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Case Study

An optimal glucose feeding strategy integrated with step-wise regulation of the dissolved oxygen level improves *N*-acetylglucosamine production in recombinant *Bacillus subtilis*

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HIGHLIGHTS

- Effect of glucose feeding on GlcNAc production by recombinant *B. subtilis* was studied.
- GlcNAc production in glucose control (5 g/L) fed-batch culture reached 26.58 g/L.
- A step-wise DO control strategy was introduced to increase GlcNAc production.
- The optimal glucose feeding and DO control strategy improved GlcNAc titer to 35.77 g/L.

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ABSTRACT

In our previous work, a recombinant *Bacillus subtilis* strain for the microbial production of *N*-acetylglucosamine (GlcNAc) was constructed through modular pathway engineering. In this study, to enhance GlcNAc production, glucose feeding approaches and dissolved oxygen (DO) control methods in fed-batch culture were systematically investigated. We first studied the effects of different glucose feeding strategies, including exponential fed-batch culture, pulse fed-batch culture, constant rate fed-batch culture, and glucose control (5 g/L, 10 g/L, 15 g/L) fed-batch culture, on cell growth and GlcNAc synthesis. We found that GlcNAc production in glucose control (5 g/L) fed-batch culture reached 26.58 g/L, which was 3.10 times that in batch culture. Next, the effect of DO level (20%, 30%, 40%, and 50%) on GlcNAc production was investigated, and a step-wise DO control strategy (0–7 h, 30%; 7–15 h, 50%; 15–50 h, 40%; 50–72 h, 30%) was introduced. With the optimal glucose and DO control strategy, GlcNAc production reached 35.77 g/L, which was 4.17 times the production in batch culture without DO control.

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1. Introduction

N-acetylglucosamine (GlcNAc) and glucosamine (GlcN), which are the building blocks of glycosaminoglycans such as hyaluronic acid and chondroitin sulfate in the human body, have long been used as pharmaceuticals and nutraceuticals to treat osteoarthritis and maintain cartilage and joint health (Chen et al., 2010). The demand for GlcNAc and GlcN as dietary supplements for joint health will continue to increase as the aging population grows worldwide. It is predicted that global GlcNAc and GlcN production will reach 46,600 tons by 2017 (Liu et al., 2013). Although GlcNAc and GlcN are synthesized in all organisms, including bacteria, yeast, filamentous fungi, plants, and animals, GlcNAc and GlcN are mainly produced through acid hydrolysis of chitin extracted from crab and shrimp shells, which is not an environmentally friendly process (Hsieh et al., 2007; Liu et al., 2013; Sitanggang et al., 2010). As demand increases, variability in raw material supply could limit this extraction method (Chen et al., 2012b).

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Moreover, GlcN from shellfish might not be suitable for people with shellfish allergies (Deng et al., 2006).

Microbial fermentation has emerged as an attractive alternative for the production of non-shellfish-derived GlcNAc and GlcN in an environmentally friendly and sustainable manner (Deng et al., 2005; Zhang et al., 2012). The fungus *Aspergillus* sp. BCRC31742 (Zhang et al., 2012) and engineered *Escherichia coli* (Deng et al., 2005) have been used to produce GlcN and GlcNAc, but several drawbacks still exist. The low product yield and low productivity of GlcN production by filamentous fungi is the main disadvantage; this decreases the economic competitiveness of fungi fermentation relative to the extraction approach (Liu et al., 2013). On the other hand, despite high yield and high productivity, *E. coli* is susceptible to phage attack, and the GlcNAc produced does not achieve generally regarded as safe (GRAS) status, which limits its application in the nutraceutical and pharmaceutical industries (Liu et al., 2014).

Unlike *E. coli*, *Bacillus subtilis*, a model organism for gram-positive bacteria, is a GRAS organism because it is free of exotoxins and endotoxins (Liu et al., 2013; Tannler et al., 2008; Westers et al., 2004; Widner et al., 2005) and does not pose a phage infection problem in industrial production. Therefore, *B. subtilis* has been used widely as an expression host for the production of industrially important biochemicals and recombinant proteins. Advances in genetic tools for genome editing and protein expression have facilitated the development of *B. subtilis* as cell factories for the production of biochemicals and heterologous proteins (Kunst and Ogasawara, 1997). For these reasons, we have performed modular pathway engineering in *B. subtilis* and generated GlcNAc-overproducing *B. subtilis* by regulating GlcNAc synthesis, glycolysis, and peptidoglycan synthesis, and we have already reported the result of fed-batch fermentation in which the highest production of GlcNAc was 31.65 g/L with glucose control at 5 g/L (Liu et al., 2014). Whereas in that work, we did not give any explanation about the reason of glucose control strategy and also did not investigate the effect of dissolved oxygen (DO) levels on GlcNAc production.

In this work, we investigated the effects of batch culture and different feeding approaches, including exponential fed-batch culture, pulse fed-batch culture, constant rate fed-batch culture, and glucose control (5 g/L, 10 g/L, 15 g/L) fed-batch culture, on the production of GlcNAc by engineered *B. subtilis* (BSGN6-P_{xylA}-*glmS*-P_{43}-*GNA1*-anti-*pfk*-*glmM* sRNA-*hfq*) in a 3-L bioreactor. We also investigated the effect of dissolved oxygen (DO) levels (20%, 30%, 40%, and 50%) on GlcNAc production. From the kinetic analysis of GlcNAc production under different DO conditions, we designed a step-wise DO control strategy. The results obtained here might facilitate the scale-up of GlcNAc production in *B. subtilis*.

