

CRISPRi allows optimal temporal control of N-acetylglucosamine bioproduction by a dynamic coordination of glucose and xylose metabolism in *Bacillus subtilis*

Yaokang Wu^{a,b}, Taichi Chen^{a,b}, Yenfeng Liu^{a,b}, Xueqin Lv^{a,b}, Jianghua Li^{a,b}, Guocheng Du^b, Rodrigo Ledesma-Amaro^c, Long Liu^{a,b,†}

^a Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China.

^b Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China.

^c Department of Bioengineering, Imperial College London, London SW7 2AZ, UK.

[†]Corresponding author: Long Liu, Tel.: +86-510-85918312, Fax: +86-510-85918309, E-mail: longliu@jiangnan.edu.cn

Abstract

Glucose and xylose are the two most abundant sugars in renewable lignocellulose sources; however, typically they cannot be simultaneously utilized due to carbon catabolite repression. N-acetylglucosamine (GlcNAc) is a typical nutraceutical and has many applications in the field of healthcare. Here, we have developed a gene repressor system based on xylose-induced CRISPR interference (CRISPRi) in *Bacillus subtilis*, aimed at downregulating the expression of three genes (*zwf*, *pfkA*, *glmM*) that control the major competing reactions of GlcNAc synthesis (pentose phosphate pathway (HMP), glycolysis, and peptidoglycan synthesis pathway (PSP)), with the potential to relieve glucose repression and allow the co-utilization of both glucose and xylose. Simultaneous repression of these three genes by CRISPRi improved GlcNAc titer by 13.2% to 17.4 ± 0.47 g/L, with the GlcNAc yield on glucose and xylose showing an 84.1% improvement, reaching 0.42 ± 0.036 g/g. In order to further engineer the synergetic utilization of glucose and xylose, a combinatorial approach was developed based on 27 arrays containing sgRNAs with different repression capacities targeting the three genes. We further optimized the temporal control of the system and found that when 15 g/L xylose was added 6 h after inoculation, the most efficient strain, BNX122, synthesized 20.5 ± 0.85 g/L GlcNAc with a yield of 0.46 ± 0.010 g/g glucose and xylose in shake flask culture. Finally, the GlcNAc titer and productivity in a 3-L fed-batch bioreactor reached 103.1 ± 2.11 g/L and 1.17 ± 0.024 g/L/h, which were 5.0-fold and 2.7-fold of that in shake flask culture, respectively. Taken together, these findings suggest that a CRISPRi-enabled regulation method provides a simple, efficient, and universal way to promote the synergetic utilization of multiple carbon sources by microbial cell factories.

Keywords: CRISPRi; N-acetylglucosamine; dynamic regulation; *Bacillus subtilis*; xylose utilization; synthetic biology

1. Introduction

With the growing concern regarding climate change, more and more chemicals and materials are being produced by microbial cell factories using renewable biomass resources (Chae et al., 2017). Glucose and xylose are the two most abundant sugars in renewable lignocellulose carbon sources. However, most microorganisms, including *Bacillus subtilis*, cannot simultaneously utilize both sugars due to carbon catabolite repression (CCR), which limits the efficient conversion of biomass resources (Görke and Stülke, 2008). In order to solve this problem caused by the native metabolic regulatory mechanisms of the cell, modifications to the glucose and xylose metabolic pathways have been extensively studied. For example, many CCR-insensitive mutants of *Escherichia coli* were generated by inactivating the phosphotransferase system (PTS) of glucose (Li et al., 2007; Nichols et al., 2001; Vargas-Tah et al., 2015). However, this modification can affect the transportation and phosphorylation of glucose, as PTS is the main way for glucose uptake (Chiang et al., 2013; Hernández-Montalvo et al., 2003). Another way to relieve the CCR of glucose on xylose metabolism is eliminating the regulation of the xylose pathway by replacing the promoters of genes in this pathway (Kim et al., 2015) or by introducing a heterologous xylose pathway (Chen et al., 2013; Gonçalves et al., 2014). However, additional optimization steps such as evolutionary engineering may be required to further balance glucose and xylose metabolism (Kim et al., 2015). Recently, Yan's group presented the SynCar mechanism, which promotes the co-utilization of glucose and xylose in *E. coli* (Y. Wu et al., 2017a, 2017b). They found that after knocking-out certain genes involved in glycolysis, glycerol or xylose were utilized as a second carbon source, maintaining cell growth and generating phosphoenolpyruvate (PEP), which can promote glucose uptake by PTS. However, this static metabolic engineering strategy may reduce the robustness of the recombinant strain and attenuate its growth (Venayak et al., 2015).

N-acetylglucosamine (GlcNAc) is a precursor of glycosaminoglycan, which plays an important role in the repair and maintenance of cartilage and joint tissue function (Tamai et al., 2003). Thus, GlcNAc is widely added to drugs and nutritious diets to treat and repair joint injuries, and it also has many applications in the fields of cosmetics and pharmaceuticals (Chen et al., 2010; L. Liu et al., 2013). At present, GlcNAc is mainly produced by

1 acid hydrolysis of chitin in shrimp shells or crab shells (Chen et al., 2010). However, the waste liquid can cause
2 serious environmental pollution and the products can cause allergic reactions. By contrast, GlcNAc production
3 by microbial fermentation from renewable biomass such as glucose and xylose is more promising. In 2005, an
4 engineered GlcNAc-producing *Escherichia coli* strain was generated, achieving a titer of 110 g/L GlcNAc (Deng
5 et al., 2005). In previous studies, we have introduced the GlcNAc synthetic pathway into the GRAS (generally
6 regarded as a safe) strain *B. subtilis* 168 (Liu et al., 2014a; Y. Liu et al., 2013), and after rewriting glucose
7 transportation and central metabolic pathways of this strain, GlcNAc titer reached 13.2 g/L in shake-flask
8 fermentation (Gu et al., 2017).

9 Three competitive pathways of GlcNAc synthesis in *B. subtilis* are the pentose phosphate pathway (HMP),
10 glycolysis (EMP), and the peptidoglycan synthesis pathway (PSP) (Gu et al., 2017), which are all essential for
11 normal cell growth and therefore cannot be deleted (Commichau et al., 2013; Peters et al., 2016). Dynamically
12 repressing these competitive pathways of GlcNAc synthesis in a switchable manner is a promising way to
13 manage the tradeoff between cell growth and product synthesis (Xu, 2018). Recently, the Clustered Regularly
14 Interspaced Short Palindromic Repeats (CRISPR) interference (CRISPRi) system was developed to repress gene
15 expression using an inactive Cas9 (dCas9) protein that can bind to a target gene and block the elongation of
16 RNA polymerase (Qi et al., 2013), and has been used in the construction of many microbial cell factories (Gao
17 et al., 2018; Kaczmarzyk et al., 2017; Kim et al., 2016; Lv et al., 2015; Westbrook et al., 2017; Zhang et al., 2017).

18 Here, we have established a novel switchable regulation method by using the CRISPRi system to
19 dynamically coordinate glucose and xylose metabolism for GlcNAc production in *B. subtilis*, without modifying
20 the genes in the native metabolic pathways of xylose and glucose. Specifically, a xylose-induced CRISPRi system
21 was constructed to dynamically regulate the competitive pathways of GlcNAc synthesis in *B. subtilis*. Therefore,
22 most glucose could be redirected towards product formation after the induction of the system. In addition, the
23 xylose used as an inducer for the expression of dCas9 is also taken up and utilized together with glucose by the
24 cell, further improving GlcNAc production. Interestingly, because the CRISPRi system has great controllability

and strong universality in different organisms, the strategy described can be easily adapted for constructing other robust microbial cell factories which utilize multiple carbon sources synergistically.

