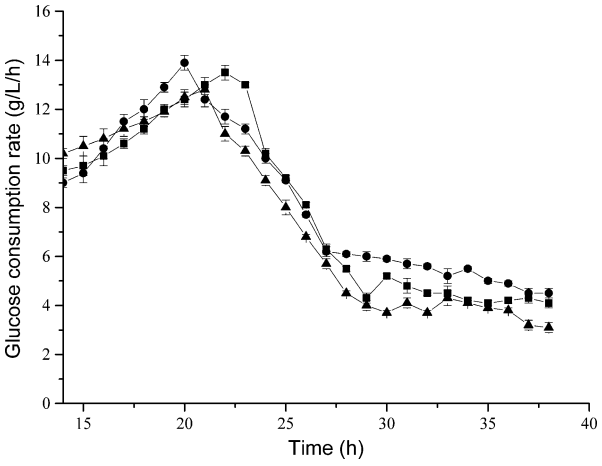

Fig. 6 Plots of the glucose consumption rates for *E. coli* with different glucose concentrations. The feeding methods were: (*filled square*) constant concentration at about 10 g/L, (*filled circle*) 5 to 20 g/L, (*filled upright triangle*) 0 to 35 g/L

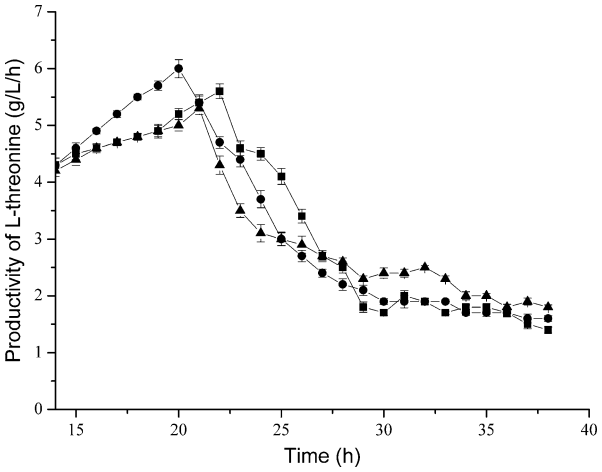

Fig. 7 Plots of productivities for L-threonine by *E. coli* with different glucose concentrations. The feeding methods were: (*filled square*) constant concentration at about 10 g/L, (*filled circle*) 5 to 20 g/L, (*filled upright triangle*) 0 to 35 g/L

productivity of L-threonine were enhanced with oscillating feeding method when the residual glucose concentration was controlled in the range of 5 to 20 g/L. However, the optimal state could not be ensured for constant operation because of the presence of many non-linear phenomena in the bioreaction system [14], despite the fact that the enhancing effects of oscillating cultivation on the biosyntheses of biomass and metabolites have been reported before [15–18].

Different feeding methods including constant glucose concentration, dissolved oxygen control (DO-control) pulse, and constant feeding rate fed-batches were performed after the initial 70 g/L sucrose was depleted at 14 h. In constant glucose concentration fed-batch fermentation, the feeding rate was adjusted according to determined glucose consumption rate to maintain the residual glucose concentration at about 10 g/L, while in the DO-control pulse fed-batch method, the feeding rate was adjusted based on the dissolved oxygen value. When glucose concentration decreased to a certain threshold (approximately 5 g/L), the DO level of the fermentation increased rapidly to 100%, suggesting the right time for glucose addition to the fermentors.

As shown in Fig. 8a, in DO-control pulse fed-batch fermentation, the glucose consumption rate reached its peak value at 19 h and declined gradually from 19 to 27 h, while the glucose consumption rate remained relatively stable after 27 h. Similarly, the plots for L-threonine productivity showed the same trend as the glucose consumption rate (Fig. 8b). The glucose consumption rates in other feeding modes were lower than that in the DO-control pulse fed-batch fermentation from 19 to 27 h. In addition, the productivity of L-threonine was lower than that in the DO-control pulse fed-batch fermentation from 27 to 40 h. The maximum values of the glucose consumption rate in constant glucose concentration, constant feeding rate, and DO-control pulse fed-batches were 13.8, 13.3, and 14.1 g/L/h, respectively. Correspondingly, the maximum productivities of L-threonine were 6.0, 5.5, and 6.2 g/L/h, respectively, while the maximum L-threonine productions were 105, 96.2, and 109.2 g/L. Compared to others feeding methods, the maximum production and productivity of L-threonine were achieved with the DO-control pulse fed-batch method. The pulse period varied in the range from 30 to 90 s. Pulse fed-batch could activate the oscillation of microorganism metabolism during the whole culture process and the medium was prone to be utilized completely.

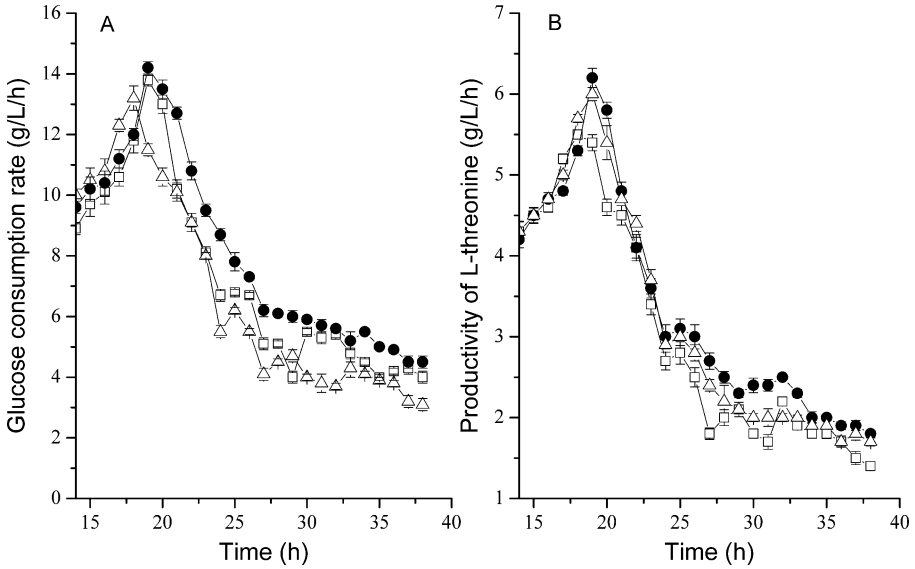

Fig. 8 Plots of the glucose consumption rates and L-threonine productivities by *E. coli* with different feeding methods. The feeding methods were: (*empty square*) constant glucose concentration fed-batch, (*empty upright triangle*) constant feeding rate fed-batch, (*filled circle*) DO-control pulse fed-batch

Effect of C/N Ratio on L-Threonine Production

Previous studies showed that ammonium sulfate and yeast extract were the optimal inorganic and organic nitrogen sources for L-threonine production, respectively [19]. It has been reported that the C/N ratio was more important than the nitrogen concentration for the increase of cell density and desired products concentration at the end of fermentation [7]. The fed-batch fermentations were performed in 5-L jar fermentors using the media of C/N ratios to examine the effect of the C/N ratio on L-threonine fermentation (Table 1). The C/N ratio was adjusted to 10, 15, 30, and 50, respectively, by adding ammonium sulfate, and the optimal ratio of ammonium sulfate to sucrose (*g*/*g*) was found to be 30. A final L-threonine production of 118 g/L was achieved after 38 h of cultivation, and the dry cell weight was 27.4 g/L with 3.10 g/L/h productivity.

In this study, the fermentation conditions such as carbon sources, initial sucrose concentration, glucose feeding methods, and the C/N Ratio for L-threonine production by *E. coli* were investigated. The optimal values of the initial sucrose concentration and C/N ratio were 70 g/L and 30, respectively. The optimal feeding method was DO-control pulse fed-batch supplied 800 g/L glucose intermittently to maintain the concentration of residual

Table 1 Effects of the C/N ratios on the production of L-threonine.

C/N ratio	10	15	30	50
Dry cell weight (g/L)	28.3±0.3	27.8±0.5	27.4±0.1	21.3±0.2
L-threonine (g/L)	108.1±0.3	112.3±0.8	118±0.5	72.5±1.0
Productivity (g/L/h)	2.84±0.04	2.95±0.02	3.10±0.03	1.72±0.08
Residual glucose (g/L)	0.8±0.1	0.6±0.1	0.4±0.1	1.3±0.3
Fermentation (h)	38	38	38	42

glucose in the range of 5 to 20 g/L. Under optimized culture conditions, a final L-threonine concentration of 118 g/L with 46% conversion ratio from glucose to threonine was achieved in a fermentor after 38 h with the productivity of 3.1 g/L/h. All these results suggested that the fermentation conditions including the concentration of sucrose, the feeding medium, the feeding method, and the C/N ratio of the medium were also important factors that affected L-threonine production by *E. coli*, in addition to strain improvement by genetic strategies. The optimization of the fermentation process enhanced the productivity of L-threonine by *E. coli* TRFC dramatically and lowered the production cost consequently.

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