Synthetic and Systems Biotechnology 5 (2020) 131–136

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/synbio

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

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ARTICLE INFO

Keywords:
Pathway bottleneck diagnosis
Cell-free synthesis system
Bacillus subtilis

ABSTRACT

Metabolic engineering is a key technology for cell factories construction by rewiring cellular resources to achieve efficient production of target chemicals. However, the existence of bottlenecks in synthetic pathway can seriously affect production efficiency, which is also one of the core issues for metabolic engineers to solve. Therefore, developing an approach for diagnosing potential metabolic bottlenecks in a faster and simpler manner is of great significance to accelerate cell factories construction. The cell-free reaction system based on cell lysates can transfer metabolic reactions from *in vivo* to *in vitro*, providing a flexible access to directly change protein and metabolite variables, thus provides a potential solution for rapid identification of bottlenecks. Here, bottleneck diagnosis of the N-acetylneuraminic acid (NeuAc) biosynthesis pathway in industrially important chassis microorganism *Bacillus subtilis* was performed using cell-free synthesis system. Specifically, a highly efficient *B. subtilis* cell-free system for NeuAc *de novo* synthesis was firstly constructed, which had a 305-fold NeuAc synthesis rate than that *in vivo* and enabled fast pathway dynamics analysis. Next, through the addition of all potential key intermediates in combination with substrate glucose respectively, it was found that insufficient phosphoenolpyruvate supply was one of the NeuAc pathway bottlenecks. Rational *in vivo* metabolic engineering of NeuAc-producing *B. subtilis* was further performed to eliminate the bottleneck. By down-regulating the expression level of pyruvate kinase throughout the growth phase or only in the stationary phase using inhibitory N-terminal coding sequences (NCSs) and growth-dependent regulatory NCSs respectively, the maximal NeuAc titer increased 2.0-fold. Our study provides a rapid method for bottleneck diagnosis, which may help to accelerate the cycle of design, build, test and learn cycle for metabolic engineering.

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

Peer review under responsibility of KeAi Communications Co., Ltd.
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https://doi.org/10.1016/j.synbio.2020.06.006
Received 3 May 2020; Received in revised form 12 June 2020; Accepted 15 June 2020

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 $(NH_4)_2SO_4$, 12.5 g/L $K_2HPO_4{\cdot}4H_2O$, 2.5 g/L KH_2PO_4, 3 g/L $MgSO_4{\cdot}7H_2O$, and 60 g/L glucose, the pH of the medium was titrated to 7.0 using saturated NH_4OH 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

