Cell-free synthesis system-assisted pathway bottleneck diagnosis and engineering in Bacillus subtilis

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1. Introduction

Identification and elimination of bottlenecks in metabolic pathways such as rate-limiting steps and allosteric regulation is one of the key issues to be solved during cell factories construction [1–3]. A number of in vivo modular pathway engineering tools and algorithms have been widely used to debottleneck pathway limitations by re-cast biosynthesis pathway into multiple modules [4–8]. Though changing the expression level of each enzyme or balancing multiple modules can effectively diagnose and eliminate bottlenecks, it becomes both time-consuming and laborious with the increase numbers of biosynthetic steps [9]. Therefore, efficient methods for bottlenecks diagnoses without genetic engineering need to be developed.

Cell-free biosynthetic system has been developed, which transferred multiple enzymatic reactions from in vivo to in vitro system, offering a convenient and flexible access to living system without the blocks of cytomembrane and cytoderm and can be potentially used for pathway diagnosis [10–14]. Currently, cell-free bioproduction of protein and chemical platforms for Escherichia coli, Streptomyces clavuligerus, Vibrio natriegens and Pseudomonas putida has been investigated [15–19]. And it has been wildly applied in protein synthesis, chemical synthesis, enzyme bottlenecks diagnoses, genetic circuits testing, mutant enzymes screening, gene expression elements characterization and environmental monitoring [20–27]. Though in vivo cellular dynamics and cellular metabolites pools are not necessarily consistent with the in vitro cellular dynamics and metabolite pools and cellular compartment, and...
cellular transport mechanisms were not likely investigated in cell-free system, in vitro testing of biosynthetic pathway has great potential to identified enzyme bottlenecks. *Bacillus subtilis* is the Gram-positive model microorganism in laboratory and industrially important chassis microorganism, which has been certified for producing Generally regarded as Safe (GRAS) grade products by the Food and Drug Administration (FDA) in the United States [28–40]. However, *B. subtilis* cell free system has not been developed for aiding pathway bottleneck analysis and further pathway engineering.

In previous work, engineered *B. subtilis* has been constructed for bioproduction of N-acetylneuraminic acid (NeuAc), which is the most common sialic acid form, playing a pivotal role in promoting infancy brain development, inflammation resistance, aging resistance, and is also precursor of many anti-virus drugs [41,42]. However, 13 enzymatic reactions of two branch pathways are involved in the biosynthesis pathway of NeuAc, making it a big challenge to identify the potential bottleneck in biosynthesis pathways in *B. subtilis*. Hence, it is a great potential that the bottleneck of NeuAc synthesis can be quickly identified by *in vitro* testing of *B. subtilis* cell-free NeuAc biosynthetic system. As a proof of concept application, *B. subtilis* cell-free NeuAc biosynthetic system was firstly constructed by optimizing cell lysis conditions, protease inhibitor addition and buffer composition in this study. Next, by adding NeuAc synthesis intermediates into cell-free reaction, the potential metabolic bottleneck was identified (Fig. 1). Finally, rational metabolic engineering of *B. subtilis* was carried out to eliminate pathway bottlenecks for improved NeuAc production in vivo.

2. Methods

2.1. Strains and plasmids

Homologous recombination with 500 bp homologous arms was used to edit the genome of *B. subtilis*. Cre-lox system was used to eliminate the resistance selection markers on genome. All strains and plasmids used in this study have been provided in Table 1.

2.2. Strains cultivation

Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) *B. subtilis* fermentation medium (BFM) medium (12 g/L yeast extract, 6 g/L tryptone, 6 g/L (NH₄)₂SO₄, 12.5 g/L K₂HPO₄·4H₂O, 2.5 g/L KH₂PO₄, 3 g/L MgSO₄·7H₂O, and 60 g/L glucose, the pH of the medium was titrated to 7.0 using saturated NH₄OH solution) was used for cell cultivation. Fermentation experiment for NeuAc production: first, colonies on the plates were picked and inoculated into 3 ml fresh LB medium. Then, it was cultured at 37 °C for 8 h with shaking at 220 rpm. Next, 2.5 ml of the culture was inoculated in a 500 ml shake flask containing 50 ml fresh BFM medium, which was then cultured at 37 °C for 48 h with shaking at 220 rpm.

