Systems metabolic engineering of *Bacillus subtilis* for efficient *N*-acetylglucosamine production

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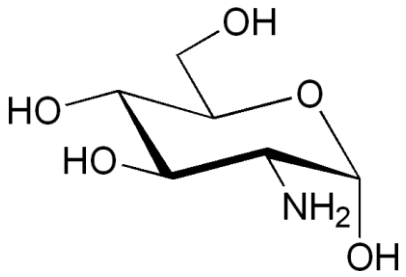
Structure and physiological function of glucosamine(GlcN)



- GlcN is derived from substitution of a hydroxyl group of glucose molecule with an amino group
- GlcN and its derivatives GlcNAc are important compounds in cartilage cells and joint tissue.
- Clinical trials with GlcN for treatment demonstrate that GlcN is necessary to repair and maintain healthy cartilage and joint function.
- The global GlcN/GlcNAc market is estimated to reach 20,000 tons in 2017 (about 5 billion USD)

Production methods of GlcN and GlcNAc

- Acidic hydrolysis of Chitin
- Enzymatic hydrolysis of Chitin
- Microbial fermentation

Acidic hydrolysis of chitin

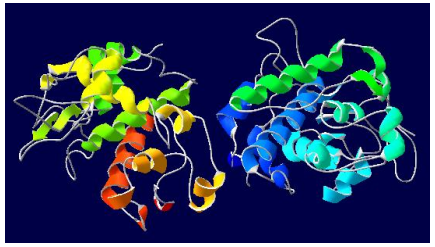
Shrimp shells

Chitin powder

Glucosamine powder

- Quantities of acid and base are needed, which cause severe pollution and are harmful for equipment
- Heavy metal pollution
- Sophisticated purification process
- Potential allergy effects

Enzymatic hydrolysis of chitin

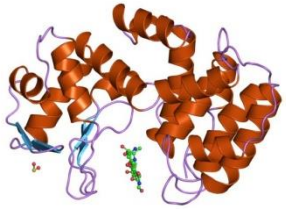


chitinase

Shrimp shells

Chitin powder

Glucosamine powder



chitosanase

- Long reaction time and low productivity
- Both of the two enzymes are needed for enzymatic hydrolysis

Microbial fermentation for GlcN/GlcNAc production

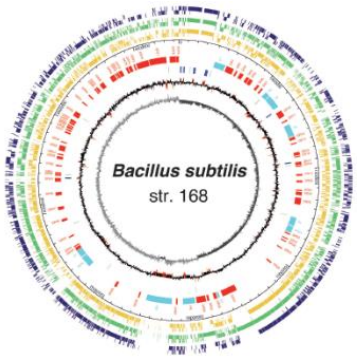
Glucosamine powder

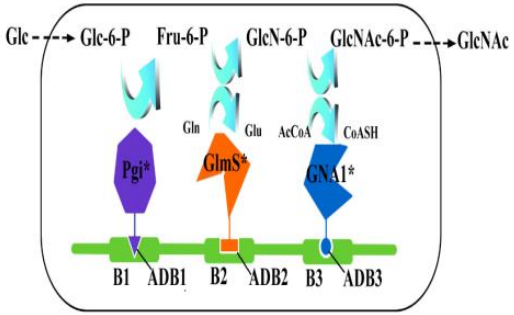
- Short fermentation period and high productivity
- No limitation by raw material supply
- Environmentally friendly
- No allergy effects

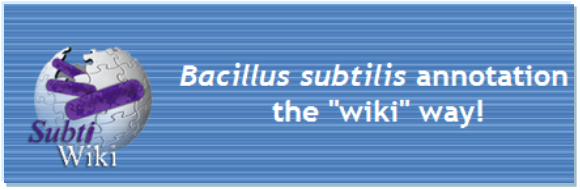
Systems engineering of *B. subtilis* for GlcNAc production

Advantages of *B. subtilis* as host

- Generally regarded as safe (GRAS)
- One of the most-well characterized gram-positive microorganisms
- There are wide arrays of tools available
- Not posing a phage infection problem during industrial production