2. Materials and Methods

2.1 Strains and reagents

E. coli JM109 was used for gene cloning. The recombinant GlcNAc synthesis *B. subtilis* strain BNY (*B. subtilis* 168 Δ nagP Δ gamP Δ gamA Δ ldh Δ pta Δ glcK Δ pckA Δ pyk::lox72, Δ nagAB::P_{veg}-yqaB, P_{veg}-glmS, pP43-GAN1) that was constructed in our previous study was used as the initial host, and all strains used in this study are listed in Table 1. Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) was used for bacterial cultivation. The shake flask fermentation medium consisted of 12 g/L yeast extract, 6 g/L tryptone, 6 g/L (NH₄)₂SO₄, 12.5 g/L K₂HPO₄·3H₂O, 2.5 g/L KH₂PO₄, 60 g/L glucose, and 10 mL/L trace metal solution. The trace metal solution contained 4 g/L FeSO₄·7H₂O, 4 g/L CaCl₂, 1 g/L MnSO₄·5H₂O, 0.4 g/L CoCl₂·6H₂O, 0.2 g/L NaMoO₄·2H₂O, 0.2 g/L ZnSO₄·7H₂O, 0.1 g/L AlCl₃·6H₂O, 0.1 g/L CuCl₂·H₂O, and 0.05 g/L H₃BO₄. And 15g/L xylose was added into fermentation broth at 4h after inoculation to induce dCas9 expression before the induction conditions were optimized. The following antibiotics were used for selections: ampicillin, 100 µg/mL; kanamycin, 50 µg/mL; chloramphenicol, 5 µg/mL; zeocin, 30 µg/mL; and spectinomycin, 100 µg/mL.

2.2 CRISPRi plasmid construction

The primers and plasmids used in this study are summarized in Supplementary Tables S1 and S2, respectively. To construct a dCas9 integrant expression plasmid, an integrant expression plasmid pLCx was constructed first. The plasmid backbone containing a pMB1 replication origin and an aadA resistance cassette was amplified from pTargetF (Addgene plasmid #62226) using primers ori-Fe and ori-Ra. Two homologous arms of the *lacA* gene were amplified from the *B. subtilis* genome using primers lacA'-Fa/lacA'-Rb and 'lacA-Fd/'lacA-Re, respectively. The chloramphenicol resistance cassette was amplified from pHT01 (Phan et al., 2015) using primers Cm-Fb and Cm-Rc, and lox71 and lox62 sites flanking this cassette were introduced. A

xylose-induced promoter P_{xyIA} and its repressor XylR were amplified from pAX01 (Nguyen et al., 2005) using primers P_{xyIA} -Fc and P_{xyIA} -Rd. These five fragments were assembled sequentially using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China), which facilitated the ligation of two or more DNA fragments through 15–20 bp overlaps. Finally, dCas9 amplified from pdCas9-bacteria (Addgene plasmid #44249) using primers dCas9-FEco31I and dCas9-RPstI was inserted between *Bam*HI and *Pst*I restriction sites of pLCx, yielding pLCx-dCas9.

To construct a sgRNA integrant expression plasmid, the downstream homologous arm of the *amyE* gene was first amplified from the *B. subtilis* genome using primers psga-R-FEcoRI and psga-R-RHindIII, and was inserted between *Eco*RI and *Hind*III restriction sites of pTargetF (Addgene plasmid #62226), resulting in psga0. Next, the upstream homologous arm of the *amyE* gene was amplified from the *B. subtilis* genome using primers psga-L1-FBamHI and psga-L1-RH, and the zeocin resistance cassette was amplified from p7z6 (Yan et al., 2008) using primers psga-L2-FH and psga-L2-RBcuI, by which lox71 and lox62 sites flanking this cassette and promoter P_{veg} were introduced. These two fragments were then assembled through fusion PCR and inserted into *Bam*HI and *Bcu*I restriction sites of psga0, yielding psga. To achieve the integrant expression of dCas9 and sgRNA, pLCx-dCas9 and psga were linearized by *Eco*91I, transformed into *B. subtilis*, and resistance marker cassettes were then evicted by recombination with lox71 and lox66 as previously described (Yan et al., 2008) (Fig. 1A). The sequences of pLCx-dCas9 and psga can be found in Supplementary Material.

Plasmid psga-GFP containing a sgRNA targeting the non-template strand of green fluorescent protein (GFP) was generated by inverse PCR of psga using primers sg-F and sg-GFP-R, and plasmids containing sgRNAs targeting other genes were constructed similarly. To construct a plasmid containing two sgRNAs, psga was linearized by PCR using primers psga-liner-F-D and psga-liner-R-A, and two sgRNA expression cassettes obtained from different sgRNA expression plasmids by primers sg-F-A/sg-R-C and sg-F-C/sg-R-D were inserted via Golden Gate assembly (Engler et al., 2009). To construct a plasmid containing three sgRNAs, primers sg-F-A/sg-R-B, sg-F-B/sg-R-C, and sg-F-C/sg-R-D were used to amplify sgRNA expression cassettes, which were then inserted into the linearized plasmid using Golden Gate assembly. It has been demonstrated that in prokaryotic

cells only the gRNA targeting the non-template strand of DNA showed obvious repression of gene expression (Qi et al., 2013). Therefore, all sgRNAs used in this study targeted the non-template strand of genes and were designed using the sgRNACas9 software (Shen, 2014).

2.3 Gene-reporter assay for CRISPRi activity

A dCas9 expression strain BNX was constructed by transforming the linearized plasmid pLCx-dCas9 into strain BNY. Plasmid psga-GFP containing a sgRNA targeting the non-coding strand of GFP, was linearized and transformed into strain BNX, yielding BNX000. The reporter-plasmid pP43-GFP (S. Yang et al., 2017a) containing GFP under the control of the strong constitutive promoter P43 was transformed into BNX and BNX000, respectively. The relative fluorescence intensities of GFP in these two strains were measured as previously described using a Multi-Mode Microplate Reader (Cytation 3; BioTek, Winooski, VT, USA) (S. Yang et al., 2017b).

2.4 Analytical methods

The shake-flask fermentation was conducted in a 250 mL Erlenmeyer flask containing around 50 mL of fermentation medium and incubated at 37 °C with shaking at 220 rpm for 48 h. The concentrations of GlcNAc, xylose, and the by-product acetoin in the fermentation broth were measured by high-performance liquid chromatography (HPLC) on an instrument equipped with an HPX-87H column (Bio-Rad, Hercules, CA, USA) and a refractive index detector. The mobile phase used was 5 mM H₂SO₄ at a flow rate of 0.6 mL/min and 40 °C. The glucose concentration in the medium was measured using a glucose-glutamate analyzer (SBA-40C; Biology Institute of Shandong Academy of Sciences). The OD₆₀₀ was measured every 12 h after inoculation and converted to dry cell weight (DCW) according to the following equation: 1 OD₆₀₀ = 0.35 g/L DCW (Liu et al., 2014b). All experiments were independently carried out at least three times.

2.5 RT-qPCR Analysis

Cells were harvested from shake flask culture and frozen immediately in liquid nitrogen. Total RNA was purified using the RNeasy Pure Kit (Qiagen Biotech, Beijing, China) according to the manufacturer's instructions. The quantity and purity of RNA were determined by optical density measurements at 260 and 280 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Subsequently, cDNA, which was obtained by reverse transcription of total RNA using a PrimeScriptTM RT-PCR Kit (Takara, Dalian, China), was used as the template for qPCRs. qPCR was performed in a 96-well plate with a total reaction volume of approximately 20 μ L using SYBR Premix Ex TaqTM (Takara) according to the manufacturer's specifications. The reactions were conducted with a LightCycler 480 II Real-time PCR instrument (Roche Applied Science, Mannheim, Germany). The *hbsU* gene was used as the internal standard (Cabrera-Valladares et al., 2012).

2.6 Fed-Batch culture in 3-L bioreactor

The production of GlcNAc via fed-batch culture of BNX122 was performed at an initial glucose concentration of 5 g/L. The fermentation medium used for fed-batch culture was optimized and consisted of 20 g/L yeast extract, 20 g/L tryptone, 10 g/L urea, 12.5 g/L $K_2HPO_4 \cdot 3H_2O$, 2.5 g/L KH_2PO_4 , and 10 mL/L trace metal solution. The trace metal solution contained 4 g/L $FeSO_4 \cdot 7H_2O$, 4 g/L $CaCl_2$, 1 g/L $MnSO_4 \cdot 5H_2O$, 0.4 g/L $CoCl_2 \cdot 6H_2O$, 0.2 g/L $NaMoO_4 \cdot 2H_2O$, 0.2 g/L $ZnSO_4 \cdot 7H_2O$, 0.1 g/L $AlCl_3 \cdot 6H_2O$, 0.1 g/L $CuCl_2 \cdot H_2O$, and 0.05 g/L H_3BO_4 . The feeding solution contained 600 g/L glucose and 300 g/L xylose. Seed culture was carried out in 500-mL shake flasks containing 75 mL of seed medium with shaking at 220 rpm and 37 °C for 12 h on rotary shakers. The seed culture (75 mL) was inoculated into a 3-L fermenter (BioFlo115, New Brunswick Scientific Co., Edison, NJ, USA) containing 1.5 L of fermentation medium. The pH was kept at 7.0 automatically via the addition of 29% NH_3 and 2M HCl, and the temperature was maintained at 37 °C. The aeration rate and agitation speed were 1.5 vvm and 800 rpm, respectively. When the optical density at 600 nm (OD₆₀₀) reached 1.5, 30 g/L glucose and 15 g/L xylose were added to the medium.