Cells cultured for cell-free system making protocol: the cultivation method is the same as that of the fermentation experiment, but the cultivation time of the cells in 500 ml shake flasks was reduced to 16 h.

2.3. Preparation of crude cell extracts

The culture from 500 ml shake flasks were firstly harvested by centrifugation at 5000 x g and 4 °C for 10 min. The centrifuged cell

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Fig. 1. Diagram of cell-free system based metabolic bottleneck diagnosis. (a) Establishing a *B. subtilis* cell-free (BCF) system. (b) Establishing a *B. subtilis* cell-free synthesis (BCFS) system for NeuAc. (C) Bottleneck identification based on BCFS system and bottleneck elimination using NCSs.
pellets were then washed three times by cell free phosphate balanced saline buffer (CFPBS buffer, 4 g/L NaCl, 1.815 g/L NaHPO₄·12H₂O, 0.1 g/L KCl, 0.135 g/L KH₂PO₄, 1 g/L MgCl₂·6H₂O, 0.31 g/L dithiothreitol) or S30A buffer (50 mM Tris–acetate buffer with 0.1 g/L KCl, 0.135 g/L 1 g/L MgCl₂·6H₂O and 0.31 g/L dithiothreitol) to remove extracellular metabolites and proteases [14]. After weighed, 1 g harvested cell pellets were resuspended in 2 ml CFPBS buffer or S30A buffer. Next, cells were lysed by sonication using an ultrasonic cell disruptor (10180302, Ningbo Xinzhi Biotechnology Co., Ltd.) in an ice-water bath for 120 min at 30% of the total power of 950 W. Short pulses (2 s) with pauses (4 s) were used to maintain low temperature. And the cell lysate was further centrifuged at 4 °C, 12000 x g for 20 min. After taking all the supernatant as crude cell extracts, the protein concentration was tested, which was about 20–25 g/L.

2.4. Cell-free reaction

First, prepare BCFS reaction buffer on ice according to Table 2 in to a 1.5 ml EP tube. After incubated at 37 °C for 10 min with shaking at 800 rpm, all the reaction substrate stock buffer (glucose, F6P (Fructose-6-P), GlcN6P (glucosamine-6-P), GlcNAc6P (N-acetylglucosamine-6-P), GlcNac, ManNac (N-acetylmannose), PEP (Phosphoenolpyruvate)) was added into the reaction system to turn on the reaction system. The reaction time was fixed to 10 min unless noted otherwise. Trichloroacetic acid solution (50 g/L) was used to terminate the reaction. Finally, the terminate reaction buffer was centrifuged at 4 °C, 12000 x g for 15 min and the supernatant was used for further liquid chromatography detection.

2.5. Analytical methods

Biomass was determined by measuring optical density at 600 nm (OD₆₀₀).

Citation 3 Multi-Mode Reader (BIOTEK, USA) was used to measuring fluorescence intensities with an excitation wavelength of 488 and an emission wavelength of 523. The background fluorescence intensities of CFPBS buffer were first subtracted from total detected fluorescence values. Then, all average fluorescence intensities were normalized from 0 to 100.

Protein concentration was detected using a BCA (Bicinchoninic Acid) protein concentration determination kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China).

NeuAc concentration was detected using a high-performance liquid chromatography (HPLC, Agilent 1200 Series, USA) with an ultraviolet absorption detector (210 nm) and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA) with a flow rate of 0.6 mL/min at 40 °C.

2.6. Model simulation

The genome-scale metabolic model of B. subtilis was used to predict the essential ions of the BCFS system [43]. First, the reaction from GlcNAc to ManNac and from ManNac to NeuAc was added to the model. After changing the objective of the model to NeuAc synthesis using changeObjective function, the theoretical maximum yield of NeuAc was calculated using optimizeCtModel function. According to the simulated results, Mn²⁺, Zn²⁺, phosphate and SO₄²⁻ was uptake by the cell for bioreaction. So Mn²⁺ and Zn²⁺ was extra tested with a concentration of 0.33 mg/L MnSO₄·H₂O and 3.8 mg/L ZnSO₄·7H₂O as the same concentration of the culture medium.