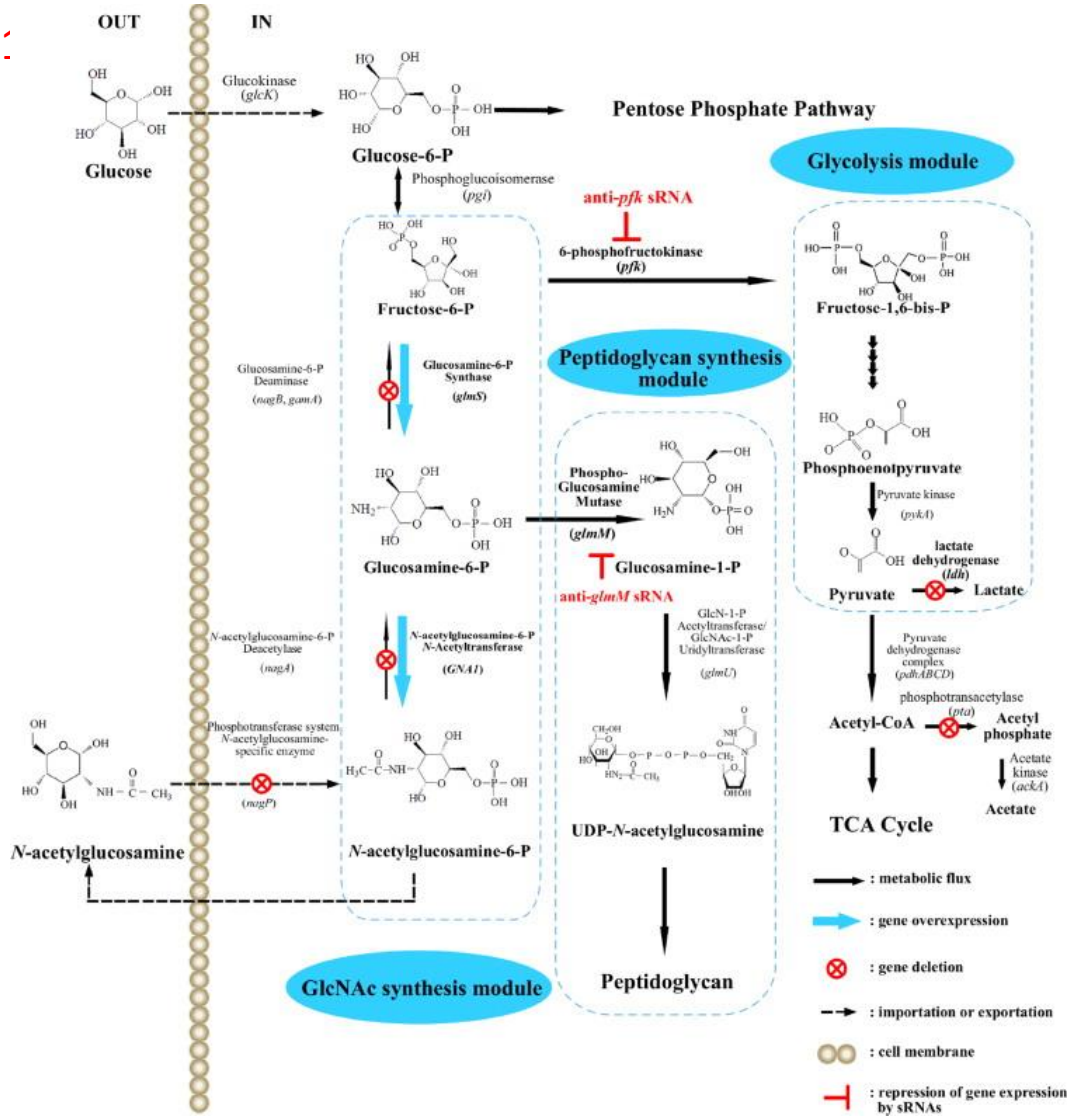




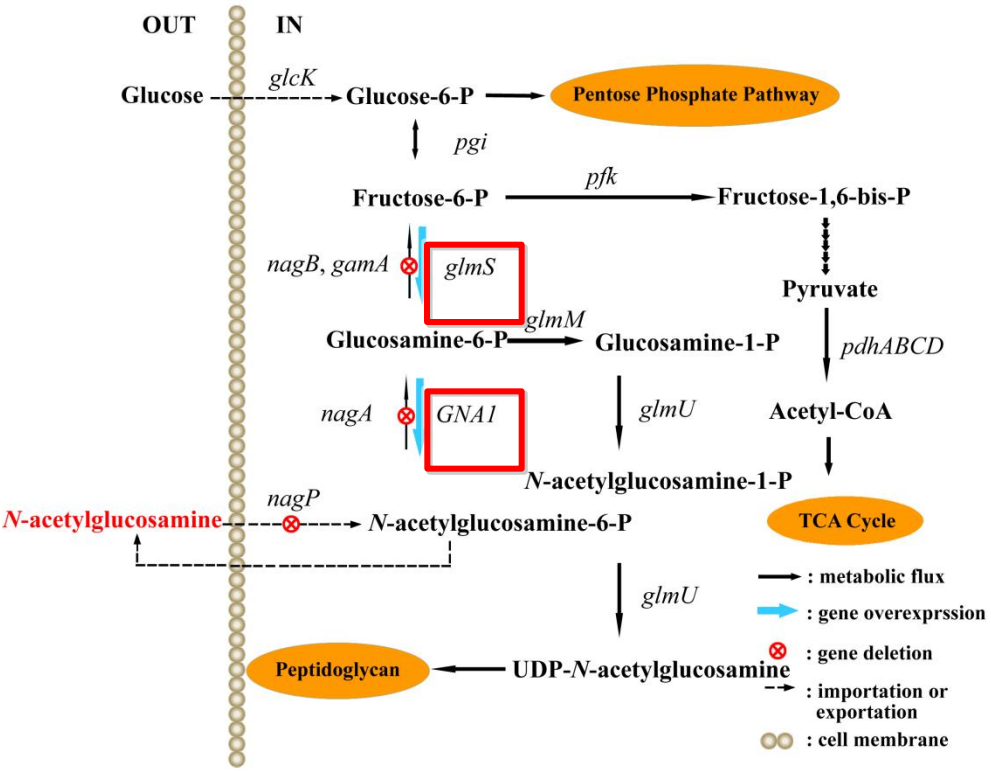


Strategies for construction of GlcNAc overproducing *B. subtilis*

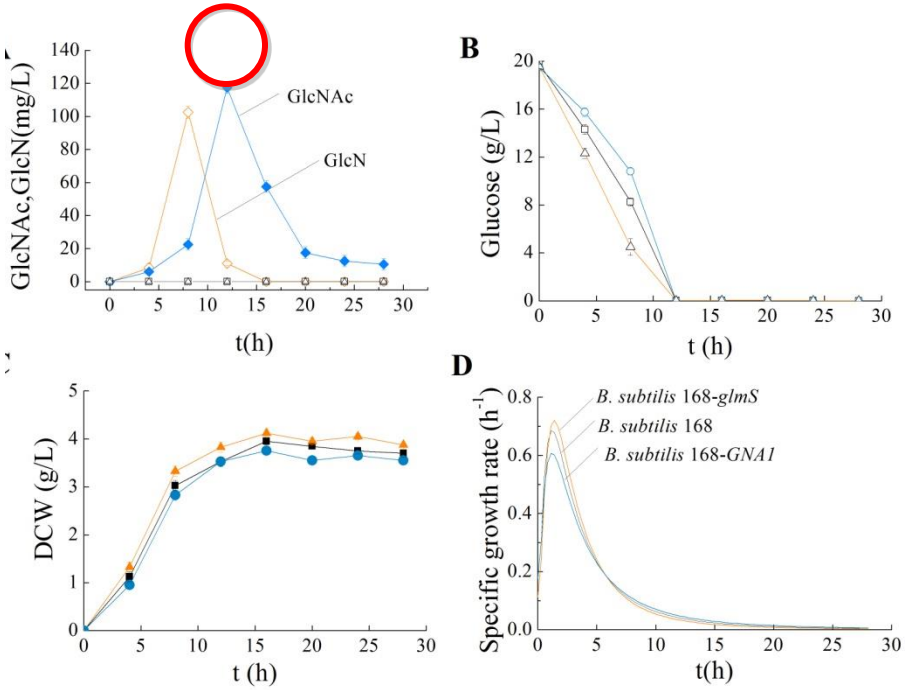
- Overexpression of GlmS and GNA1 two key enzymes
- Knockout of *nagP, nagA, nagB* and *gamA* genes
- Directed evolution of GlmS and GNA1
- Blocking acidic by-products accumulation
- Expression of anti-pfk and anti-glmM sRNAs
- Respiration chain engineering
- Flux balance analysis and by-products deletion



Engineering GlcNAc pathway—overexpression of *glmS and GNA1*

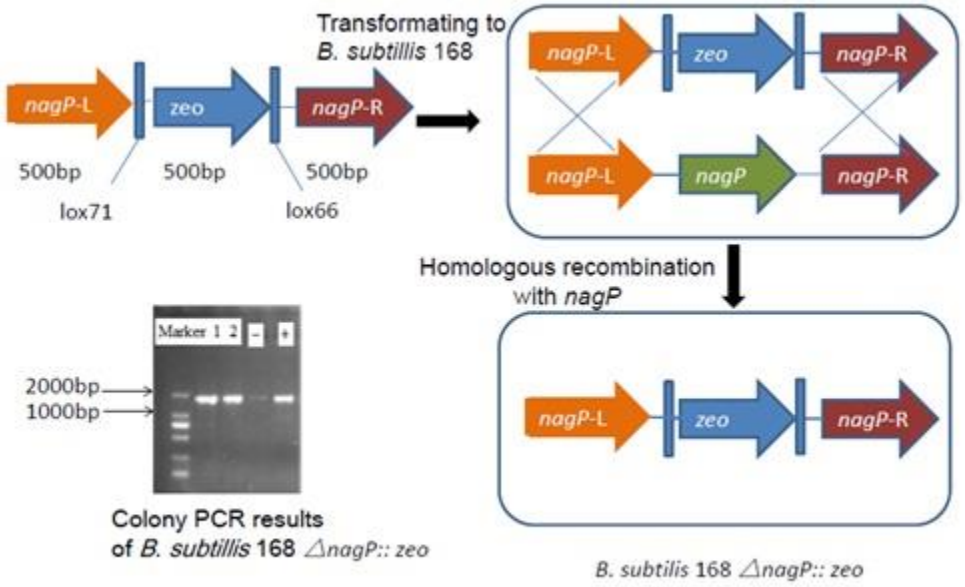


Metabolic pathway of GlcNAc

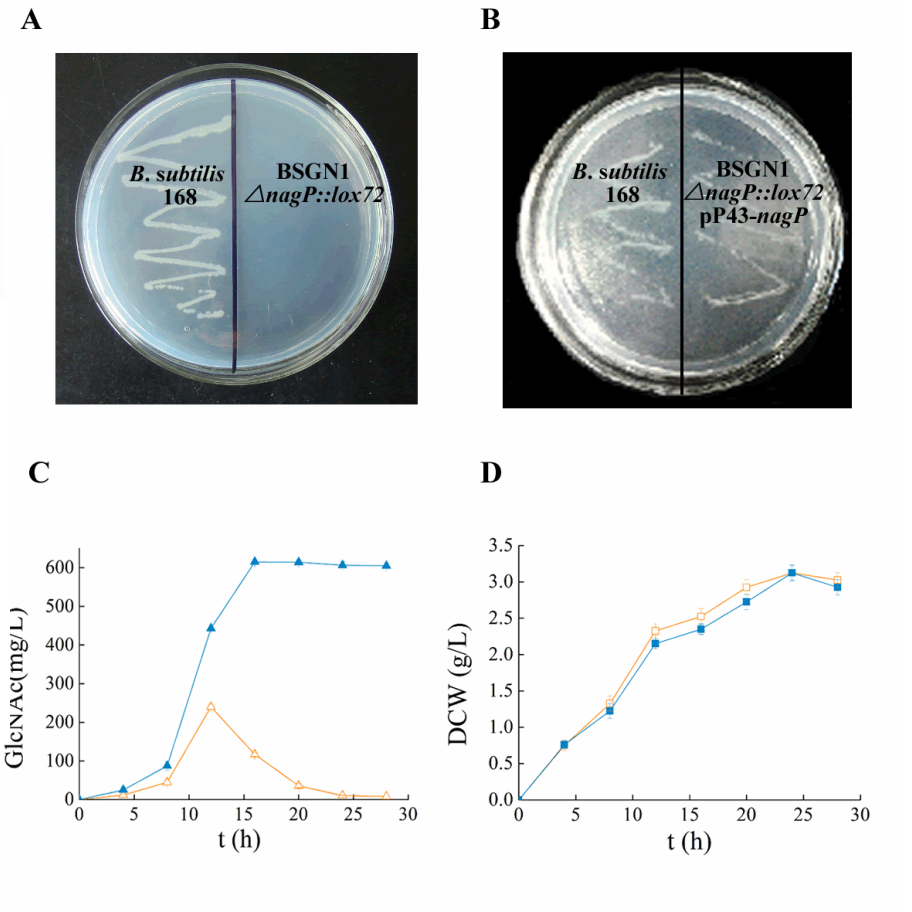


Accumulation of GlcN and GlcNAc was realized by overexpression of GlmS and GNA1 (230 mg/L), but GlcN and GlcNAc decreased due to catabolic reactions.