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 30 g/L. In fed-batch cultivation with control of glucose concentration, the glucose concentration was maintained at approximately 20 g/L by feeding with concentrated glucose and xylose. The feeding rates were adjusted every 2 h based on the concentration of residual glucose in the fermentation medium, which was measured using a glucose-glutamate analyzer (SBA-40E, Biology Institute of Shandong Academy of Sciences, Jinan, China).

2.7 Statistical analysis

All data were expressed as mean $\pm$ SD. Differences were determined by 2-tailed Student's t-test between two groups, or one-way ANOVA followed by post-hoc Tukey's test for multiple groups. Statistical significance is indicated as * for $p < 0.05$ and ** for $p < 0.01$.

3. Results

3.1 CRISPRi-mediated gene knockdown in *B. subtilis*

The CRISPRi system was developed as shown in Fig. 1A and is described in Materials and Methods. Briefly, plasmids pLCx-dCas9 and psga were constructed to integrate dCas9 expressed by a strong inducible promoter P_{xyIA} and sgRNA-controlled by a strong constitutive promoter P_{veg} , into the *lacA* and *amyE* loci of the *B. subtilis* genome, respectively. There are two main reasons for using this xylose-inducible promoter: firstly, it can reduce the metabolic burden of expressing cas9 constitutively; secondly, the inducer xylose may also serve as a supplementary carbon for biomass production after repressing the competing reactions of GlcNAc. To examine the capability of the CRISPRi system built here for transcriptional repression in *B. subtilis*, pLCx-dCas9 was transformed into a GlcNAc producing strain BNY, yielding BNX and then psga-GFP containing a GFP-targeting sgRNA was transformed into BNX, resulting in BNX000. A fluorescence-based reporter plasmid, pP43-GFP, was transformed into BNX and BNX000, and the relative fluorescence intensity was determined under various concentrations of the inducer xylose. As shown in Fig. 1B, the repressive effect was strengthened with increasing xylose concentration. A 33.1-fold repression was observed when 5 g/L xylose was used, and a 70.1-

fold repression was achieved if the concentration of xylose was increased to 20 g/L. Interestingly, using 15 g/L xylose, a 65.8-fold repression was reached, suggesting that this concentration is sufficient to fully induce the expression of dCas9. These results indicated that the CRISPRi system constructed here worked well and could be used for the following studies.

3.2 Engineering the simultaneous weakening of GlcNAc competing pathways and the co-utilization of glucose and xylose

With the CRISPRi working efficiently in *B. subtilis*, we designed a dynamic and temporal regulation system that, upon addition of xylose, uncouples the cross-talk between the pathways involved in glucose and xylose consumption. This simultaneously allows 1) the abolition of CCR, which could facilitate the co-consumption of glucose and xylose and 2) the channeling of the glucose flux directly towards GlcNAc by repressing competing reactions (Fig. 2A).

As shown in Fig. 2A, there are three major competitive pathways, namely HMP, EMP, and PSP, which drain the carbon flux from the metabolism of glucose and xylose away from the production of GlcNAc (Gu et al., 2017). Because all these three pathways are essential for normal cell growth, a dynamic regulation strategy was employed to balance the carbon flux between cell growth and GlcNAc synthesis using an inducible CRISPRi system. The *zwf* gene encoding glucose 6-phosphate dehydrogenase, *pfkA* encoding phosphofructokinase, and *glmM* encoding phosphoglucosamine mutase that control the major rate-limiting steps of HMP, PEP, and PSP, respectively, were chosen as regulatory targets (Fig. 2A). Because *pfkA* and *pyk* encoding pyruvate kinase were in the same operon in the *B. subtilis* genome, strain BNY in which *pyk* has been knocked out was chosen as the initial host, in order to exclude the effects of repression on these two genes together by sgRNA targeting *pfkA*. It has been reported that sgRNA targeting the beginning of the coding region and the region inside the initial RNAP complex on the promoter were both efficient for silencing gene expression (Qi et al., 2013). However, the specific promoter regions of these genes were unknown, so plasmids *psga-zwf1*, *psga-pfkA1*, *psga-glmM1*, *psga-zwf1-pfkA1*, and *psga-zwf1-pfkA1-glmM1* containing a single sgRNA or multiple sgRNAs targeting the

beginning of the coding region of each gene were constructed and integrated into genome of BNX, resulting in BNX100, BNX010, BNX001, BNX110, and BNX111, with the repression of these genes being validated by RT-qPCR (Fig. 2B). A comparison with strain BNY without the CRISPRi system and strain BNX, expressing dCas9 and the guide RNA targeting GFP, revealed similar GlcNAc titers (from 15.0 ± 0.54 g/L to 15.2 ± 0.38 g/L), with a slight reduction of GlcNAc yield on glucose and xylose (from 0.27 ± 0.005 g/g to 0.22 ± 0.002 g/g), suggesting that the CRISPRi system consumed some resources of the cell (Fig. 2C). Hence, strain BNX000 was used as the control for evaluation.

In a previous study, Chen et al. found that wild *B. subtilis* cannot metabolize xylose when glucose was present in the culture (Chen et al., 2010). However, as shown in Fig. 2C, the control strain BNX000 is able to consume around 5 g/L xylose when 60 g/L glucose and 15 g/L xylose were added, which may be caused by the introduction of the GlcNAc synthesis pathway. This pathway consumed some intracellular fructose-6-P, which is an immediate precursor for the synthesis of the main CCR regulator 1,6-bisphosphate (FBP) in *B. subtilis* (Görke and Stülke, 2008). This further supports our hypothesis that the repression of each of these three genes, *zwf*, *pfkA*, and *glmM*, could reduce glucose catabolism and promote xylose utilization. The recombinant strain BNX100 with repression of *zwf* showed a 27.0% reduction in glucose consumption and a 50.9% improvement in xylose consumption, compared with strain BNX000, suggesting that xylose can be used as a supplementary carbon source if the major pathway by which glucose flows into HMP is repressed. Compared with strain BNX000, the maximal DCW of this strain reduced by 35.1%, while the titer of GlcNAc did not show obvious change (from 15.2 ± 0.38 g/g to 14.2 ± 1.46 g/g), with the yield of GlcNAc improving by 43.4% to 2.13 ± 0.001 g/g dry cell. Furthermore, the yields of by-product acetoin of these two strains did not present a clear difference (from 1.06 ± 0.065 g/g dry cell to 1.01 ± 0.061 g/g dry cell). The constructed recombinant strain BNX010 with repressed *pfkA* expression presented a 76.2% reduction in glucose consumption and a 2.0-fold improvement in xylose consumption compared with strain BNX000, while the maximal DCW of this strain was reduced by 34.3% to 6.7 ± 0.17 g/L. As *glcK* encoding glucokinase was knocked out in the initial host strain BNY, the strains used in this study can only utilize glucose by PEP-dependent PTS.

Hence, the significantly weakened glucose utilization of this strain may be caused by the reduced PEP level after repressing EMP from glucose. Despite the titer of GlcNAc reduced by 26.9% to 11.1 ± 0.74 g/L, the yield of GlcNAc on glucose and xylose increased by 91.3% to 0.44 ± 0.023 g/g, demonstrating that the fructose-6-P produced was mainly directed into the GlcNAc synthesis pathway. The by-product acetoin was not detected in the broth of this strain, suggesting that carbon overflow was relieved by repressing *pfkA*. The recombinant strain BNX001, repressed for *glmM*, showed a 9.9% reduction in glucose consumption and a 64.9% improvement in xylose consumption. Even though the exponential growth phase was delayed for around 12 h (data not shown), the maximal DCW of this strain was similar to that of the control strain. Besides, the GlcNAc titer of this strain (14.1 ± 2.06 g/L) was similar to that of the control strain. The by-product acetoin produced by this strain compared with the control strain showed an increase of 14.6%, suggesting that a higher carbon flux was directed into EMP when PSP was repressed. In addition, unusual cell lysis was not observed, although *B. subtilis* is well known for cell lysis under certain conditions.

Because EMP and HMP were connected to each other through the intermediates fructose-6-P and glyceraldehyde-3P, the effects of simultaneous repression of *zwf* and *pfkA* were investigated. As shown in Fig. 2C, the consumption of glucose by strain BNX110 showed a 39.5% reduction compared with BNX000, which was higher than that of BNX100 but lower than that of BNX0110. In addition, the xylose consumed by strain BNX110 improved by 50.7% compared with BNX000, which was also higher than that of BNX100 but lower than that of BNX010. Although there was no significant increase in the GlcNAc titer of strain BNX110 (15.5 ± 0.25 g/L), the yield of GlcNAc on glucose and xylose increased by 49.3% to 0.34 ± 0.016 g/g, and the titer of by-product acetoin decreased by 64.2%, demonstrating that more sugars can be converted into GlcNAc after blocking EMP and HMP at the same time. When *zwf*, *pfkA*, and *glmM* were repressed simultaneously, the titer of GlcNAc increased by 13.2% to 17.4 ± 0.47 g/L, and the yield of GlcNAc on glucose and xylose reached 0.42 ± 0.036 g/g, representing an improvement of 84.1%. Compared with strain BNX000, BNX111 showed a 9.1% reduction in biomass and a 54.7% reduction in acetoin production. Together, these results suggest that

1 simultaneous repression of these three genes using CRISPRi can tune the metabolic coordination of glucose
2 and xylose consumption, which may promote more efficient transformation of these sugars into GlcNAc.

3 4 3.3 Combination and optimization of sgRNAs

5 It has been reported that sgRNAs targeting different positions of a gene can produce different repressive
6 strengths (Qi et al., 2013). This trait has been used in the construction of several microbial cell factories (Wang
7 et al., 2017; J. Wu et al., 2017; Wu et al., 2015). Here, we have designed three sgRNAs targeting the beginning,
8 middle, or end of the coding region for each gene - *zwf*, *pkfA*, and *glmM* - in order to fine-tune their repressive
9 strength (Fig. 3A). Therefore, we constructed 27 sgRNA arrays containing different combinations of these
10 sgRNAs within the psga plasmid (Fig. 3B). These plasmids were linearized and transformed into the strain BNX,
11 which generated 27 recombinant strains that were used in shake-flask fermentations. As shown in Fig. 4, the
12 titers of GlcNAc ranged from 15.9 ± 0.31 g/L to 18.7 ± 0.45 g/L, with consumptions of glucose from 29.83 ± 0.48
13 g/L to 55.5 ± 0.51 g/L, and consumptions of xylose from 5.93 ± 0.2 g/L to 11.28 ± 0.71 g/L. After comparing the
14 carbon consumptions and GlcNAc synthesis of different strains, the highest yielding strain, BNX122, was
15 selected for subsequent experiments. In comparison with strain BNX000, this strain is able to produce 21.4%
16 more GlcNAc (18.7 ± 0.45 g/L), and the yield of GlcNAc on glucose and xylose improved by 88.5% (0.44 ± 0.023
17 g/g). In addition, the biomass and by-product acetoin of this strain were reduced by 29.6% and 57.2%,
18 respectively, suggesting that more carbon flux was directed into the GlcNAc synthesis pathway by using this
19 sgRNA combination. As expected, the repressive strength of this combination for *zwf*, *pkfA*, and *glmM* were in
20 high, middle, and middle levels, respectively (Fig. S1).

21 22 3.4 Dynamic coordination of glucose and xylose metabolism for GlcNAc production

23 In this study, xylose had two functions. On the one hand, it was used to induce the expression of dCas9
24 protein, which can weaken the three major competitive pathways of GlcNAc synthesis and promote the
25 efficient conversion of glucose and xylose into GlcNAc. On the other hand, xylose can be absorbed and utilized

1 by the cell together with glucose, which alleviated the adverse effects on cell growth caused by the weakened
2 glucose catabolism. Therefore, the effects of the time of addition and amounts of xylose on strain BNX122 were
3 investigated to dynamically coordinate the glucose and xylose metabolism for GlcNAc production.

4 In order to explore the effects of xylose addition time, 15 g/L xylose was added at different time points
5 after inoculation, and time courses of sugar utilization, cell growth, and GlcNAc synthesis were obtained (Fig.
6 S2). As shown in Fig. 5A, GlcNAc production reached 17.4 ± 0.50 g/L, 17.9 ± 0.36 g/L, 20.5 ± 0.85 g/L, $20.4 \pm$
7 0.88 g/L, and 17.1 ± 0.10 g/L, when xylose was added at 0 h, 3 h, 6 h, 9 h, and 12 h after inoculation, respectively,
8 with all cases producing higher titers than that of the control strain. When xylose was added at 6 h, GlcNAc
9 titers (20.5 ± 0.85 g/L) and its yields on glucose and xylose (0.46 ± 0.010 g/g) were both higher than for other
10 induction times. Moreover, the biomass (7.2 ± 0.30 g/L) and the by-product acetoin (2.4 ± 0.25 g/L) were both
11 lower than at other times. This suggests that by adding xylose later (9 h and 12 h) more glucose is used for
12 growth and by-product formation. This phenomenon was reported in a study conducted by Soma et.al, in which
13 they used a metabolic toggle switch to redirect metabolic flux from a central metabolic pathway toward a
14 synthetic pathway (Soma et al., 2014).

15 In order to further optimize the efficient co-utilization of glucose and xylose, the effects of the addition of
16 different xylose amounts were investigated by adding 0 g/L, 5 g/L, 10 g/L, 15 g/L, 20 g/L, 25 g/L, and 30 g/L
17 xylose at 6 h after inoculation. As shown in Fig. 5B, with increasing xylose concentration, the consumption of
18 glucose gradually decreased, and accordingly, the consumption of xylose increased. Due to the known leaky
19 expression of P_{xyIA} (Liu et al., 2014a), even without adding xylose, GlcNAc titer and yield on glucose were both
20 slightly increased compared with the control strain, while biomass and by-product acetoin showed little change.
21 The highest GlcNAc titer was achieved by adding 15 g/L xylose, indicating that an appropriate glucose and
22 xylose ratio should be ensured to balance intracellular metabolism and coordinate glucose and xylose
23 metabolism for GlcNAc production. Interestingly, about 30 g/L glucose was consumed when 15 g/L xylose was
24 added to the broth. This proportion was similar to that of lignocellulosic biomass taking up 60–70 and 30–40%
25 of their hydrolysates (Kwak et al., 2017).