2. Methods

2.1. Microorganisms and media

The engineered *B. subtilis* strain BSGN6-P_{xylA}-*glmS*-P_{43}-*GNA1*-anti-*pfk*-*glmM* sRNA-*hfq* (Δ*nagP*Δ*gamP*Δ*gamA*Δ*nagA*Δ*nagB*Δ*ldh* Δ*pta*::*lox72*, co-overexpression of *glmS*, *GNA1*, and sRNA-encoding genes), abbreviated BSGN hereafter, was used in this study. The construction of engineered *B. subtilis* was described previously (Liu et al., 2014).

The seed medium was Luria–Bertani broth or agar plates containing (g/L): tryptone 10, yeast extract 5, and NaCl 10. The fermentation medium contained (g/L): corn steep liquor 50, yeast extract 20, $K_2HPO_4{\cdot}3H_2O$ 12.5, KH_2PO_4 2.5, $MgSO_4{\cdot}7H_2O$ 3, $CaCO_3$ 5, glucose 60, and 15 mL of trace metal solution. The trace metal solution contained (per liter of 5 M HCl) (g/L): $FeSO_4{\cdot}7H_2O$ 4.0, $Cacl_2$ 4.0, $MnSO_4{\cdot}5H_2O$ 1.0, $Cocl_2{\cdot}6H_2O$ 0.4, $NaMoO_4{\cdot}2H_2O$ 0.2, $ZnSO_4{\cdot}7H_2O$ 0.2, $Alcl_3{\cdot}6H_2O$ 0.1, $Cucl_2{\cdot}H_2O$ 0.1, and H_3BO_4 0.05. The feeding solution contained (g/L): glucose 800.

2.2. Culture conditions

Seed culture was carried out in 500-mL shake flasks containing 45 mL of seed medium with shaking at 200 rpm and 37 °C for 9 h on rotary shakers. The seed culture (45 mL) was inoculated into a 3-L fermentor (BioFlo115; New Brunswick Scientific Co., Edison, NJ, USA) with an initial 1.5 L of fermentation medium. The pH was kept at 7.4 automatically via the addition of 29% NH_3 and 2 M HCl, and the temperature was maintained at 37 °C. When the optical density at 600 nm (OD600) reached 0.4, xylose was added to the medium to a final concentration of 5 g/L to induce gene expression under the control of the xylose-inducible PxylA promoter. Therefore, the induction time and concentration of inducer in all of the following experiments were controlled at the same level, and the basic control of all synthesis gene expression in each batch and fed- batch condition was at the same level.

2.3. Batch culture

Batch culture was carried out in fermentation medium with an initial glucose concentration of 60 g/L. The total broth volume was 1.5 L after inoculation. Two six-bladed disk turbines provided agitation. The aeration rate and agitation speed were 1.5 vvm and 600 rpm, respectively. The temperature was maintained at 37 °C. Samples were collected every 2 h after inoculation for analysis of the residual glucose concentration, dry cell weight (DCW), and GlcNAc concentration.

2.4. Fed-batch culture

All fed-batch cultures were initiated as a batch culture with an initial glucose concentration of 20 g/L. The feeding solution was pumped into the fermentor using a computer-coupled peristaltic pump after 7 h of batch fermentation. The pH, temperature, aeration rate, and agitation speed were the same as those used for batch fermentation.

In exponential glucose feeding fed-batch culture, the substrate feeding rate F (mL/h) was calculated according to the mass balance using Eq. (1):

$$F = \mu_{set} V_0 X_0 \exp(\mu_{set} t)/Y_{X/S} S_0 \quad (1)$$

V_0 is the initial culture volume (L), μ_{set} is the specific growth rate (h^{-1}) which is set as 0.5 h^{-1}, 0.3 h^{-1}, 0.2 h^{-1}, and 0.1 h^{-1} when t was 0–2 h, 2–4 h, 4–6 h, and 6–23 h, respectively. X_0 is the initial cell concentration (g/L), $Y_{X/S}$ is the theoretical cell yield on glucose (0.2 g DCW/g glucose), S_0 is the glucose concentration in the feeding solution (800 g/L), and t is the culture time after the initiation of glucose exponential feeding (h). Exponential feeding stopped when DCW ceased to increase. The total feeding solution volume was 400 mL.

In pulse fed-batch culture, whenever the residual glucose concentration fell to 0–5 g/L, the feeding solution was pumped into the fermentor to restore the glucose concentration to 20 g/L. A total of 412 mL of feeding solution was added to the fermentor during the culture period.

In fed-batch culture with a constant feeding rate, the feeding solution was pumped into the fermentor at a rate of 12 mL/h when the residual glucose concentration decreased to 0–5 g/L. A total of 768 mL of feeding solution was added to the fermentor.

In fed-batch cultivation with control of glucose concentration, the glucose concentration was maintained at approximately 5, 10, or 15 g/L by feeding with concentrated glucose (800 g/L). The glucose feeding rates were adjusted every hour based on the

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