Engineering GlcNAc pathway—*nagP* gene knockout

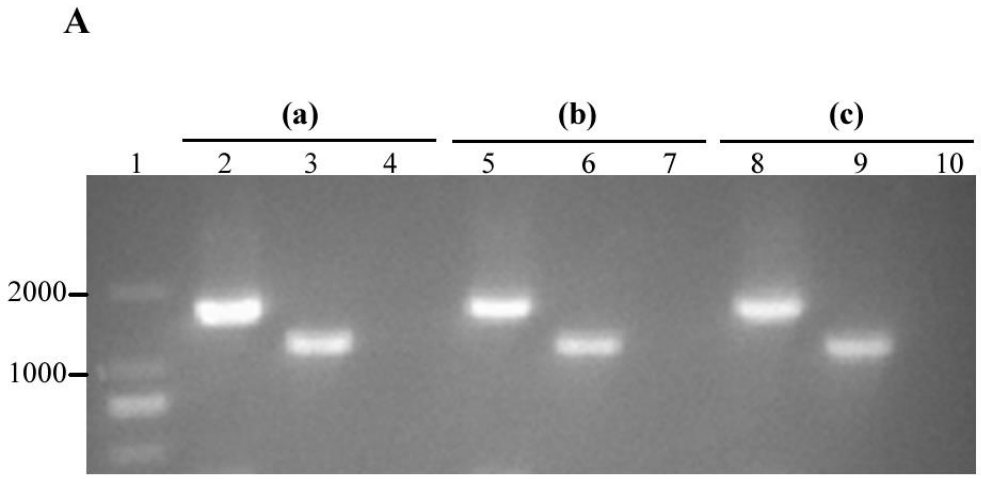


***nagP* knockout workflow**

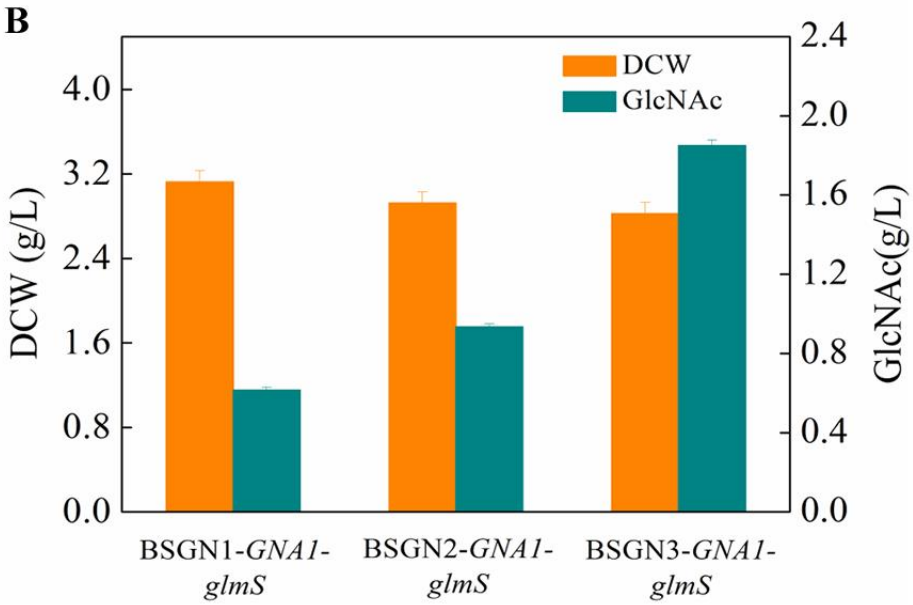


Knockout of *nagP* blocked extracellular importation, which facilitated GlcNAc accumulation, and GlcNAc titer reached 620 mg/L

Engineering GlcNAc pathway—*nagA, nagB* and *gamA* knockout

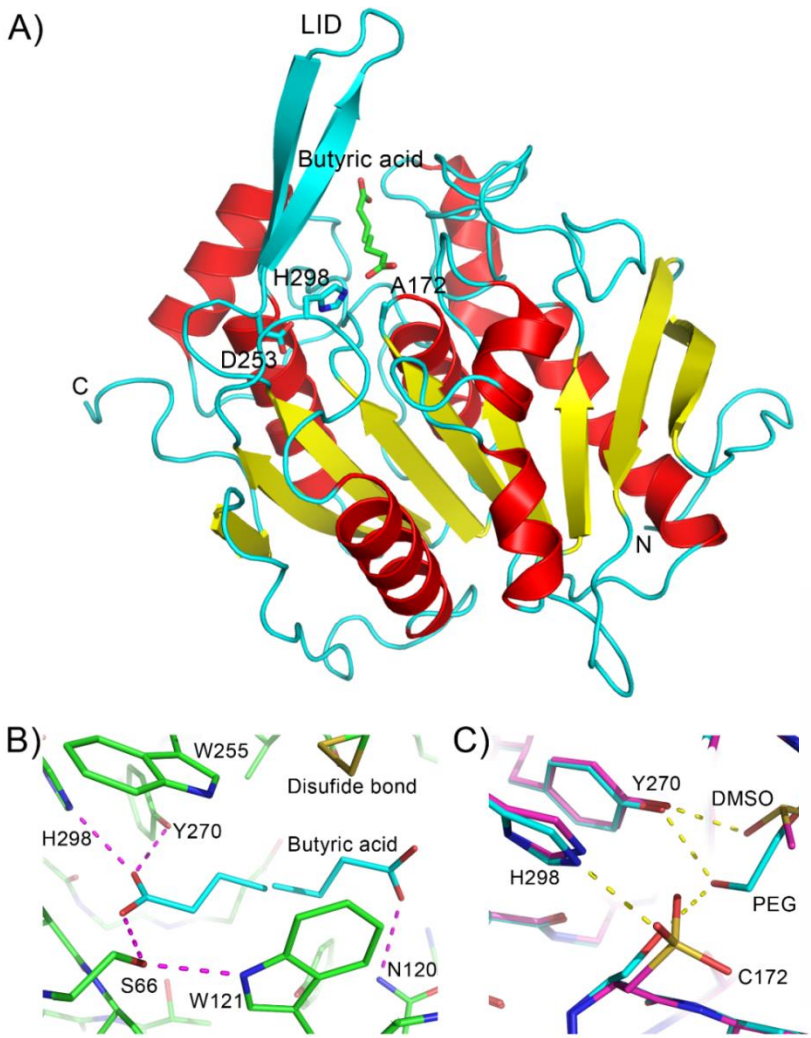


Colony PCR for verification of *nagA, nagB* and *gamA* knockout

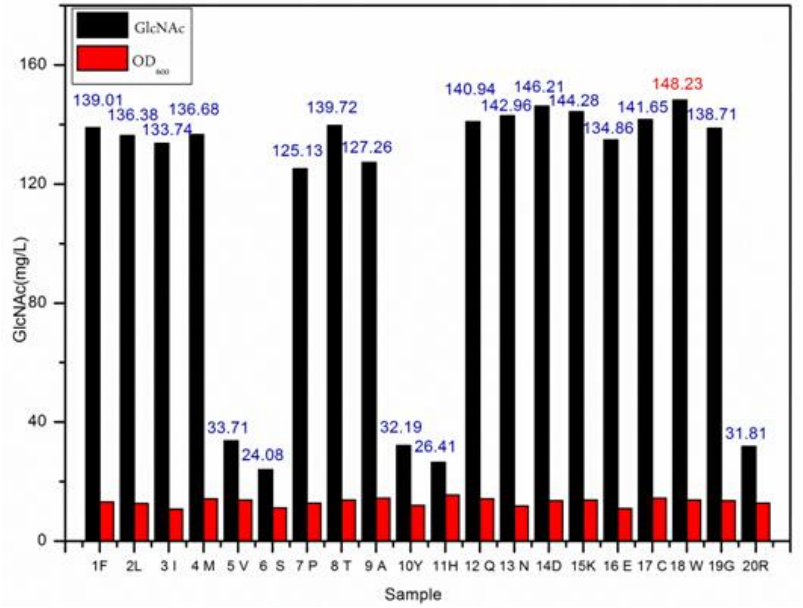


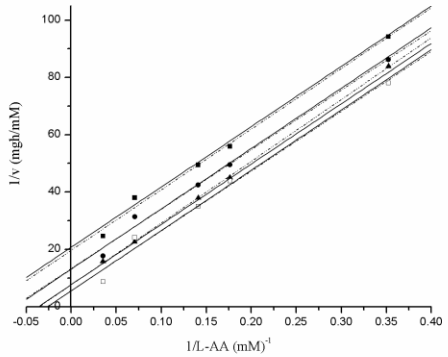
By further blocking intracellular GlcANc catabolic pathway, GlcNAc titer was increased to 1.8 g/L, which was 17-fold higher than that of control.