3.5 RT-qPCR analysis of key genes in glucose and xylose metabolism

As mentioned above, when xylose was added at 0 h, 6 h, and 12 h after inoculation, the cells showed significant differences in their capacity to utilize xylose, with xylose consumptions at 1.29, 2.17, and 1.33 times than that of the control strain, respectively. As shown in Fig. 6, *B. subtilis* lacks a xylose-specific transporter, so xylose is imported into the cell by an arabinose transporter, AraE, regulated by the repressor AraR and the catabolite control protein A, CcpA (Fujita, 2009; Krispin and Allmansberger, 1998). The intracellular xylose is transformed into xylulose-5-P by xylose isomerase XylA and xylulokinase XylB, and then enters the HMP. XylA and XylB are in the same operon, which is controlled by the repressors XylR and CcpA (Kraus et al., 1994). Therefore, in the presence of glucose and xylose, the global regulatory factor CcpA regulates the expression of xylose utilization-related genes, making the cell use glucose primarily (Fujita, 2009). In this study, we found that when EMP, HMP, and PSP were weakened, xylose could be absorbed together with glucose. Hence, we speculated that the transcriptional regulation of *araE* and *xylAB* controlled by CcpA should be relieved to a certain extent. Therefore, the transcriptional levels of glucose and xylose metabolism-related genes, namely *ptsGHI*, *pfkA*, *zwf*, *araE*, *xylAB*, *tkl*, *ywjH*, *ywlF*, and *rpe* in strain BNX122 were determined at 24 h when 15 g/L xylose was added at 0 h, 6 h, and 12 h after inoculation, while strain BNX000 was induced by the same amount of xylose at 4 h after inoculation and used as a control. As shown in Fig. 6, the transcriptional levels of *ptsGHI*, *zwf*, and *pfkA*, which are related to glucose metabolism, showed significant downregulation in strain BNX122, while the transcriptional levels of *araE*, *xylAB*, and *tkl* showed significant upregulation. When xylose was added at 6 h after inoculation, *araE*, *xylAB*, and *tkl* were upregulated 13.4-fold, 294.4-fold, and 4.1-fold, respectively, which was consistent with the changes found in xylose utilization. Further investigation is needed to explore why adding xylose at this time is most favorable for the use of xylose. In addition, the changes in the expression of *pgi*, *ywlF*, and *ywjH* were all less than 2-fold, which is in accordance with a previous study showing that these genes are expressed constitutively (Ludwig, 2001).

3.6 Production of GlcNAc by BNX122 in a 3-L bioreactor

Based on the flask culture results, the recombinant strain BNX122 was used to produce GlcNAc in a 3-L fermenter via fed-batch culture, and two feeding strategies were implemented. Fig. 7A and Fig. 7B show the time courses of the GlcNAc production in pulse fed-batch culture and fed-batch culture with control of glucose concentration, respectively. In pulse fed-batch fermentation, a total of 500 mL of feeding solution was added to the fermentor during the culture period, and the maximum DCW and GlcNAc were 12.3 ± 0.49 g/L and 85.8 ± 1.58 g/L, respectively. The GlcNAc yield on glucose and xylose and productivity were 0.40 ± 0.011 g/g and 0.93 ± 0.017 g/L/h, respectively. In fed-batch cultivation with control of glucose concentration, a total of 650 mL of feeding solution was added to the fermentor during the culture period, and the maximum DCW and GlcNAc reached 13.2 ± 0.21 g/L and 103.1 ± 2.12 g/L, which were 1.07-fold and 1.2-fold of that in pulse fed-batch fermentation, respectively. The GlcNAc yield on glucose and xylose and productivity reached 0.44 ± 0.016 g/g and 1.17 ± 0.024 g/L/h, which were 1.1-fold and 1.26-fold of that in pulse fed-batch fermentation, respectively. These results demonstrate that fed-batch cultivation with control of glucose concentration was favorable for GlcNAc production by strain BNX122, and that this feeding strategy might be useful for the scale-up of GlcNAc production by this strain. To examine its stability, the recombination strain BNX122 was grown for 60 generations in a series of 14-mL disposable, round-bottom culture tubes. After 60 generations, the strain presented the same productivity as the starting strain in the 3-L fermenter, suggesting that this engineered strain is extremely genetically stable and suited to large-scale production.

4. Discussion

Wild strains such as *B. subtilis* cannot use glucose and xylose at the same time due to CCR (Fujita, 2009), and much work has been carried out to resolve this issue (Wu et al., 2016). In this study, we found that repression of EMP, HMP, or PSP in *B. subtilis* by CRISPRi could promote the synergic utilization of glucose and xylose (Fig. 2), improving both the titer and yield of the target product GlcNAc significantly (Fig. 5). This result is consistent with previous reports stating that eliminating parts of the central carbon metabolic pathways could promote synergic utilization of a second carbon source in *E. coli* (Y. Wu et al., 2017a, 2017b).

Furthermore, the transcriptional changes of xylose metabolism-related genes *araE*, *xylAB*, and *tkt* also illustrate that CCR by glucose was relieved in the CRISPRi-regulated strain (Fig. 6B). In addition, this is the first report of using a mix of glucose and xylose, the two major components of lignocellulose (Ha et al., 2011; Nichols et al., 2018; Wei et al., 2013), for the production of GlcNAc.

In the recombinant strain BNY, EMP, HMP, and PSP compete with GlcNAc synthesis for the same precursors, such as glucose-6-P, fructose-6-P, and glucosamine-6-P, which strongly limits GlcNAc titer and yield (Fig. 2A). Because EMP, HMP, and PSP are all necessary for normal cell growth and cannot be knocked-out directly, a synthetic sRNA engineering strategy and initiation codon-optimization strategy were used in our previous studies to weaken these competing pathways, with both methods proving to be effective for the improvement of GlcNAc synthesis (Gu et al., 2017; Liu et al., 2014a). However, there are some drawbacks to both methods. The plasmid-based sRNA engineering strategy possessed lower stability compared with our CRISPRi system which was integrated into the genome, limiting its application in large-scale production. As for the initiation codon-optimization strategy, its regulatory strength cannot be controlled and modification of the initiation codons of each gene to be regulated is required. In this study, we found that improvement to the GlcNAc titer ranged from 2.4% to 21.5%, and can be acquired using different combinations of sgRNAs (Fig. 4), suggesting that tuning the strength of repression is an important factor. By using CRISPRi this can be easily controlled by changing the sgRNA targeting site (Gao et al., 2018; Lv et al., 2015; Qi et al., 2013). In addition, only one sgRNA array was enough to employ regulation of multiple genes, and the array can be easily built through multi-fragment assembly strategies, such as Golden Gate in this case (Fig. 3).

Since it was introduced in 2013, the CRISPRi system has been successfully developed and applied in many organisms, including *E. coli*, *Corynebacterium glutamicum*, *B. subtilis*, and other (Mougiakos et al., 2018; Peters et al., 2016; Westbrook et al., 2016). Unlike the CRISPRi systems previously constructed in *B. subtilis* (Peters et al., 2016; Westbrook et al., 2016), we have introduced lox71 and lox66 sites into the CRISPRi plasmids (Fig. 1A), which can remove the resistance genes used for screening by Cre/lox-based site-specific recombination, avoiding influence on the subsequent genetic manipulation. In this study, only sgRNAs targeting the non-

template strand of *zwf*, *pfkA*, and *glmM* were designed and assembled together. In future studies, sgRNAs targeting the template strand and the promoter region could also be considered and more sgRNAs could be assembled together using the Golden Gate method for the construction of a larger regulation libraries of multiple genes. Moreover, a biosensor-based directed evolution method may be used to screen for desired mutants, by coupling growth-related phenotypes with production synthesis (P. Yang et al., 2017). In summary, the method employed here is simple, efficient, and universal, because only a dCas9 protein and three sgRNAs need to be introduced into the cell without genetic modification of any gene involved in glucose and xylose metabolism, and because it can be applied to the construction of other robust microbial cell factories, enabling multiple carbon sources to be utilized simultaneously.

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- 12

Table 1 Strains used in this study

Strain	Characteristics
<i>Escherichia coli</i> JM109	<i>recA1, endA1, thi, gyrA96, supE44, hsdR17Δ (lac-proAB) /F'[traD36, proAB⁺, lacI^q, lacZΔ M15]</i>
BNY	<i>B. subtilis</i> 168Δ <i>nagPΔgamPΔgamAΔldhΔptaΔglcKΔpckAΔpyk::lox72, ΔnagAB::P_{veg}-yqaB, P_{veg}-glmS, pP43-GAN1</i>
BNX	BNY derivative, Δ <i>lacA::xylR-P_{xy}/A-dCas9</i>
BNX/pP43-GFP	BNX derivative, pP43-GFP
BNX000	BNX derivative, Δ <i>amyE::sg_{GFP}</i>
BNX000/pP43-GFP	BNX000 derivative, pP43-GFP
BNX100	BNX derivative, Δ <i>amyE::sg_{zwf1}</i>
BNX010	BNX derivative, Δ <i>amyE::sg_{pfkA1}</i>
BNX001	BNX derivative, Δ <i>amyE::sg_{glmM1}</i>
BNX110	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA1}</i>
BNX111	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA1}-sg_{glmM1}</i>
BNX121	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA2}-sg_{glmM1}</i>
BNX131	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA3}-sg_{glmM1}</i>
BNX211	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA1}-sg_{glmM1}</i>
BNX221	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA2}-sg_{glmM1}</i>
BNX231	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA3}-sg_{glmM1}</i>
BNX311	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA1}-sg_{glmM1}</i>
BNX321	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA2}-sg_{glmM1}</i>
BNX331	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA3}-sg_{glmM1}</i>
BNX112	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA1}-sg_{glmM2}</i>
BNX122	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA2}-sg_{glmM2}</i>
BNX132	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA3}-sg_{glmM2}</i>
BNX212	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA1}-sg_{glmM2}</i>
BNX222	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA2}-sg_{glmM2}</i>
BNX232	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA3}-sg_{glmM2}</i>
BNX312	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA1}-sg_{glmM2}</i>
BNX322	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA2}-sg_{glmM2}</i>
BNX332	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA3}-sg_{glmM2}</i>
BNX113	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA1}-sg_{glmM3}</i>
BNX123	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA2}-sg_{glmM3}</i>
BNX133	BNX derivative, Δ <i>amyE::sg_{zwf1}-sg_{pfkA3}-sg_{glmM3}</i>
BNX213	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA1}-sg_{glmM3}</i>
BNX223	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA2}-sg_{glmM3}</i>
BNX233	BNX derivative, Δ <i>amyE::sg_{zwf2}-sg_{pfkA3}-sg_{glmM3}</i>
BNX313	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA1}-sg_{glmM3}</i>
BNX323	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA2}-sg_{glmM3}</i>
BNX333	BNX derivative, Δ <i>amyE::sg_{zwf3}-sg_{pfkA3}-sg_{glmM3}</i>

Figure captions

Fig. 1. Repression of GFP reporter with CRISPRi. (A) Schematic of the two cassettes used to implement CRISPRi in *B. subtilis* and its mechanism. (B) Relative fluorescence intensity in strains BNX and BNX000 under various concentrations of inducer xylose. Numbers above each bar report the fluorescence folds change relative to the control strain. All data were the average of three independent studies with standard deviations. The * indicates $p < 0.05$.

Fig. 2. Weakening major competitive pathways on GlcNAc production in *B. subtilis* by CRISPRi. (A) The essential genes in GlcNAc synthesis pathway and three major competitive pathways. The red dashed lines indicate the CRISPRi-dependent repression. (B) Relative normalized expression of genes *zwf*, *pfkA*, and *glmM* in strains BNX111. (C) Effects of weakening major competitive pathways on GlcNAc production. HMP, pentose phosphate pathway; EMP, glycolysis pathway; PSP, peptidoglycan synthesis pathway. All data were the average of three independent studies with standard deviations. The ** and * indicate $p < 0.01$ and 0.05 relative to the control strain BNX000, respectively, except when another pair is specifically indicated.

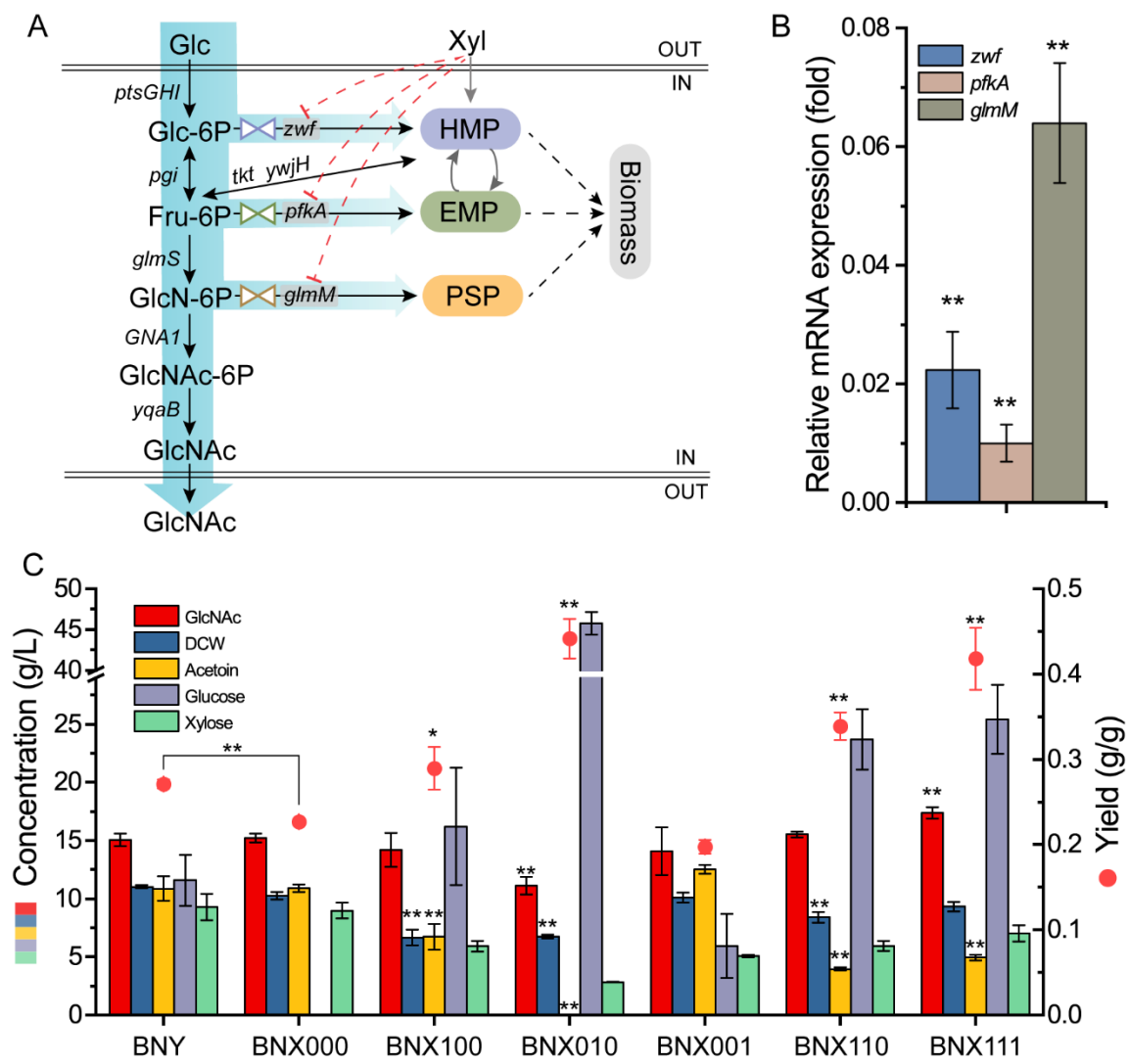
Fig. 3. Combination and optimization of sgRNAs. (A) sgRNAs chosen for the essential genes controlling three major competitive pathways on GlcNAc production. (B) Construction of sgRNA arrays by Golden Gate assembly.

Fig. 4. Coordination of the regulation of essential genes controlling three major competitive pathways on GlcNAc production. All data were the average of three independent studies with standard deviations. The * and ** indicate $p < 0.05$ and $p < 0.01$ relative to control strain BNX000, respectively.

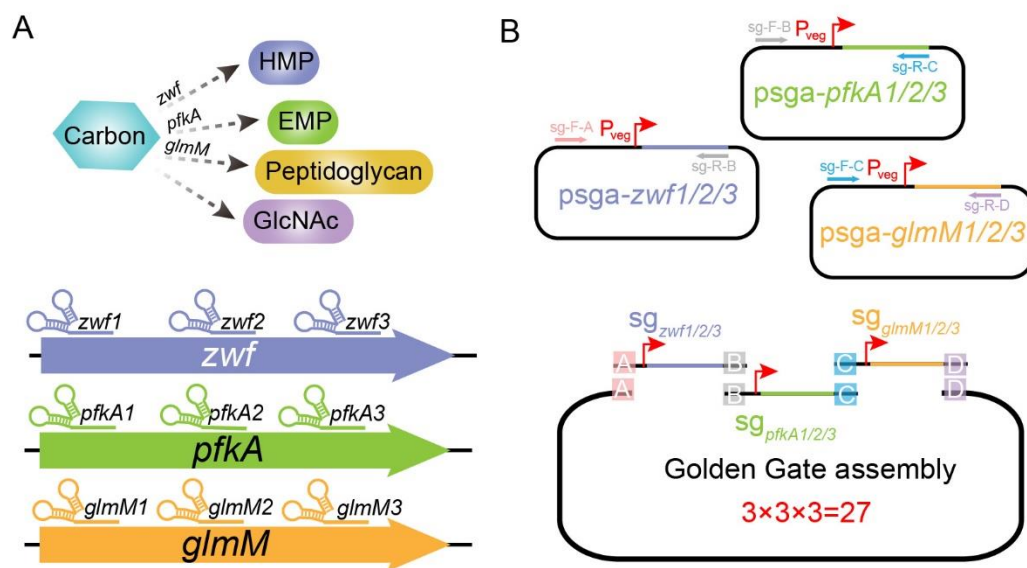
Fig. 5. Dynamic coordination of glucose and xylose metabolism for GlcNAc production. (A) Effects of addition time of xylose on GlcNAc production. (B) Effects of addition amount of xylose on GlcNAc production. The dotted lines represent the addition amount of xylose. All data were the average of three independent studies with standard deviations. The ** and * indicate $p < 0.01$ and 0.05 relative to the control strain BNX000, respectively, except when another pair is specifically indicated.

Fig. 6. RT-qPCR analysis of key genes in glucose and xylose metabolism. Each graph represents the relative normalized expression of the gene in strains BNX122 with xylose added at 0 h, 3 h, and 6 h after inoculation. All data were the average of three independent studies with standard deviations. The ** and * indicate $p < 0.01$ and 0.05 relative to the control strain BNX000, respectively. Xyl: Xylose, Xylu: Xylulose, X5P: Xylulose-5-P, G3P: Glyceraldehyde-3-P, E4P: Erythrose-4-P, S7P: Sedoheptulose-7-P, R5P: Ribose-5-P, F6P: Fructose-6-P, CcpA: catabolite control protein A.

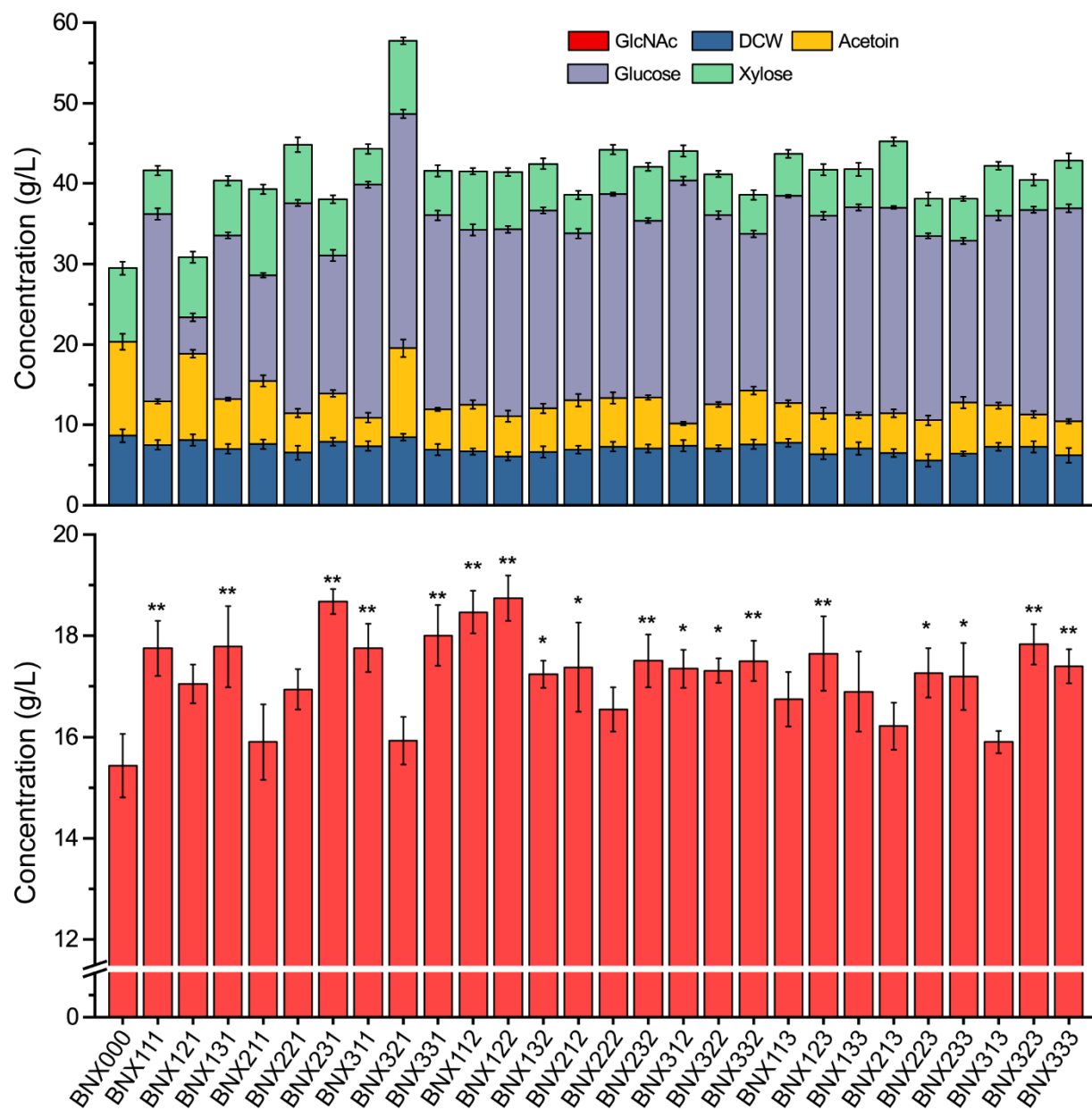
Fig. 7. Production of GlcNAc by BNX122 in a 3-L fermenter. (A) Time course of the GlcNAc production in pulse fed-batch fermentation. (B) Time course of the GlcNAc production in fed-batch cultivation with control of glucose concentration. All data were the average of three independent studies with standard deviations.