3. Results

3.1. Developing a B. subtilis cell-free system

In order to construct a B. subtilis cell-free (BCF) system, optimization of cell lysis methods was first carried out. As a Gram-positive bacterium, B. subtilis’s cell wall is more difficult to be disrupted compared to Gram-negative bacterium E. coli. When cells are lysed by sonication, the volume of the bacterial suspension and the processing time affect both the degree of cell disruption and protein activity. To monitor the protein degradation in the lysate, B. subtilis that expresses green fluorescent protein (GFP) intracellularly was used. By monitoring the
fluorescence intensity in the supernatant, changes in protein content in the lysate can be easily characterized. First, the lysis volume is optimized. When the lysis volume is reduced, the bacterial suspension can fully contact the ice bath, which can slow down the protein denaturation, but the local high temperature around the ultrasonic probe will also accelerate the protein denaturation. As the lysis volume increases, the system temperature may rise due to insufficient ice bath. However, the damage of protein activity by local high temperature around the ultrasonic probe can be weakened. Three gradient volumes were selected for testing: 1 ml, 7.5 ml and 15 ml. At the same time, we took samples at different sonication times as insufficient time may cause incomplete cell lysis and excessive time may cause protein degradation or inactivation. It can be seen from the results that the maximum amount of active protein can be obtained after a 25-min sonication of 15 ml bacterial suspension (Fig. 2a).

At the same time, the protease of B. subtilis needs to be considered when establishing the BCF system. B. subtilis has a strong ability to secrete extracellular proteases, and a large amount of extracellular protease will be produced after the logarithmic growth phase, which can seriously affect the stability of the cell-free reaction system. Therefore, additional protease inhibitors are needed to slow down the degradation of key proteins by protease during the reaction. To inhibit a variety of proteases, including serine proteases, cysteine proteases, aspartic proteases and metalloproteases, protease inhibitor cocktails are chosen to achieve inhibition of various proteases. After adding the protease inhibitor cocktail, the protein degradation rate was slowed down, and the decrease in fluorescence intensity was reduced by 8.2% at 1.5 h (Fig. 2b).

3.2. Establishing a B. subtilis cell-free NeuAc synthesis system

The B. subtilis cell-free synthesis (BCFS) system suitable for NeuAc in vitro synthesis was further established. First, the cell culture time was carefully selected. During the fermentation process, the cell activity at different growth stages varies greatly. Harvesting cells at the time point when the NeuAc production rate is highest can accelerate the rate of NeuAc synthesis during the cell-free reaction. Therefore, the growth curve and the NeuAc biosynthesis curve of the NeuAc biosynthesis strain were measured, and the NeuAc specific synthesis rate per cell per hour was calculated (Fig. 3a). The fermentation curve showed that the NeuAc ratio synthesis rate reached the maximum in the middle of the logarithmic growth (16 h), which was 0.005 g/L/OD_{600}/h (Fig. 3a). Therefore, the bacterial cell harvest time was fixed at 16 h.

Next, the NeuAc extracellular biosynthesis buffer was optimized to ensure efficient cell-free reactions. First, two commonly used enzymatic reaction buffers were selected for further testing of cell-free reactions: cell-free phosphate balanced saline (CFPBS) and S30A buffer. In the BCF system, some ions are considered to be the key factors affecting enzyme activity, such as Mg^{2+} and K^+, which are already components of two buffers. However, for the biosynthesis of NeuAc by B. subtilis, there may be additional ions necessary for NeuAc synthesis. To search for these specific ions, a genome-scale metabolic model of B. subtilis was used [43].

After adding the NeuAc biosynthetic pathway to the model, the objective of the model was changed to NeuAc synthesis. The simulation results show the absorption of Mn^{2+}, Zn^{2+}, and SO4^{2-}. Therefore, additional addition of these ions can only slightly promote NeuAc synthesis. The possible reason is that the cell lysate itself contains enough essential ions, or that these predicted ions play a key role in other cell functions, but have little effect on NeuAc synthesis. In summary, a BCFS system that can efficiently produce NeuAc has been established, and its NeuAc biosynthesis rate (25.633 g/L/h) is 305-fold than that of fermentation (0.084 g/L/h), which laid the foundation for further diagnosis potential bottlenecks in biosynthesis pathways for NeuAc.

3.3. In vitro diagnosing NeuAc biosynthesis bottlenecks

The optimized BCFS system was further used to diagnose the potential metabolic bottleneck of the NeuAc synthesis pathway (Fig. 4a). The low flux of enzymatic reactions or competition of other metabolic pathways for intermediates can greatly limit the synthesis rate of the target metabolites, thus the synthesis rate of the target metabolite can be increased by adding the corresponding intermediates. NeuAc is synthesized from two direct precursors, including ManNAc and PEP (Fig. 4a). Therefore, all intermediates in the ManNAc synthesis pathway and PEP in the endogenous glycolysis pathway were added separately to the NeuAc cell-free synthesis system at different concentrations. The results showed that the NeuAc titer increased significantly by 94% to 0.621 g/L when 1 g/L of PEP was added, while there was no significant change in NeuAc titer when all other intermediate metabolites were added, revealed that insufficient PEP may be the potential bottlenecks in biosynthesis pathways for NeuAc (Fig. 4b).
3.4. In vivo eliminating NeuAc biosynthesis bottlenecks

In vivo experiments were then carried out to verify and eliminate the metabolic bottleneck diagnosed by the BCFS system. Since PEP is in the high-flux glycolysis pathway when glucose is the substrate, the main reason for the lack may be efficient conversion to pyruvate. Therefore, blocking the formation of pyruvate from PEP can theoretically increase the accumulation of PEP. However, knocking out pyruvate kinase (Pyk) will greatly limit the TCA cycle and further have a greater impact on cell growth [44,45]. Therefore, the expression of Pyk to an appropriate level is the potential optimal way to achieve a balance between cell growth and NeuAc biosynthesis.

Among all the available gene expression regulatory elements, N-terminal coding sequences (NCSs) were selected to regulate the expression level of pyk due to its easy-to-operate characteristics, wide range of expression regulation levels and dynamic patterns [42]. First, five inhibitory NCSs (N groeS, N pepF, N ydiC, N psdD, N lipA) and five growth-dependent regulatory NCSs (N hbs, N xyfG, N menK, N yoaD, N yigN) were chosen for down-regulating pyk expression throughout the growth phase or only in the stationary phase by fused to the 5’ end of the pyk gene on genome, respectively (Fig. 4c). Fermentation experiments showed that all engineered strains have achieved different degrees of increase in NeuAc titer and the N ydiC fusion strain reached a titer of 3.015 g/L, which is 2.05-fold of the control. Maximum OD600 of 5.53 was obtained, which decreased 15.84% compared to the control. As our experimental results indicated, the existence of metabolic bottlenecks in the NeuAc synthesis pathway in B. subtilis was demonstrated and further eliminated.

4. Conclusion

Our study provides an example to take easy access to in vivo reactions and achieve quick identification of metabolic bottlenecks. A highly efficient NeuAc cell-free synthesis system based on B. subtilis cell lysate was developed and used to diagnose the potential metabolic bottleneck of the NeuAc synthesis pathway in B. subtilis. The experimental results revealed that insufficient PEP severely limit the synthesis of NeuAc. We also verified the identified metabolic bottlenecks in vivo, achieving an increase of NeuAc production level. In addition, the method of constructing and optimizing the cell-free reaction system can be extended to other microorganisms and for other applications in metabolic engineering, such as efficient synthesis of cytotoxic proteins, efficient synthesis of cytotoxic compounds, kinetic monitoring of intermediates, characterization of gene expression regulatory elements and efficient gene amplification.
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