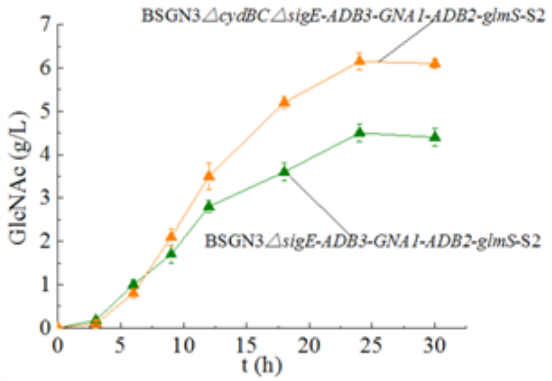
Directed evolution of GlmS for improvement of catalytic efficiency



Structure model of GlmS

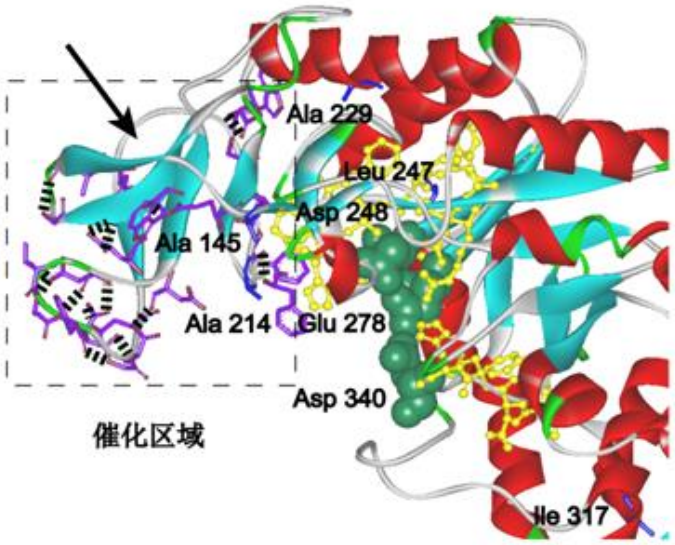




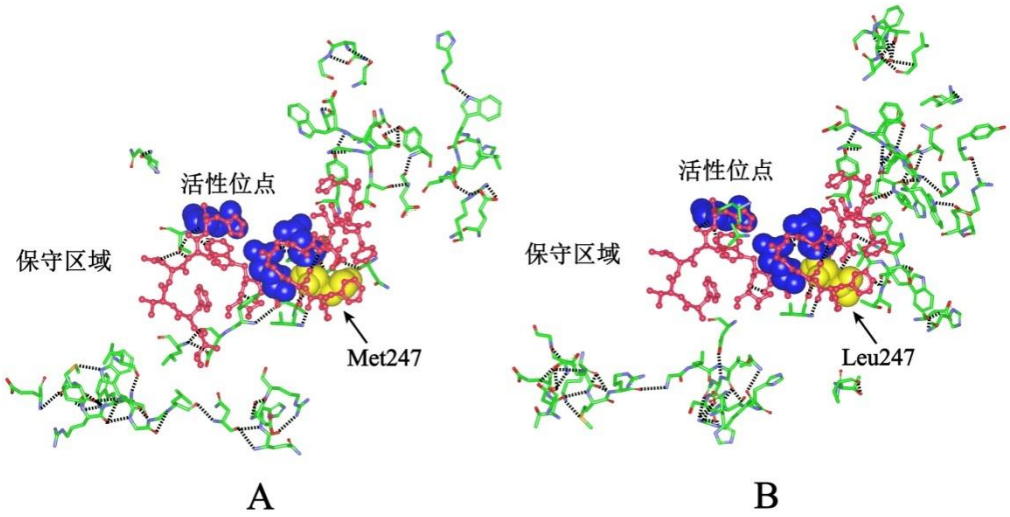


The catalytic efficiency of GlmS mutant was improved by 3-fold, resulting in the increase of GlcNAc yield

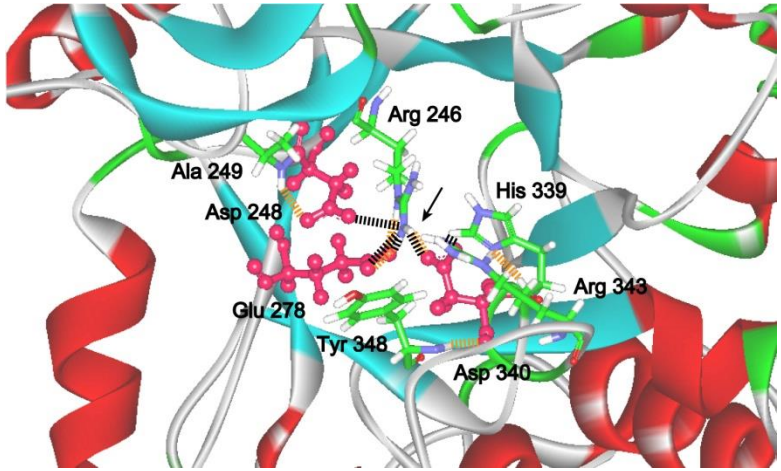
Directed evolution of GNA1 for improvement of catalytic efficiency