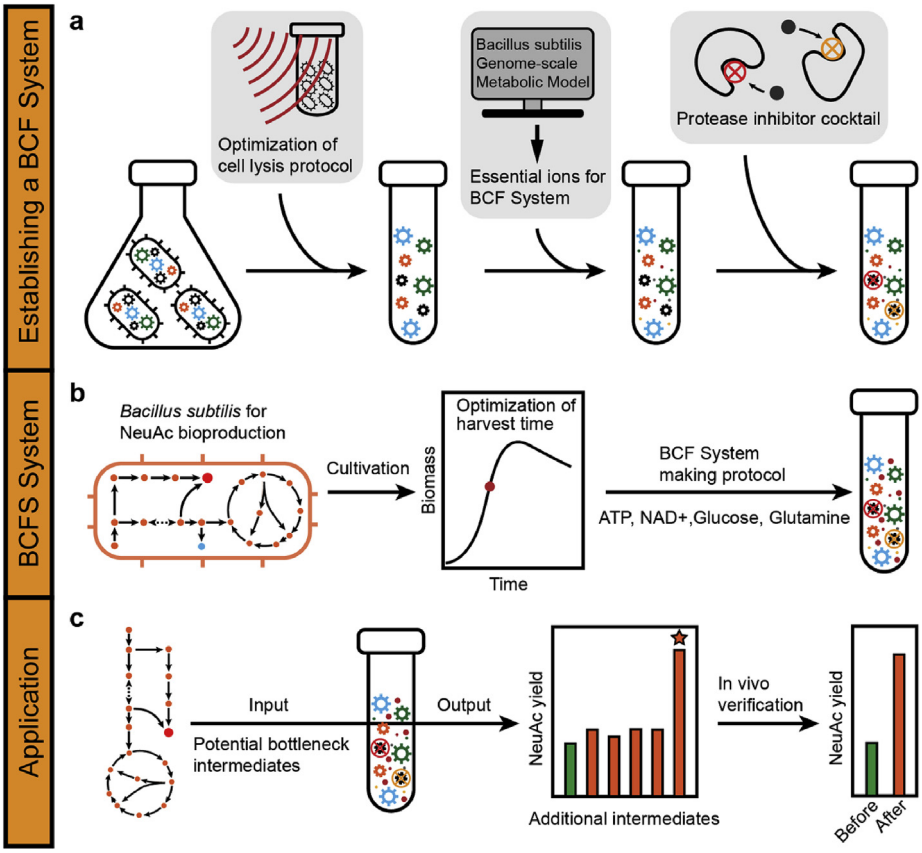

Fig. 1. Diagram of cell-free system based metabolic bottleneck diagnosis. (a) Establishing a *B. subtilis* cell-free (BCF) system. (b) Establishing a *B. subtilis* cell-free synthesis (BCFS) system for NeuAc. (C) Bottleneck identification based on BCFS system and bottleneck elimination using NCSs.

Table 1
Strains and plasmids used in this study.

Names	Characteristics	Source or Reference
Strains		
BSU168	*B. subtilis* 168 *trpC2*	BGSC 1A1
BSU168- pP43NMK-GFP	*B. subtilis* 168 *trpC2* harboring pP43NMK-GFP	Tian et al. [42]
BSGN6	*B. subtilis* 168 derivate	Liu et al. [46]
B6AN	BSGN6 harboring plasmid pP43NMK-AGE-NEUB	Zhang et al. [41]
B6AN-N*groeS-pyk*	B6AN derivate, with N*groeS* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*pepF-pyk*	B6AN derivate, with N*pepF* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*ydiC-pyk*	B6AN derivate, with N*ydiC* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*rpsD-pyk*	B6AN derivate, with N*rpsD* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*lipA-pyk*	B6AN derivate, with N*lipA* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*hbs-pyk*	B6AN derivate, with N*hbs* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*yxjG-pyk*	B6AN derivate, with N*yxjG* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*menK-pyk*	B6AN derivate, with N*menK* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*yoaD-pyk*	B6AN derivate, with N*yoaD* inserted into the 5′ end of the *pyk* gene	This work
B6AN-N*yvgN-pyk*	B6AN derivate, with N*yvgN* inserted into the 5′ end of the *pyk* gene	This work
Plasmids		
pP43NMK-GFP	pP43NMK derivate with *gfp* cloned under the control of P_{43} promoter	Tian et al. [42]
pP43NMK-AGE-NEUB	pP43NMK derivate with *age* and *neuB* cloned under the control of P_{43} promoter	Zhang et al. [41]

pellets were then washed three times by cell free phosphate balanced saline buffer (CFPBS buffer, 4 g/L NaCl, 1.815 g/L $Na_2HPO_4{\cdot}12H_2O$, 0.1 g/L KCl, 0.135 g/L KH_2PO4, 1 g/L $MgCl_2{\cdot}6H_2O$, 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 $MgCl_2{\cdot}6H_2O$ 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-6P), GlcN6P (glucosamine-6P), GlcNAc6P (N-acetylglucosamine-6P), 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.

Table 2
BCFS reaction system.

Compound	Stock concentration	Volume	Final concentration
ATP (x20)	60 mM	62.5 μL	3 mM
NAD^+ (x100)	57.5 mM	12.5 μL	0.575 mM
ADP (x50)	150 mM	25 μL	3 mM
Sodium phosphate buffer (Na_2HPO_4 and NaH_2PO_4, pH = 7.0)	100 mM	100 μL	10 mM
Crude cell extracts		1000 μL	
Total		~1.2 mL	

2.5. Analytical methods

Biomass was determined by measuring optical density at 600 nm (OD_{600}).

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 optimizeCbModel function. According to the simulated results, Mn^{2+}, Zn^{2+}, phosphate and $SO4^{2+}$ was uptake by the cell for bioreaction. So Mn^{2+} and Zn^{2+} was extra tested with a concentration of 0.33 mg/L $MnSO_4{\cdot}H_2O$ and 3.8 mg/L $ZnSO_4{\cdot}7H_2O$ 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

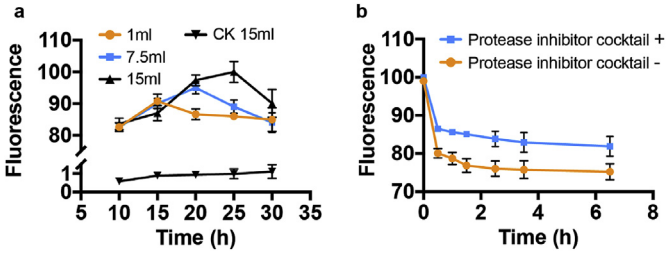

Fig. 2. Optimization of the BCF system making protocol. (a) Cell suspension volume optimization for sonication characterized by fluorescence intensity. "CK 15 ml" indicate wild type *B. subtilis* 168 was lysed by sonication in a volume of 15 ml as blank control. (b) Effect of protease inhibitor cocktail on protein degradation. "+", additional added; "-", no additional added. Triplicate fermentation experiments were carried out, and error bars represent standard deviation (SD).

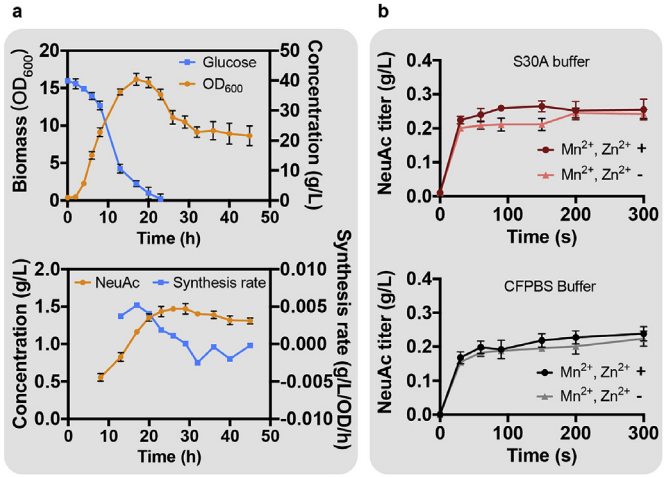

Fig. 3. Optimization of the BCFS system making protocol. (a) Growth curve and the NeuAc biosynthesis curve of the NeuAc biosynthesis strain. (b) Optimization of cell-free reaction buffer. CFPBS buffer, cell-free phosphate balanced saline buffer; "+", additional added; "-", no additional added. Triplicate fermentation experiments were carried out, and error bars represent standard deviation (SD).

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 increase, 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 kind 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 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 was performed in both buffers to verify its effectiveness. The experimental results show that the highest titer of NeuAc in S30A buffer is 11.4% higher than that in CFPBS, reaching 0.265 g/L (Fig. 3b). However, the additional addition of predicted 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).

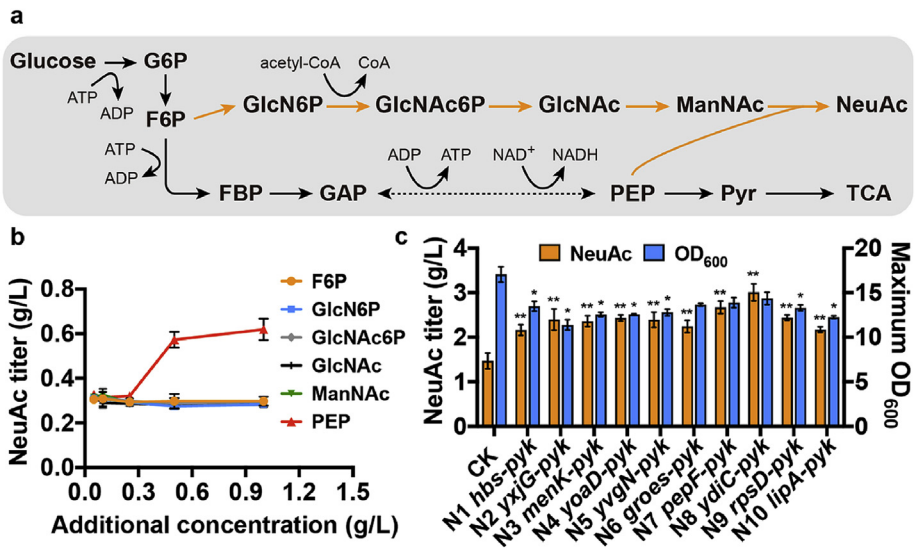

Fig. 4. Diagnosing and eliminating metabolic bottleneck of NeuAc synthesis pathway. (a) NeuAc biosynthetic pathway in engineered *B. subtilis*. (b) NeuAc titer when additional intermediate metabolites are added in BCFS system. (c) NeuAc titer after fused different NCSs to the 5 'end of the pyruvate kinase coding sequence on genome. G6P, Glucose-6P; F6P, Fructose-6P; FBP, Fructose-1,6-2P; GAP, glyceraldehyde-3-P; GlcN6P, glucosamine-6P; GlcNAc6P, N-acetylglucosamine-6P; GlcNAc, N-acetylglucosamine; ManNAc, N-acetylmannose; PEP, phosphoenolpyruvate; Pyr, pyruvate. Triplicate fermentation experiments were carried out, and error bars represent standard deviation (SD). The * and ** symbol indicate $P < 0.05$ and $P < 0.01$ relative to control strain, respectively.

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, reducing 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 *rpsD*, N *lipA*) and five growth-dependent regulatory NCSs (N *hbs*, N *yxjG*, N *menK*, N *yoaD*, N *yvgN*) was chosen for down-regulating *pyk* expression level 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 OD_{600} of N *ydiC* fusion strain was 14.35, which decreased 15.84% compared to that of control. As our experimental results indicated, the existence of metabolic bottlenecks in the NeuAc biosynthetic 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.

CRediT authorship contribution statement

Rongzhen Tian: Data curation, Methodology, Software, Writing - original draft. **Minghu Wang:** Data curation, Methodology, Writing - original draft. **Jintian Shi:** Data curation, Methodology. **Xiaolong Qin:** Data curation, Methodology. **Haoyu Guo:** Data curation, Methodology. **Xuanjie Jia:** Data curation, Methodology. **Jianghua Li:** Data curation, Writing - review & editing. **Long Liu:** Conceptualization, Writing - review & editing. **Guocheng Du:** Conceptualization, Writing - review & editing. **Jian Chen:** Supervision, Writing - review & editing. **Yanfeng Liu:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgements

This study is financially supported by the National Key Research and Development Program of China (2018YFA0900300), National Natural Science Foundation of China (31972854), Key Research and Development Program of Jiangsu Province (BE2019628), Fundamental Research Funds for the Central Universities (JUSRP52020A), National First-class Discipline Program of Light Industry Technology and Engineering (LITE2018-16), Postgraduate Research & Practice Innovation Program of Jiangsu Province (JNKY19_015).

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