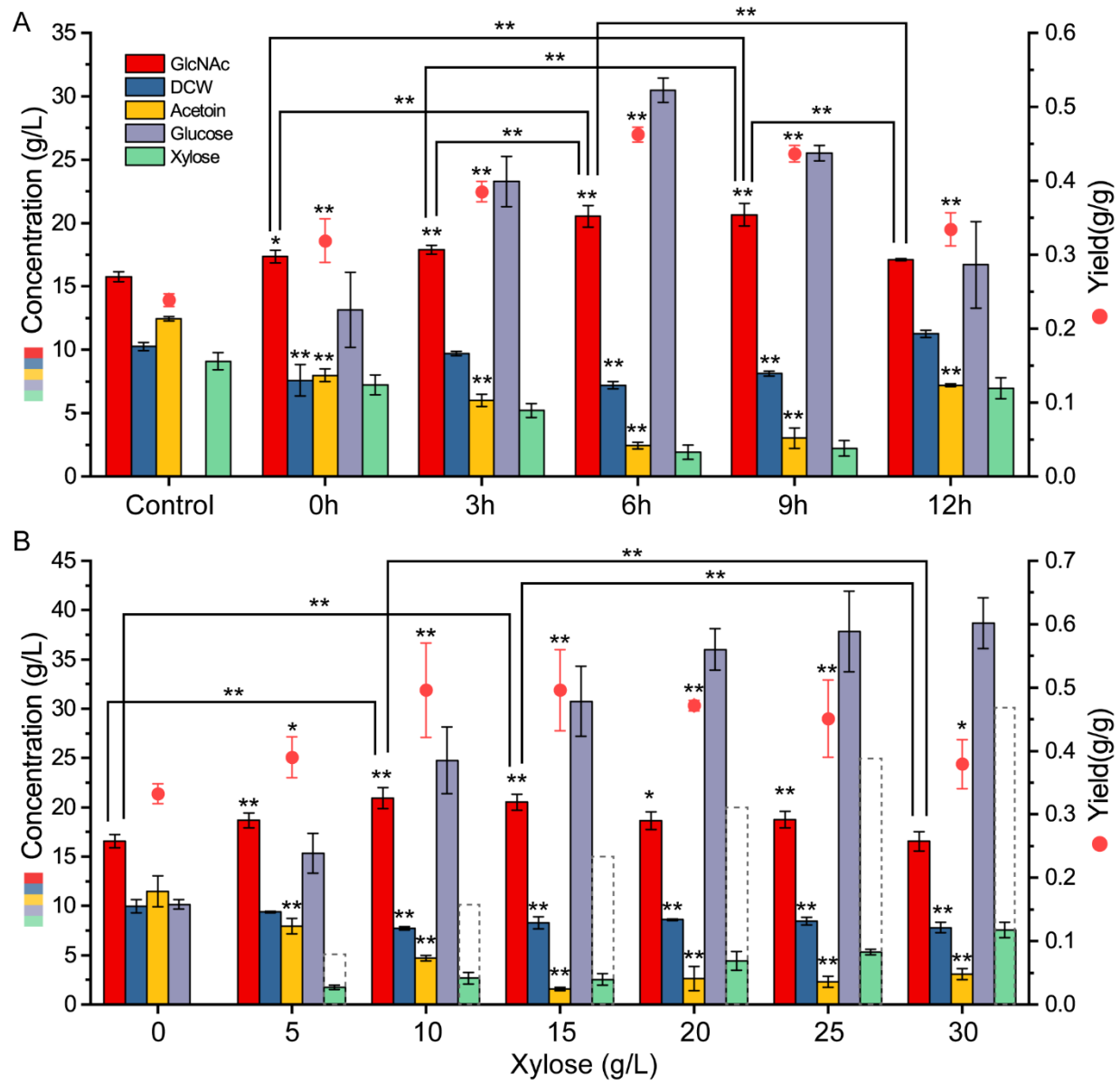
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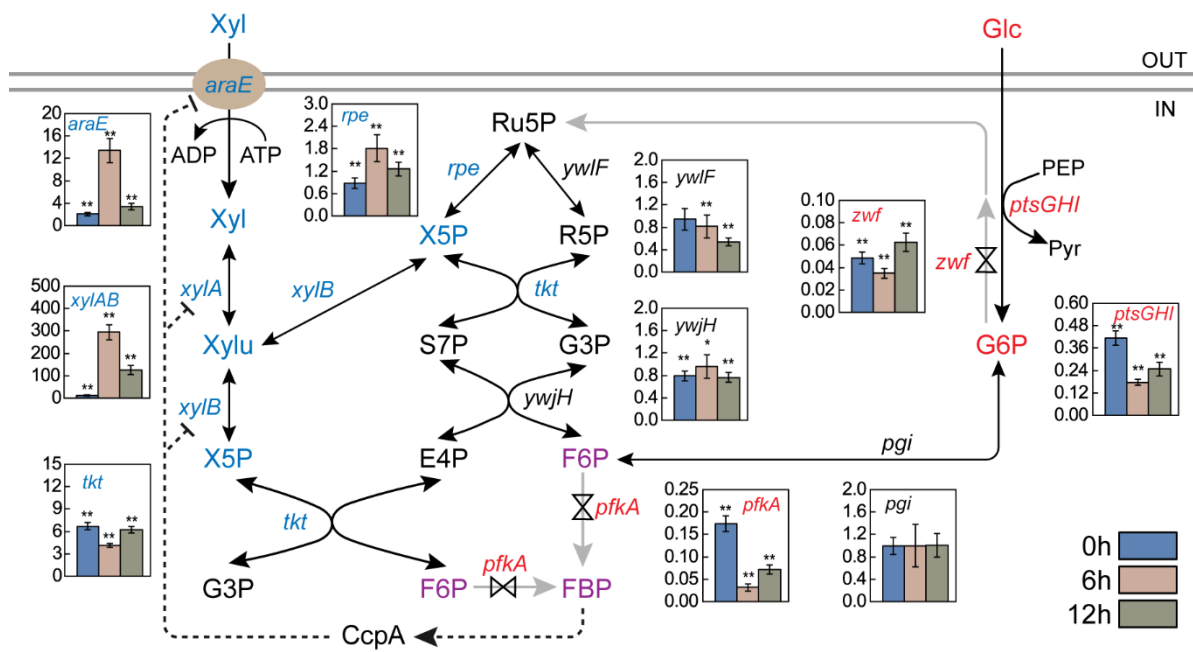
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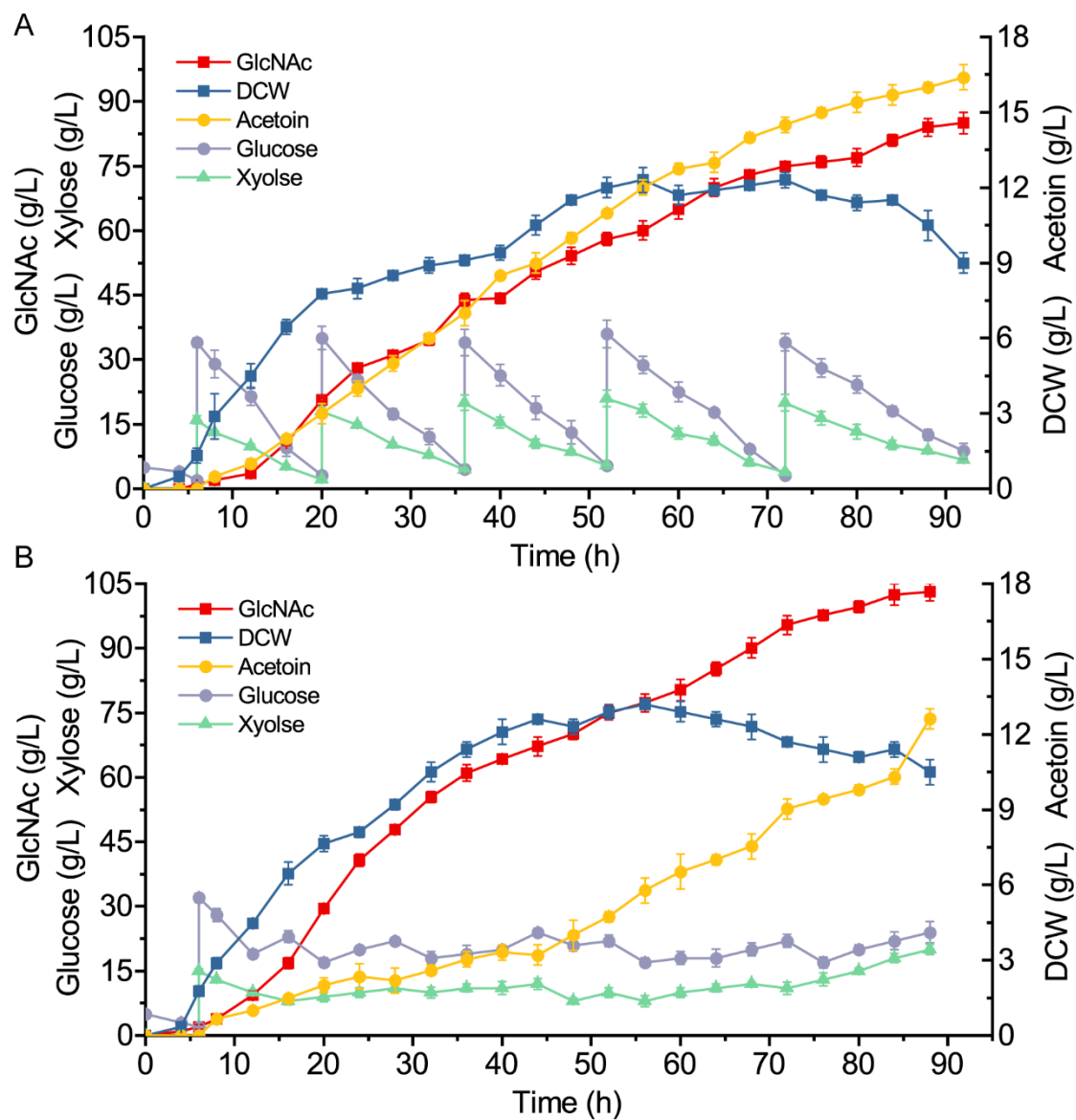
(Fig. 4)



(Fig. 5)



(Fig. 6)



(Fig. 7)