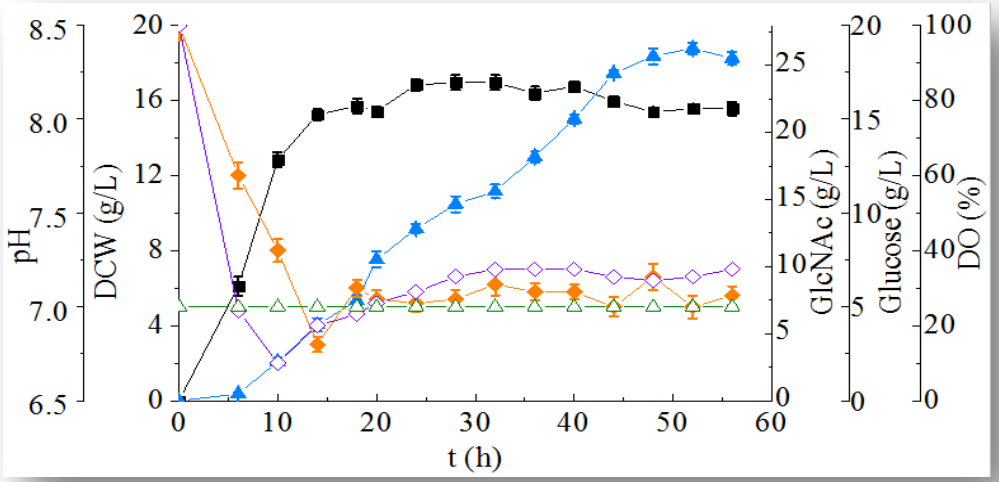
Structure model of GNA1



Comparison of hydrogen-bonding

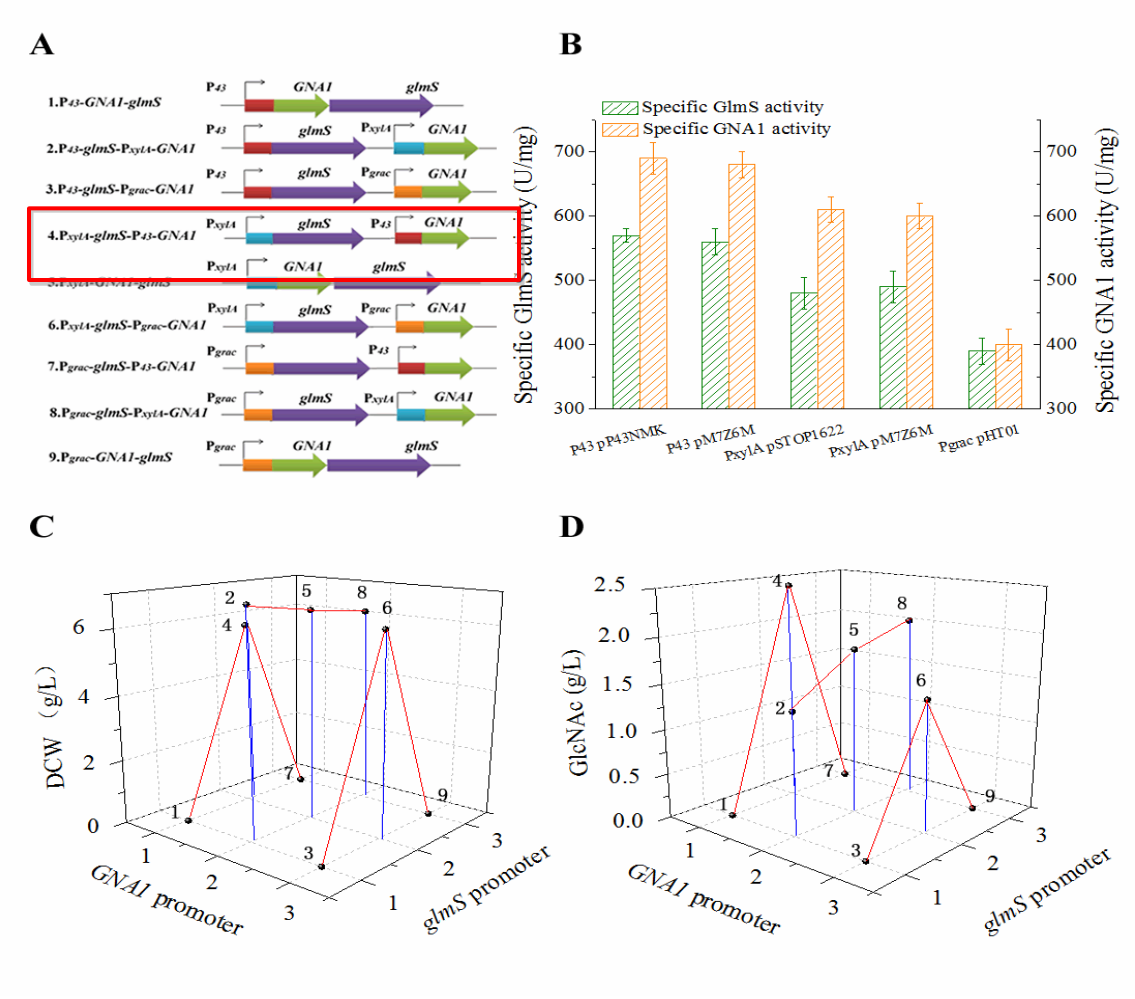


The salt bridges of GNA1 mutant



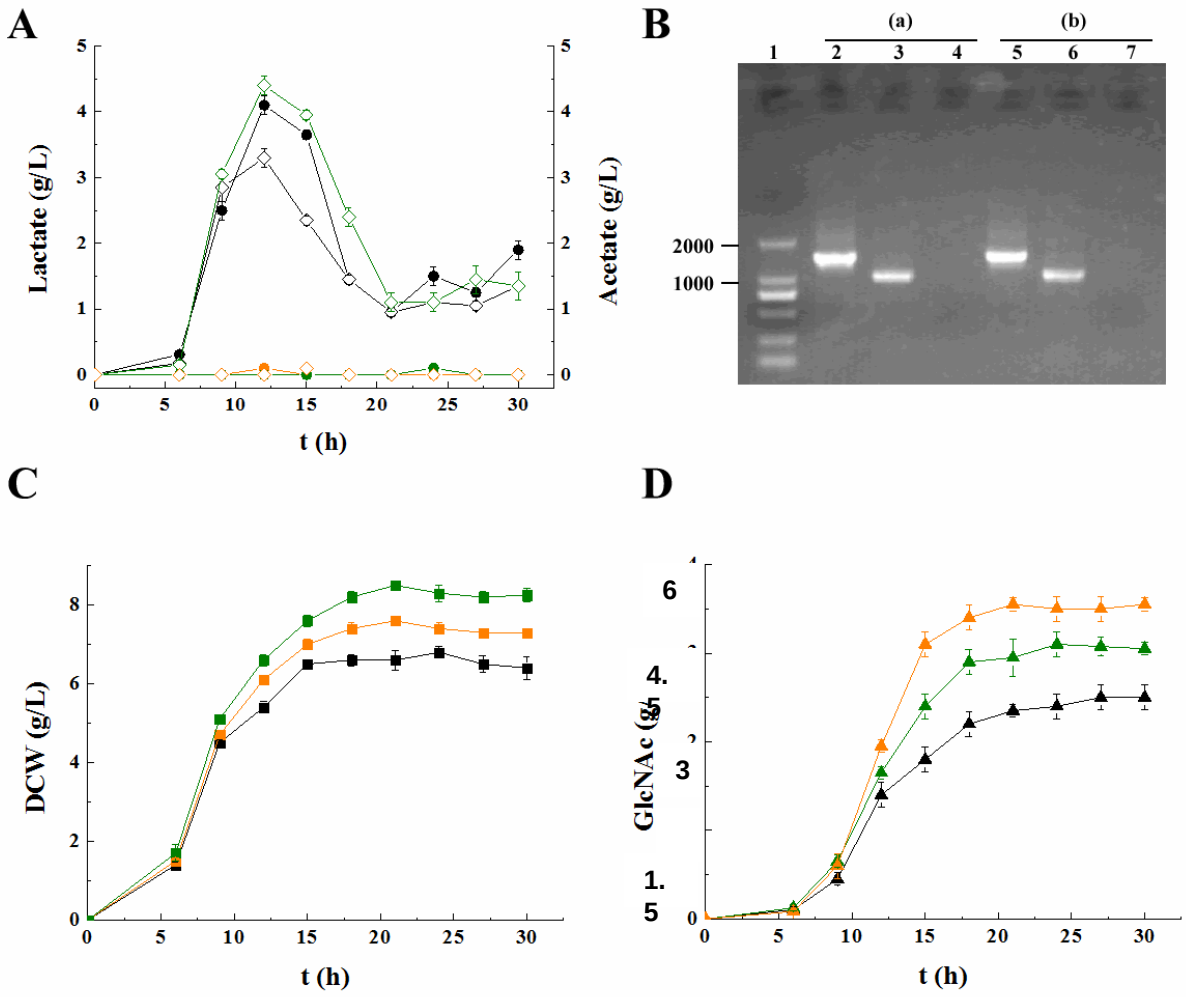
GlcNAc production process with (GlmS*GNA1*)

Engineering GlcNAc network—two promoter system for expression of GNA1 and GlmS



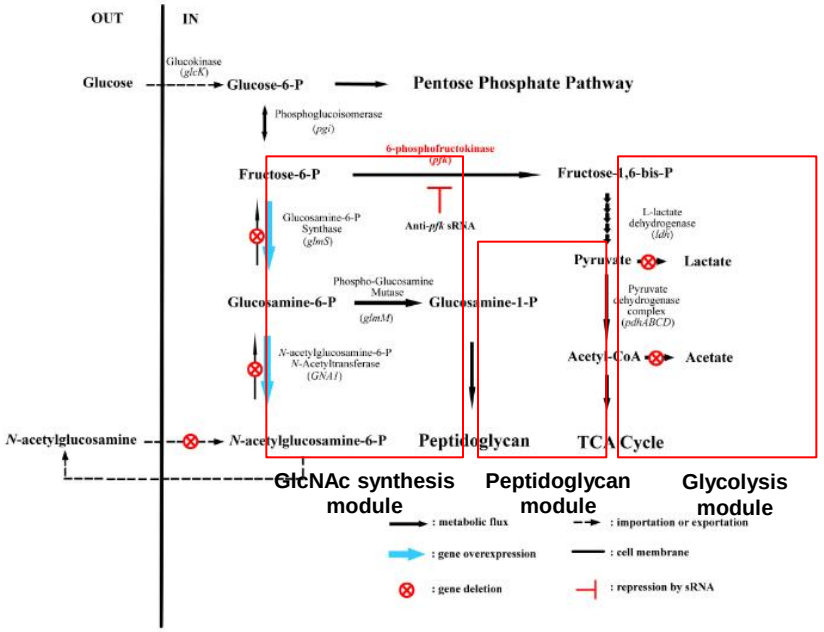
Based on two promoter system for expression of GNA1 and GlmS, GlcNAc titer reached 2.51 g/L, increased by 30%.

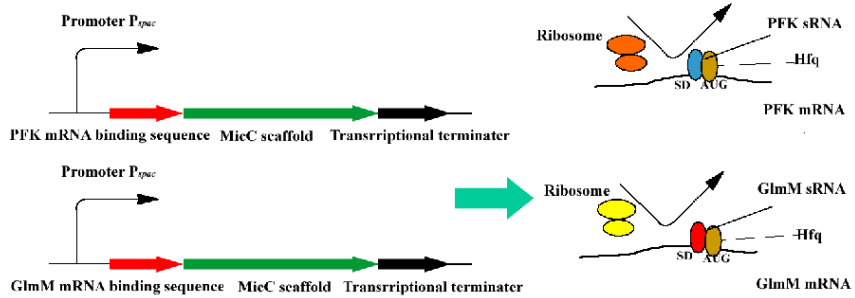
Engineering GlcNAc network—Blocking formation of lactate and acetate



After blockage of lactate and acetate formation, GlcNAc titer reached 4.5 g/L and 5.2 g/L, respectively, which was 2-fold higher compared with strain without blocking acidic by-products formation.

Engineering GlcNAc network—construction of synthetic sRNA

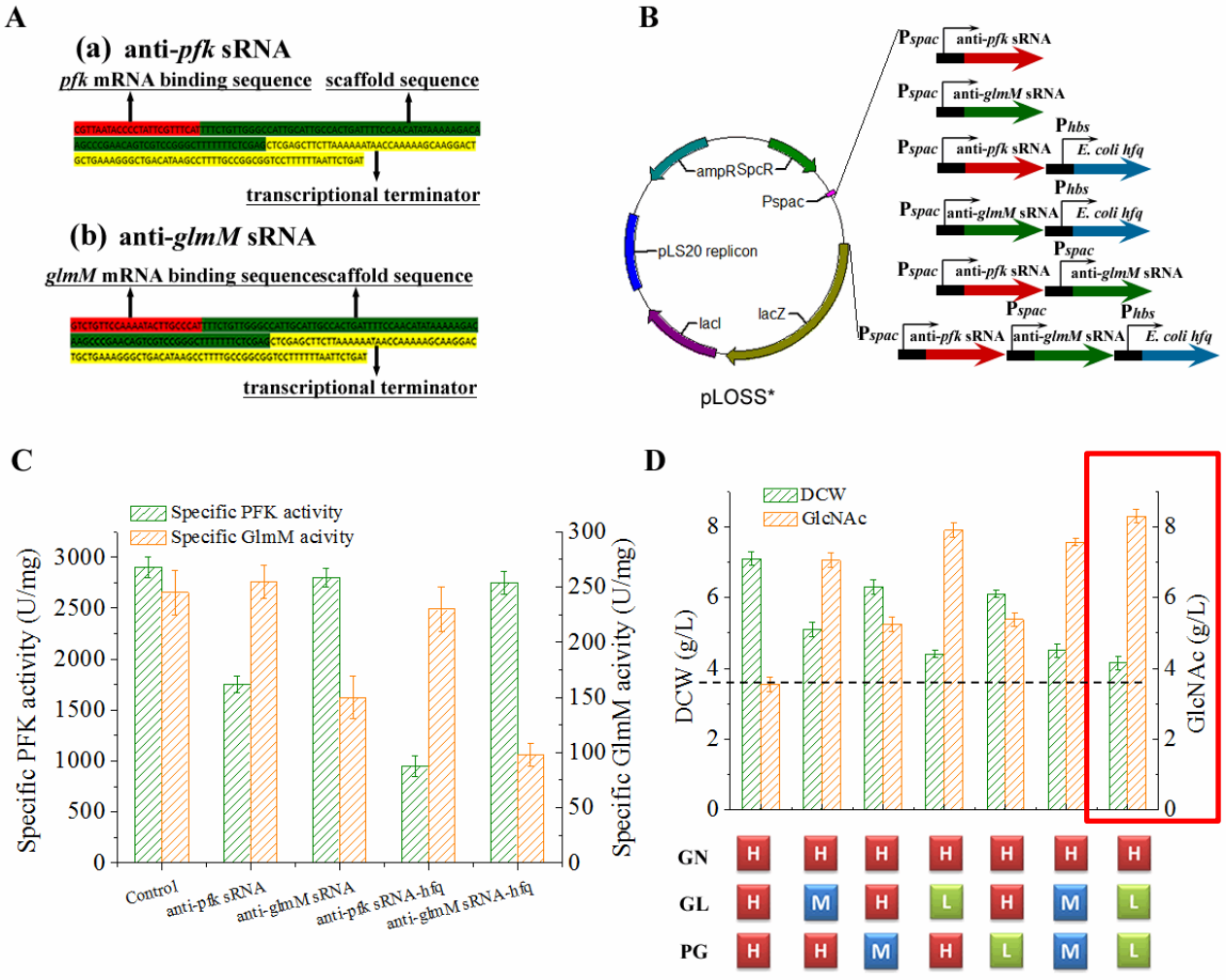




- Expressing anti-*pfk* sRNA controlled activity of glycolysis module at medium level (60%)
- Expressing anti-*glmM* sRNA controlled activity of peptidoglycan module at medium level (60%)
- Co-overexpressing anti-*pfk* sRNA, anti-*glmM* sRNA, and Hfq controlled activities of glycolysis peptidoglycan module at low level (30%)

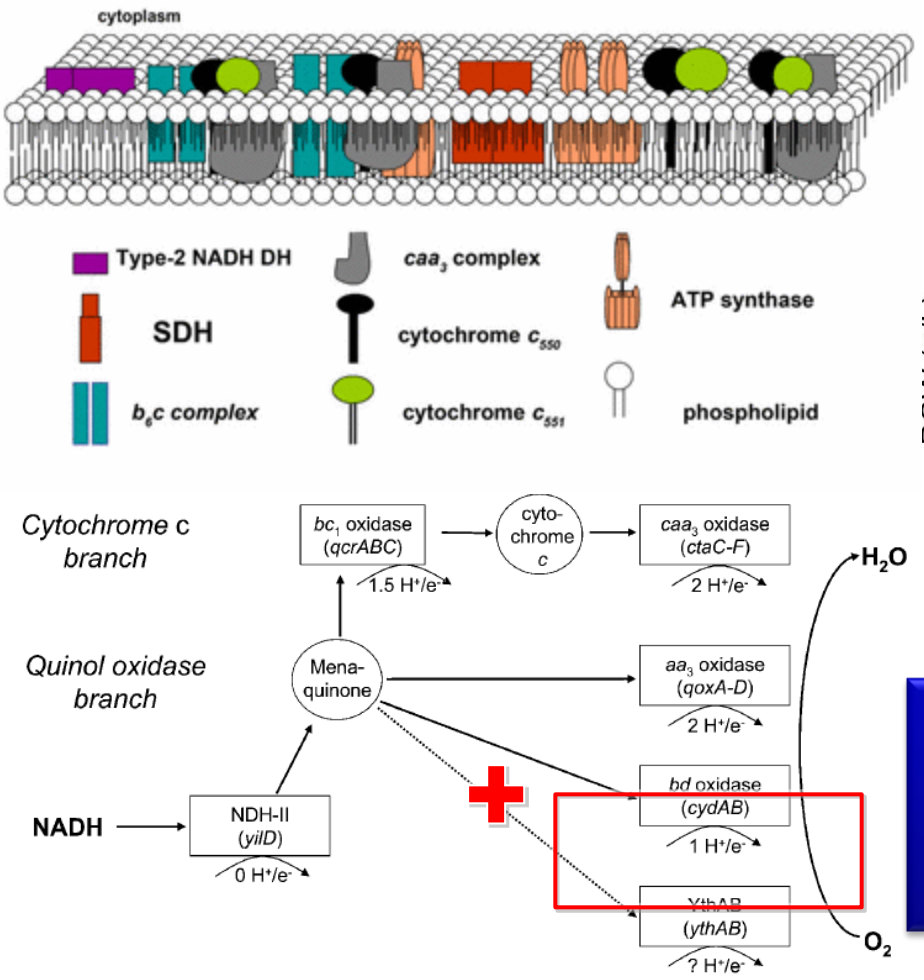
sRNA can effectively control competitive pathway of GlcNAc synthesis (glycolysis module and peptidoglycan module).

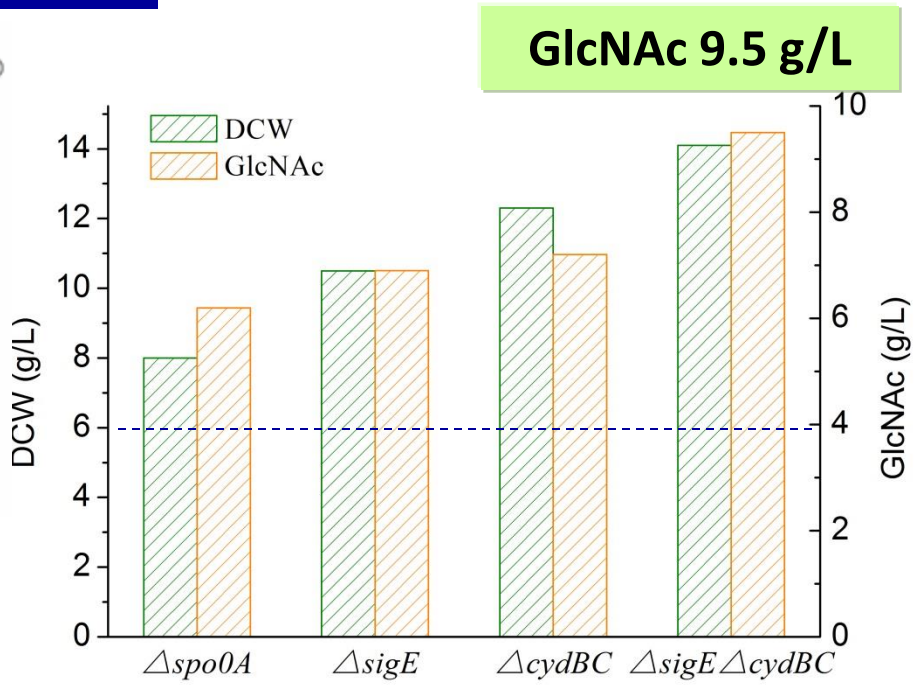
Engineering GlcNAc network—sRNA-based modular pathway engineering



GlcNAc titer reached 8.30 g/L with yield on cell 2.00 g/g DCW by assembly and optimization of various modules via a module engineering approach.

Physiology property optimization—Blocking sporulation and respiration chain engineering

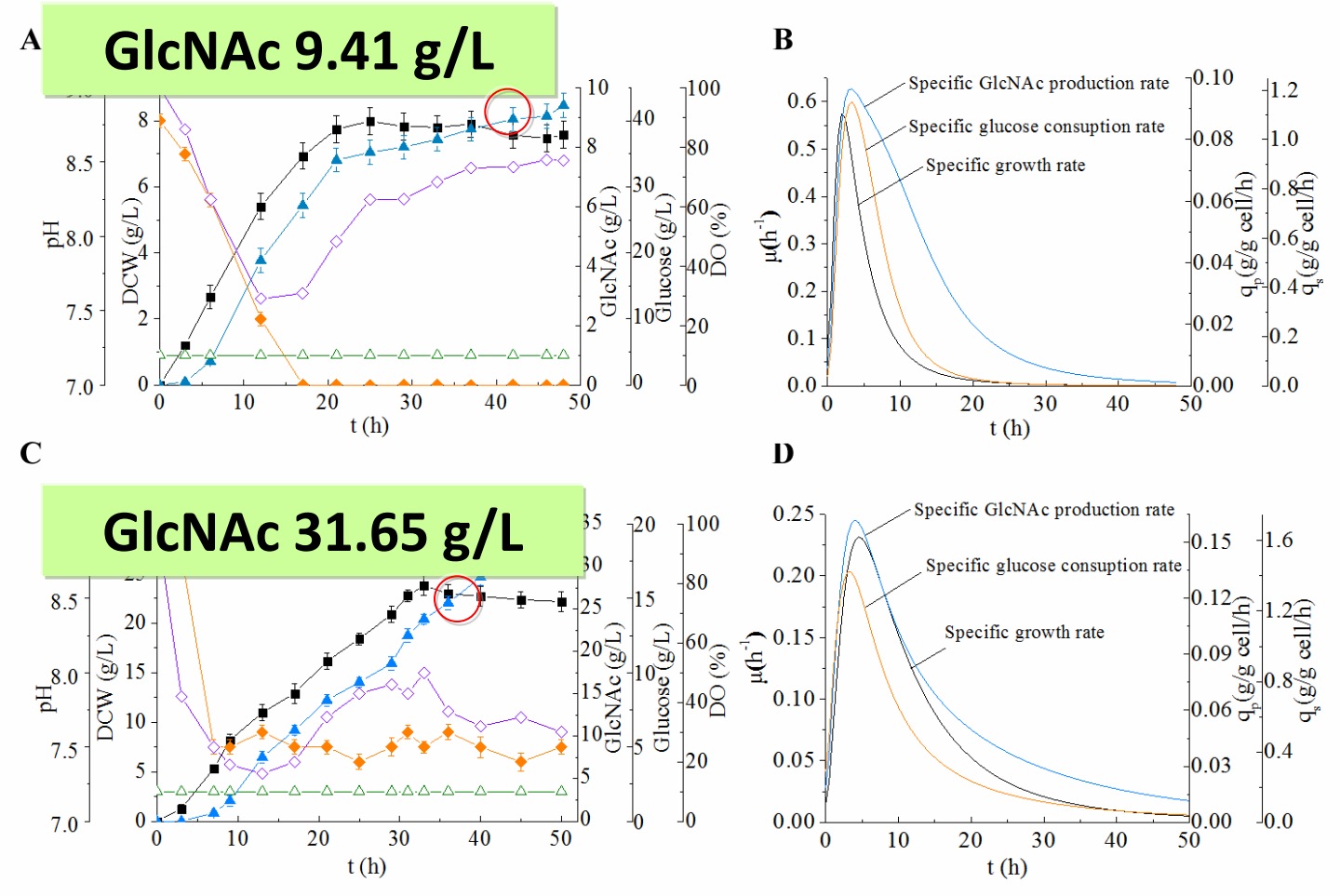




GlcNAc titer increased 10% by knockout of *sigE* gene for blocking sporulation with reduced maintenance metabolism.

Further respiration chain engineering, knockout of *cydAB* gene, blocked the inefficient respiratory chain and diverted the electron flux through a more efficient respiratory chain. GlcNAc titer was further enhanced to 9.5 g/L.

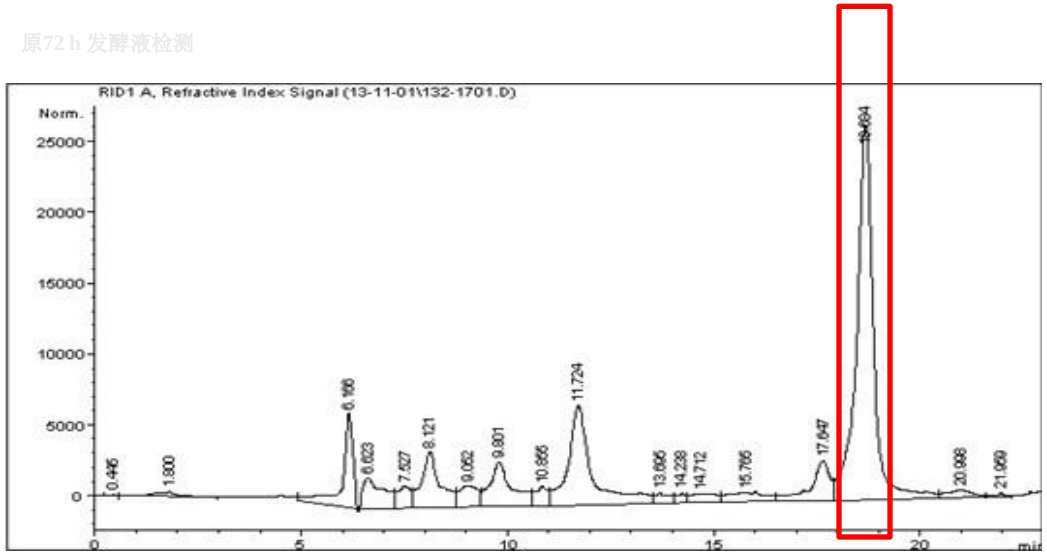
Batch and fed-batch fermentation in a 3 L bioreactor



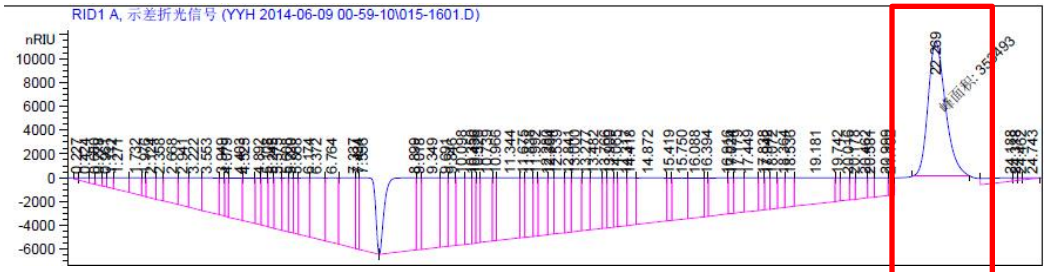
In fed-batch culture, GlcNAc titer reached 31.65 g/L with specific production rate 0.054 g/g DCW/h and productivity 0.63 g/L/h.

Blocking by-product acetoin formation

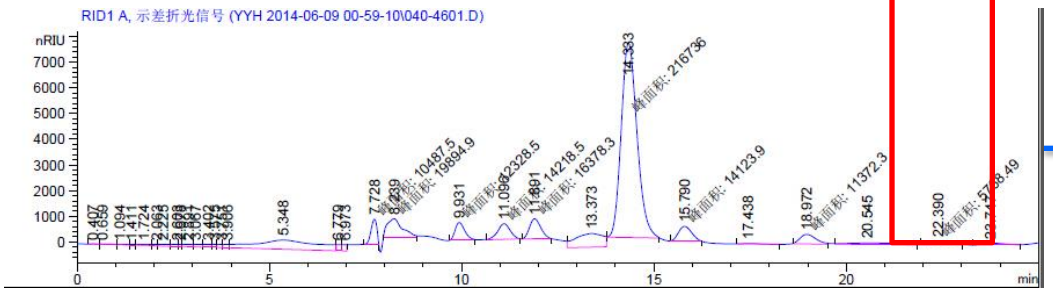
原72 h 发酵液检测

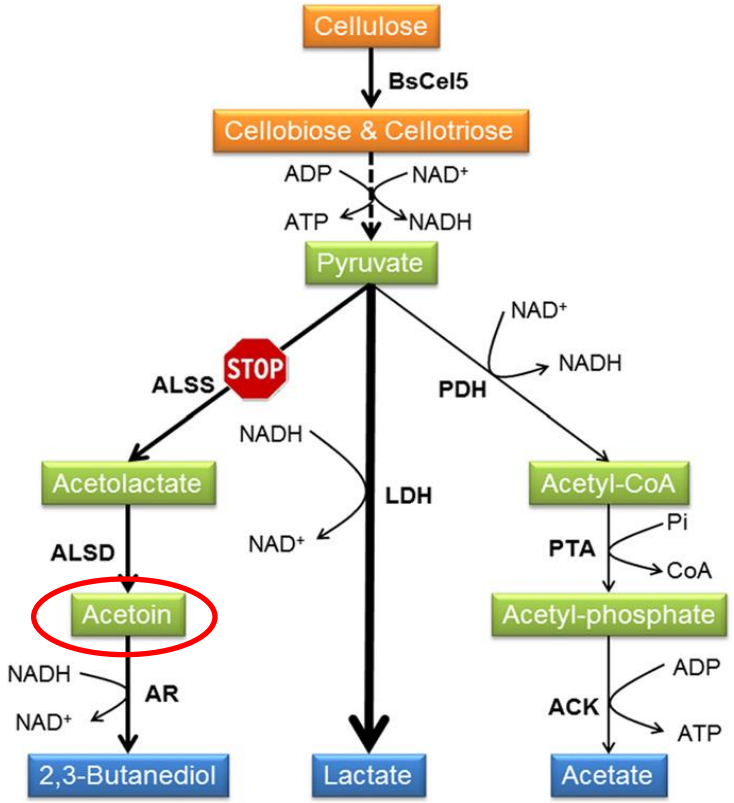


乙偶姻标样



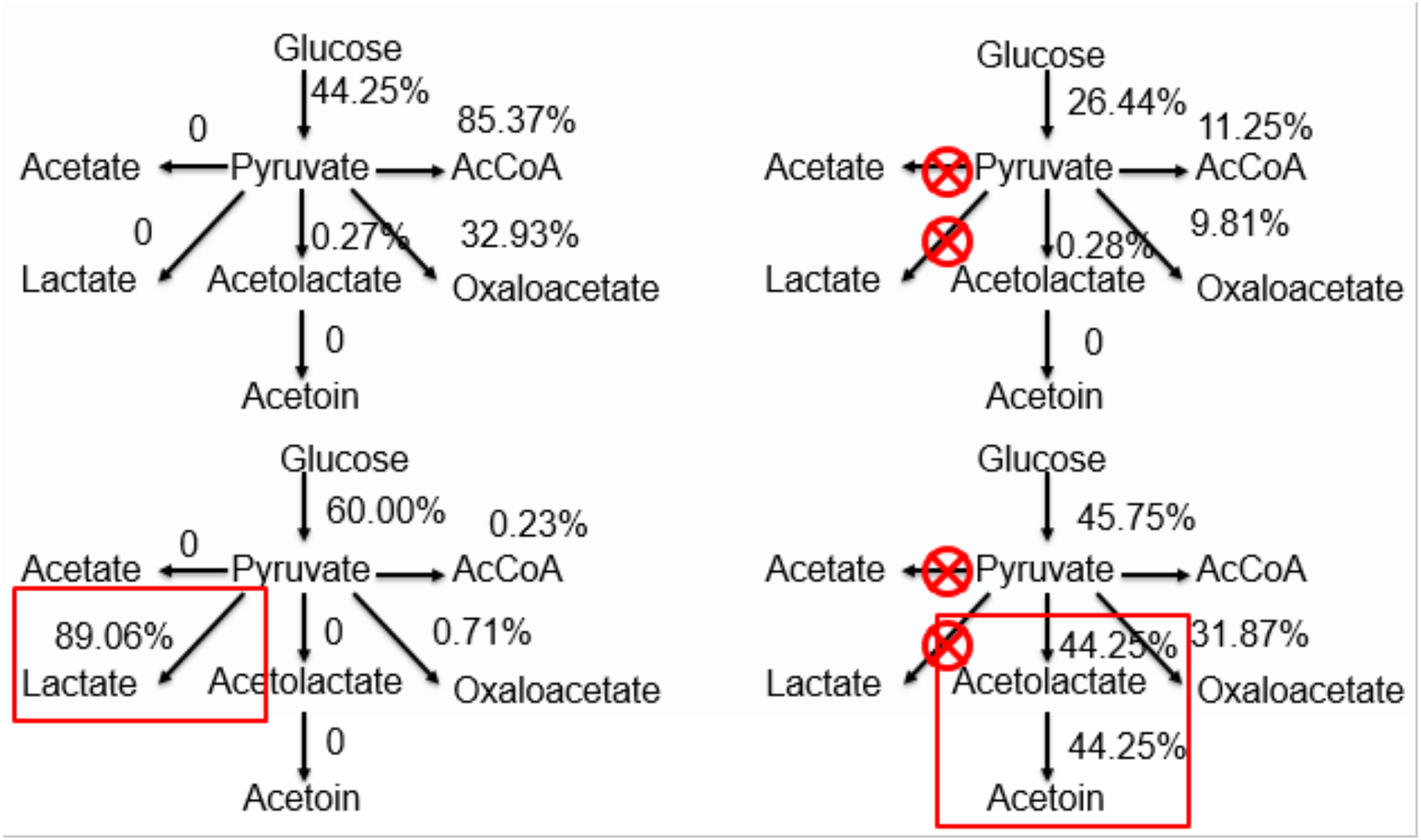
阻断乙偶姻之后72h 发酵液检测



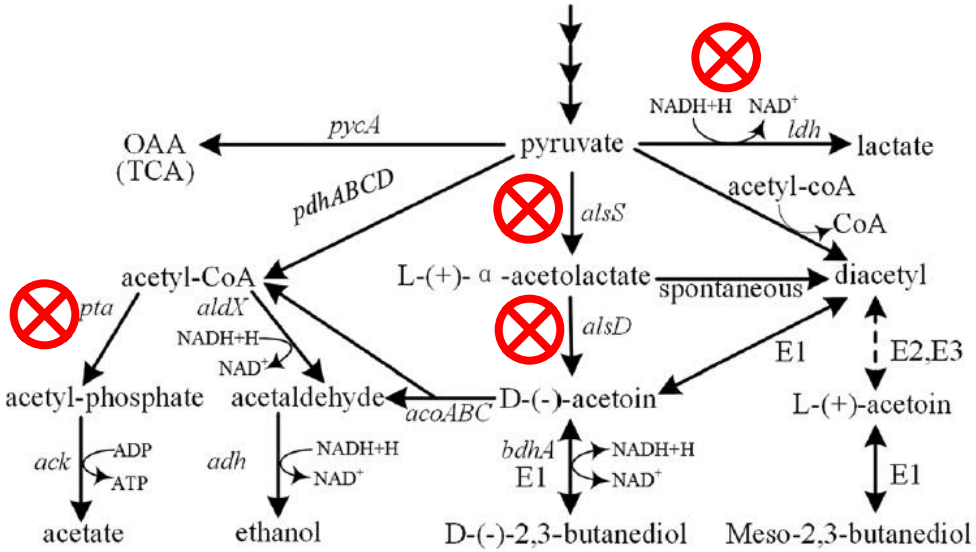


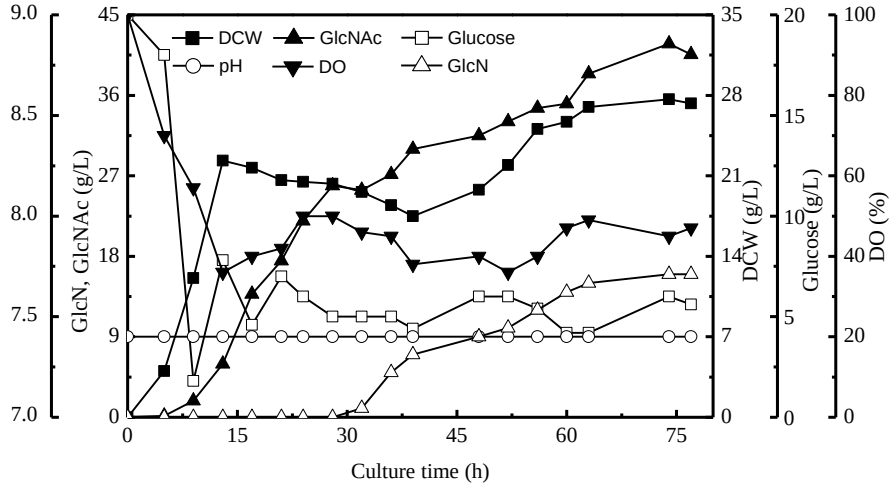
Acetoin formation was entirely blocked

Flux balance analysis of key metabolic nodes



Effect of blocking acetoin formation on GlcNAc production





	B6GECG	B6△alsSD 1	B6△alsSD 2
Glucose consumption (g/L)	313	260	240
GlcNAc titer (g/L)	31.16	**41.77**	**41.41**
Dry cell weight (g/L)	23.38	27.65	27.33
GlcNAc productivity (g/L h)	0.433	0.847	0.858
Yield on glucose (%)	9.95	**16.06**	**17.25**

Related publications

- Zhu et al., *Bioresource Technology*. 2015. 177: 387-392.
- Liu et al., *Applied Microbiology and Biotechnology*. 2015. 99: 1109-1118.
- Liu et al., *Metabolic Engineering*. 2014. 23: 42-52.
- Liu et al., *Metabolic Engineering*. 2014. 24: 61-69.
- Liu et al., *Metabolic Engineering*. 2013. 19: 107-115.
- