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SHORT COMMUNICATION

Optimization of glucose feeding approaches for enhanced glucosamine and *N*-acetylglucosamine production by an engineered *Escherichia coli*

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Abstract In this work, a recombinant *Escherichia coli* was constructed by overexpressing glucosamine (GlcN) synthase and GlcN-6-P *N*-acetyltransferase for highly efficient production of GlcN and *N*-acetylglucosamine (GlcNAc). For further enhancement of GlcN and GlcNAc production, the effects of different glucose feeding strategies including constant-rate feeding, interval feeding, and exponential feeding on GlcN and GlcNAc production were investigated. The results indicated that exponential feeding resulted in relatively high cell growth rate and low acetate formation rate, while constant feeding contributed to the highest specific GlcN and GlcNAc production rate. Based on this, a multistage glucose supply approach was proposed to enhance GlcN and GlcNAc production. In the first stage (0–2 h), batch culture with initial glucose concentration of 27 g/l was conducted, whereas the second culture stage (2–10 h) was performed with exponential feeding at $\mu_{set} = 0.20\ h^{-1}$, followed by feeding concentrated glucose (300 g/l) at constant rate of 32 ml/h in the third stage (10–16 h). With this time-variant glucose feeding strategy, the total GlcN and GlcNAc yield reached 69.66 g/l, which was enhanced by 1.59-fold in comparison with that of batch culture with the same total glucose concentration. The time-dependent glucose feeding approach developed here may be useful for production of other fine chemicals by recombinant *E. coli*.

Keywords Glucosamine · *N*-acetylglucosamine · Fed-batch culture · *Escherichia coli* · Optimal feeding strategy

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Introduction

Glucosamine (GlcN, 2-amino-2-deoxy-D-glucose) and its *N*-acetylated derivative *N*-acetylglucosamine (GlcNAc, 2-acetami-do-2-deoxy-D-glucose) are synthesized in all organisms such as bacteria, yeast, filamentous fungi, plants, and animals [1–3]. GlcN provides good regeneration of human joint cartilage and has been widely applied in clinical trials to relieve symptoms of osteoarthritis [4, 10, 11]. GlcN is currently extracted from crab and shrimp shells by acid hydrolysis of chitin, which is not environmentally friendly, and such production may become limited by variable raw material supply. Moreover, GlcN from shellfish may not be suitable for those people with shellfish allergies [3].

In comparison with the extraction approach, microbial production of GlcN has advantages such as less environmental pollution, stable raw material, and lack of allergy factors [5, 6]. However, in the literature there are few studies regarding production of GlcN via microbial fermentation [2–4]. The fungus *Aspergillus* sp. BCRC31742 was used to produce GlcN, and the maximum yield of glucosamine reached 7.48 g/l [4]. However, the main

problem with filamentous fungi fermentation is the long cultivation time (5–7 days), which decreases the GlcN productivity and thus reduces the commercial competitiveness of microbial fermentation in comparison with the traditional extraction approach. A recombinant *Escherichia coli* was constructed by metabolic engineering means to develop an economically competitive fermentation process for GlcN and GlcNAc production, and over 110 g/l GlcNAc was obtained under the optimal conditions [2, 3].

Previous research has indicated that GlcN can impose severe inhibition of cell growth, and thus GlcN cannot be obtained at high yield from microbial fermentation with wild-type strains [3]. However, as the *N*-acetylated derivative of GlcN, GlcNAc is stable and noninhibitory to cells, and can be hydrolyzed easily to GlcN under mild acidic conditions. Therefore, inhibition of cells by GlcN can be alleviated by extending the GlcN pathway to GlcNAc by overexpressing GlcN-6-P *N*-acetyltransferase [2, 3].

In this work, the GlcN synthase encoding gene (*glmS*) from *E. coli* BL21 and the GlcN-6-P *N*-acetyltransferase encoding gene from *Saccharomyces cerevisiae* S288C were cloned and overexpressed in *E. coli* ATCC 25947(DE3) according to the literature [2]. Then, the influence of different culture modes (batch culture, constant-rate glucose feeding, interval glucose feeding, and exponential glucose feeding) on GlcN and GlcNAc production by the recombinant *E. coli*-glmS-gna1 was investigated. Based on kinetic analysis of the different culture modes, a time-dependent glucose supply strategy was proposed, and production of GlcN and GlcNAc was enhanced significantly. The multistage glucose feeding approach developed here may be useful for microbial production of other fine chemicals by recombinant *E. coli*.

Materials and methods

Microorganism and media

E. coli-glmS-gna1 was constructed by cloning and expressing *glmS* and *gna1* genes in *E. coli* ATCC 25947(DE3) according to the method described in the literature [2]. The *glmS* gene was amplified from *E. coli* BL21 genome, and *gna1* gene was amplified from *Saccharomyces cerevisiae* S288C genome. The *glmS* fragments were inserted between the restriction sites *Sac*I and *Hin*dIII of the plasmid pET-28a, and *gna1* fragments were inserted between the restriction sites *Not*I and *Xho*I of the plasmid pET-28a, yielding the recombined plasmid pET-28a-glmS-gna1. Recombinant *E. coli*-glmS-gna1 was constructed by transforming the plasmid pET-28a-glmS-gna1 into *E. coli* ATCC 25947(DE3).

Seed medium consisted of (in g/l): yeast extract 5, peptone 10, NaCl 10, agar 20, pH 7.0. Fermentation medium consisted of (in g/l): glucose 100, peptone 12, yeast extract 24, glycerol 4 (v/w), KH_2PO_4 2.31, K_2HPO_4 12.54. All media were sterilized at 121°C for 15 min and supplemented with 50 μg/ml kanamycin.

Culture conditions

Seed culture was carried out in 500-ml Erlenmeyer flasks containing 75 ml seed medium. One hundred microliters of frozen glycerol stock (kept at −80°C) was inoculated and cultured at 37°C and 200 rpm for 12 h on rotary shakers. Seed culture (150 ml) was inoculated into a 3-l fermentor (BioFlo 110; New Brunswick Scientific Co.) for fermentation. The pH was automatically controlled at 7.00 ± 0.05 by adding NaOH solution (5 M), and the temperature was maintained at 37°C. The dissolved oxygen (DO) level was maintained above 20% of saturation by cascading the agitation speed (400–900 rpm) and aeration rate (1–4 vvm). To induce protein expression, 5 g/l lactose was aseptically added at optical density (OD_{600}) of 0.6.

Batch culture

Batch culture was carried out in fermentation medium with initial glucose concentration of 100 g/l. The broth volume was 1.5 l. Sampling was done every 2 h for analysis of residual glucose concentration, acetic acid concentration, dry cell weight (DCW), and GlcN and GlcNAc concentration since inoculation.

Constant-rate glucose feeding culture

Fed-batch culture with constant-rate glucose feeding was carried out with initial glucose concentration of 27 g/l. The initial broth volume for fed-batch culture was 1.1 l. After 2 h of batch culture, feeding of concentrated glucose (250, 300, and 350 g/l) was started at feeding rate of 32 ml/h by a computer-controlled pump. To keep the total glucose in batch and fed-batch culture equal, the feeding volume was 480, 400, and 342.9 ml, respectively.

Interval glucose feeding fed-batch culture

Interval glucose feeding fed-batch culture was carried out with initial glucose concentration of 27 g/l. The initial broth volume was 1.1 l. After 2 h of batch culture, concentrated glucose (300 g/l) was fed into the fermentor at feeding rate of 50 ml/h by a computer-controlled pump. The total feeding time was 8 h, which was started at 2, 4, 6, 8, 10, 12, 14, and 16 h and lasted for 1 h every time.

The feeding rate of 50 ml/h was selected to make the total glucose in batch and fed-batch culture equal.

Exponential glucose feeding fed-batch culture

Exponential glucose feeding fed-batch culture was conducted with initial glucose concentration of 27 g/l. The initial broth volume was 1.1 l. After 2 h of batch culture, concentrated glucose (300 g/l) was exponentially fed into the fermentor using a computer-controlled pump. The feeding rate F was calculated according to the mass balance equation (1), and the specific growth rate μ_{set} was controlled at 0.2 h^{-1}. The total feeding volume was 400 ml.

$$F(t) = \frac{\mu_{set} X_0 V_0 \exp(\mu_{set} t)}{Yx/sS_0}, \quad (1)$$

where F is the feeding rate (l/h), μ_{set} is the set specific growth rate (0.2 h^{-1}), X_0 is the initial cell concentration (g/l), V_0 is the initial culture volume (1), t is the culture time after initiation of glucose exponential feeding (h), Yx/s is the yield coefficient estimated from batch culture (0.13 g DCW/g glucose), and S_0 is the glucose concentration in the feeding solution (g/l).

Multistage glucose supply strategy

The multistage glucose feeding strategy was performed as follows. The initial glucose concentration was 27 g/l, and the initial broth volume was 1.1 l. Seed culture (10% v/v) was inoculated into the fermentation medium. After 2 h of batch culture (the first stage), glucose (300 g/l) was supplied in exponential feeding mode for 8 h, with cell growth controlled at specific growth rate (μ_{set}) of 0.20 h^{-1} (the second stage). In the third stage, constant-rate feeding of 300 g/l glucose at rate of 32 ml/h was conducted for 6 h.

Analytical methods

Five milliliters of culture broth was centrifuged at 10,000 × g for 10 min, and the supernatant was taken out for glucose, acetate acid, GlcN, and GlcNAc analysis. DCW was determined by drying cells at 65°C to constant weight. Residual glucose in the fermentation broth was measured by glucose–glutamate analyzer SBA-40C (Biology Institute of Shandong Academy of Sciences, Jinan, China). Acetic acid concentration was measured by high-performance liquid chromatography (HPLC, 1100 series; Agilent, Santa Clara, CA) with a reverse phase column (Zorbax SB-Aq) with injection volume of 5 µl, and a mobile phase consisting of 0.1% phosphoric acid aqueous solution at flow rate of 1.0 ml/min. The column temperature was maintained at 30°C, and the detection wavelength was 210 nm. GlcN concentration was assayed by a modification of the colorimetric method of Elson and Morgan [5]. GlcNAc concentration was determined by HPLC (Agilent 1200 series, USA) with a refractive index detector (RID). HPLC was carried out using a NH_2 column (4.6 × 250 mm) packed with 5-mm particle size packing material. After filtration through a 0.45-µl membrane filter, 10 µl of sample was injected into the column. The mobile phase was 70% acetonitrile with flow rate of 0.7 ml/min, and the column temperature was maintained at 30°C.

Statistical analysis

All experiments were performed at least three times, and results are expressed as mean ± standard deviation (SD). Statistical analysis was performed with Student's t test. P value <0.01 was considered statistically significant.

Results and discussion

Batch culture of *E. coli*-glmS-gna1 for production of GlcN and GlcNAc

Figure 1a shows the kinetics of GlcN and GlcNAc production by *E. coli*-glmS-gna1 with batch culture. DCW increased with culture time, and decreased in the decline phase due to cell death. The DCW of batch culture reached a maximum value of 14.60 g/l at 10 h, and the average specific growth rate was 0.09 h^{-1}. In *E. coli*, glucose is the carbon skeleton for GlcN and GlcNAc synthesis, so different glucose fed-batch modes exert certain influence on the yield of GlcN and GlcNAc. GlcN and GlcNAc yield increased with culture time, and decreased in the later stages probably due to transportation of GlcN and GlcNAc from the fermentation broth into the cell [6, 8, 9]. GlcN and GlcNAc yield reached maximum values of 22.0 and 22.70 g/l at 16 and 18 h, respectively, and the productivity of GlcN and GlcNAc was 0.92 and 0.95 g/(l h), respectively. Total yield of GlcN and GlcNAc reached a maximum value of 43.78 g/l at 16 h. The yield of GlcN on cell ($Y_{GlcN/X}$) and the yield of GlcNAc on cell ($Y_{GlcNAc/X}$) were 1.52 and 1.55, respectively (Table 1).

Influence of constant-rate glucose feeding on GlcN and GlcNAc production

Figure 1b–d shows the influence of constant-rate glucose feeding on GlcN and GlcNAc production. Feeding with different concentrations of glucose had significant effects on cells growth, and the DCW reached a maximum value of 13.60, 15.20, and 16.20 g/l at 10, 14, and 16 h, respectively, when feeding with 250, 300, and 350 g/l glucose. DCW

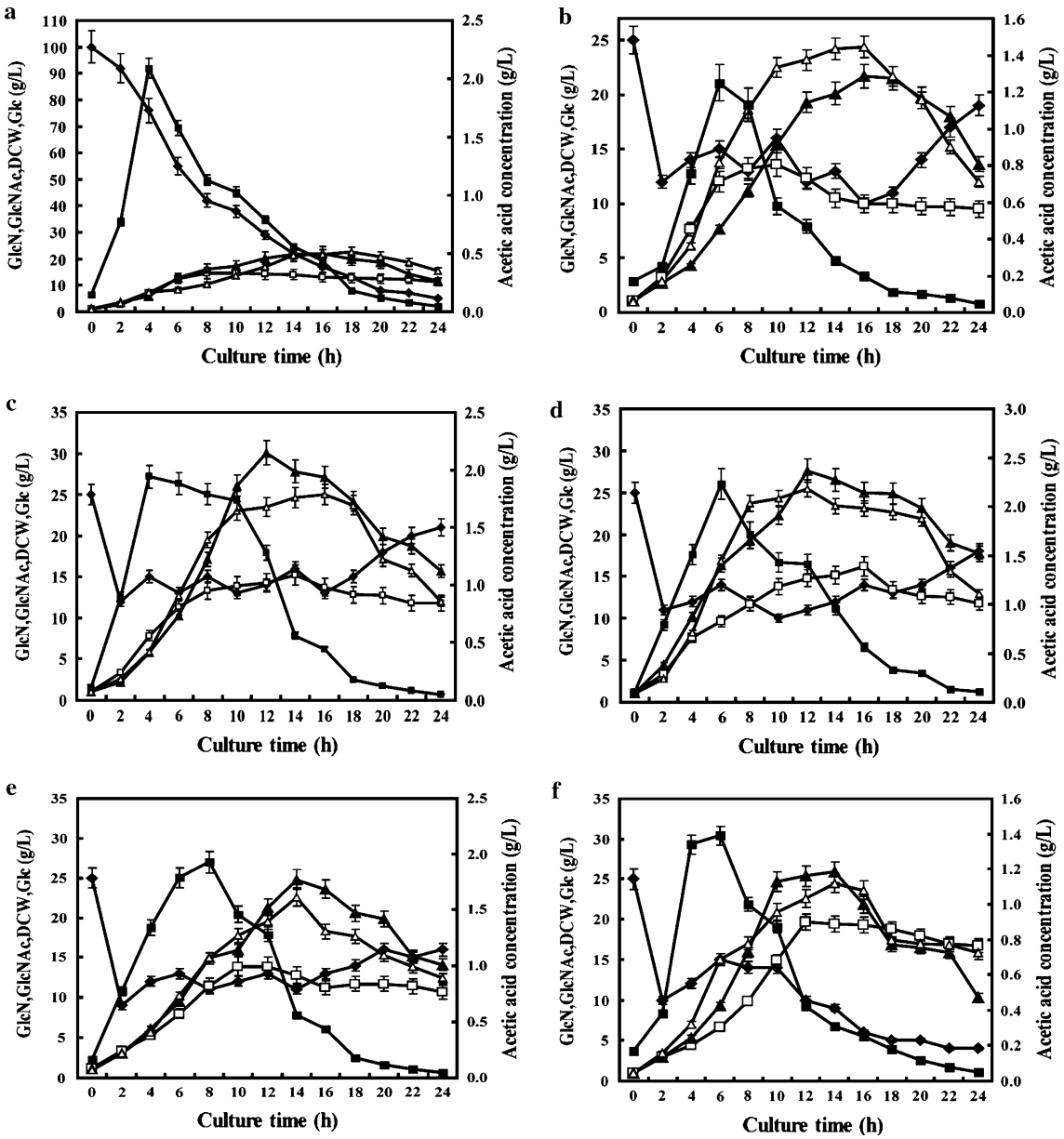

Fig. 1 Influence of different glucose feeding strategies on GlcN and GlcNAc production by *E. coli*-glms-gna1. Batch culture (**a**). Constant-rate feeding with glucose of 250 g/l (**b**), 300 g/l (**c**), 350 g/l (**d**). Interval feeding with 300 g/l glucose (**e**). Exponential feeding with 300 g/l glucose (**f**). *Filled diamonds* residual glucose concentration (g/l), *open squares* DCW (g/l), *filled triangles* GlcN concentration (g/l), *open triangles* GlcNAc concentration (g/l), *filled squares* acetic acid concentration (g/l)

increased with the increase of fed glucose concentration, and the time at which DCW was maximum was also delayed when the concentration of feeding glucose increased from 250 to 350 g/l. Feeding with glucose at different concentrations also had significant effects on GlcN and GlcNAc yield. GlcN yield reached a maximum value of 21.7, 30.1, and 27.6 g/l at 16, 12, and 12 h when feeding with 250, 300, and 350 g/l glucose, respectively. Feeding with 300 g/l glucose resulted in the maximum GlcN yield (30.1 g/l), which indicated that feeding low-concentration glucose (250 g/l) may not provide enough carbon source for cell growth and GlcN synthesis, whereas feeding high-concentration glucose (350 g/l) may be inhibitory to cell growth owing to production of excessive acetic acid (2.23 g/l). In addition, feeding with 300 g/l glucose caused maximum GlcN productivity of 1.25 g/(l h), and the time at which GlcN yield was maximum was also brought forward by high-concentration glucose feeding.

The GlcNAc yield reached a maximum value of 24.4, 25.0, and 25.50 g/l at 16, 16, and 12 h when feeding with 250, 300, and 350 g/l glucose, respectively. Feeding with 350 g/l glucose achieved maximum GlcNAc productivity

Table 1 Comparison of GlcN and GlcNAc production by *E. coli*-glms-gna1 with different glucose feeding approaches

Fermentation parameters	Batch culture	Constant-rate feeding (250 g/l)	Constant-rate feeding (300 g/l)	Constant-rate feeding (350 g/l)	Interval feeding mode	Exponential feeding mode	Three-stage feeding
Glucose consumption (g/l)	95.04 ± 3.33	81.17 ± 3.02	79.32 ± 2.47	82.43 ± 3.19	84.09 ± 3.29	96.22 ± 3.71	92.36 ± 3.56
Maximum DCW (g/l)	14.60 ± 0.68	13.60 ± 0.41	15.20 ± 0.77	16.20 ± 0.68	13.90 ± 0.59	19.70 ± 0.96	20.44 ± 0.96
Maximum GlcN concentration (g/l)	22.00 ± 0.75	21.70 ± 0.71	30.10 ± 1.52	27.60 ± 1.34	24.80 ± 1.43	25.90 ± 1.09	35.10 ± 1.16
Maximum GlcNAc concentration (g/l)	22.70 ± 0.86	24.40 ± 1.54	25.00 ± 1.33	25.50 ± 1.62	22.60 ± 1.03	24.50 ± 1.58	34.56 ± 1.28
Maximum acetic acid concentration (g/l)	2.09 ± 0.08	1.45 ± 0.052	1.94 ± 0.08	2.23 ± 0.09	1.93 ± 0.08	1.39 ± 0.06	1.33 ± 0.07
Average specific glucose consumption rate (h^{-1})	0.52 ± 0.02	0.26 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.29 ± 0.01	0.32 ± 0.01	0.24 ± 0.01
Average specific growth rate (h^{-1})	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
Average specific GlcN production rate (h^{-1})	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.16 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	0.14 ± 0.01
Average specific GlcNAc production rate (h^{-1})	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.17 ± 0.01	0.14 ± 0.01
Cell productivity (g/l h)	0.61 ± 0.04	0.57 ± 0.02	0.63 ± 0.04	0.68 ± 0.03	0.58 ± 0.02	0.82 ± 0.02	0.85 ± 0.02
GlcN productivity (g/l h)	0.92 ± 0.05	0.90 ± 0.07	1.25 ± 0.05	1.15 ± 0.04	1.03 ± 0.04	1.08 ± 0.05	1.46 ± 0.06
GlcNAc productivity (g/l h)	0.95 ± 0.03	1.02 ± 0.05	1.04 ± 0.03	1.06 ± 0.03	0.94 ± 0.02	1.02 ± 0.04	1.44 ± 0.08
GlcN yield on glucose (mol/mol)	0.23 ± 0.01	0.27 ± 0.01	0.38 ± 0.02	0.34 ± 0.02	0.30 ± 0.01	0.27 ± 0.02	0.38 ± 0.02
GlcNAc yield on glucose (mol/mol)	0.19 ± 0.01	0.25 ± 0.01	0.26 ± 0.01	0.25 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.31 ± 0.03
Yield of GlcN on cell ($Y_{GlcN/X}$)	1.51 ± 0.06	1.60 ± 0.08	1.98 ± 0.08	1.70 ± 0.08	1.78 ± 0.08	1.31 ± 0.08	1.72 ± 0.09
Yield of GlcNAc on cell ($Y_{GlcNAc/X}$)	1.55 ± 0.07	1.79 ± 0.11	1.64 ± 0.10	1.57 ± 0.09	1.63 ± 0.07	1.24 ± 0.06	1.69 ± 0.06

of 1.06 g/(l h). It seemed that feeding glucose at high concentration could increase GlcNAc yield and bring forward the time at which the GlcNAc yield was maximum.

The total GlcN and GlcNAc yield reached maximum values of 46.10, 53.60, and 53.10 g/l at 16, 12, and 12 h when feeding with 250, 300, and 350 g/l glucose, respectively. This indicated that feeding high-concentration glucose was favorable for increasing total GlcN and GlcNAc yield (Table 1).

Influence of interval glucose feeding on GlcN and GlcNAc production

Figure 1e shows the influence of interval glucose feeding on GlcN and GlcNAc production. The highest DCW was 13.90 g/l at 12 h. The highest GlcN yield with the interval feeding mode was 24.80 g/l at 14 h. In terms of maximum GlcNAc yield, there were not significant differences between batch culture and interval feeding. The highest GlcNAc yield of interval feeding modes was 22.60 g/l at 14 h with productivity of GlcN and GlcNAc of 1.03 and 0.94 g/(l h), respectively. The total yield of GlcN and GlcNAc reached a maximum value of 47.40 g/l at 14 h. The yield of GlcN on cell ($Y_{GlcN/X}$) and the yield of GlcNAc on cell ($Y_{GlcNAc/X}$) were 1.78 and 1.63, respectively (Table 1).

Influence of exponential glucose feeding on GlcN and GlcNAc production

Figure 1f shows the influence of exponential glucose feeding on GlcN and GlcNAc production. The highest DCW of the exponential feeding mode was 19.70 g/l at 12 h. Compared with other fed-batch culture modes, exponential feeding achieved the maximum DCW due to the fact that exponential feeding can provide a suitable quantity of glucose for cell growth. The average specific growth rate of exponential feeding (0.11 h^{-1}) was higher than in the other culture modes. The highest GlcN yield with exponential glucose feeding was 25.90 g/l at 14 h. The average specific GlcN production rate with exponential feeding reached 0.15 h^{-1}, which was close to the value gained by constant-rate feeding with 350 g/l glucose (0.16 h^{-1}). The highest GlcNAc yield with exponential

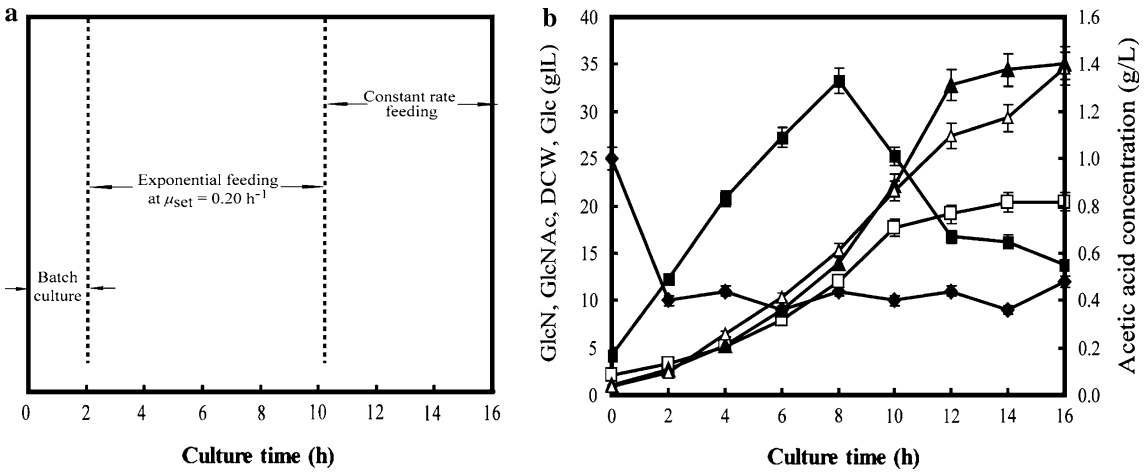

Fig. 2 Scheme of three-stage glucose feeding strategy (**a**) and GlcN and GlcNAc fermentation results by *E. coli*-glms-gna1 (**b**). *Filled diamonds* residual glucose concentration (g/l), *open squares* DCW (g/l), *filled triangles* GlcN concentration (g/l), *open triangles* GlcNAc concentration (g/l), *filled squares* acetic acid concentration (g/l)

feeding was 24.50 g/l at 14 h. The total yield of GlcN and GlcNAc reached a maximum value of 50.40 g/l at 14 h (Table 1).

Enhanced GlcN and GlcNAc production by a multistage glucose supply strategy

The glucose feeding strategy is critical because it affects the metabolic fluxes, and consequently the cell concentration, the specific productivity of GlcN and GlcNAc, and especially the formation of by-products such as acetic acid [7]. So, feeding strategies must be optimized for efficient synthesis of GlcN and GlcNAc. It seemed that a single fed-batch culture mode was not the most suitable method for GlcN and GlcNAc production by recombinant *E. coli*-glms-gna1. Exponential feeding resulted in relatively high cell growth rate and low acetate formation rate, while the highest specific GlcN and GlcNAc production rate were obtained with constant feeding. Based on this, a three-stage glucose supply approach was proposed to enhance GlcN and GlcNAc production. In the first stage (0–2 h), batch culture with initial glucose concentration of 27 g/l was conducted, whereas the second culture stage (2–10 h) was performed with exponential feeding at $\mu_{set} = 0.20\ h^{-1}$, followed by feeding concentrated glucose (300 g/l) at constant rate of 32 ml/h in the third stage (10–16 h) (Fig. 2a). Results of the time-variant glucose feeding strategy showed that DCW reached a maximum value of 20.44 g/l at 16 h. GlcN and GlcNAc concentration reached respective maximum values of 35.10 and 34.56 g/l at 16 h, whereas the maximum value of acetic acid concentration was only 1.33 g/l, which was far below the values reached by other glucose feeding modes. The total yield of GlcN and GlcNAc reached 69.66 g/l, which was enhanced by 1.59-fold in comparison with batch culture with the same total glucose concentration (Fig. 2b). Productivity of GlcN and GlcNAc reached maximum values of 1.46 and 1.44 g/(l h) respectively, which were higher than the values of batch culture mode by 58.7% and 51.6% respectively, and $Y_{GlcN/X}$ and $Y_{GlcNAc/X}$ reached maximum values of 1.72 and 1.69 g/l h, respectively, which were higher than the values of batch culture mode by 13.9% and 9.0%, respectively (Table 1).

Conclusions

In this work, the effects of different glucose feeding strategies including constant-rate feeding, interval feeding, and exponential feeding on GlcN and GlcNAc production were explored. It was found that exponential feeding was favorable for cell growth and decreasing acetate formation, while with constant feeding, the highest specific GlcN and GlcNAc production rates were obtained. Accordingly, a multistage glucose supply strategy was proposed. In the first stage (0–2 h), batch culture with initial glucose concentration of 27 g/l was conducted, whereas in the second culture stage (2–10 h), exponential feeding at $\mu_{set} = 0.20\ h^{-1}$ was performed, and in the third stage (10–16 h), feeding of concentrated glucose (300 g/l) at constant rate of 32 ml/h was carried out. With this growth-phase-based glucose feeding strategy, the total production of GlcN and GlcNAc reached 69.66 g/l, which was enhanced by 1.59-fold in comparison with that of batch culture with the same total glucose concentration, verifying the effectiveness of the proposed multistage glucose feeding strategy proposed here.

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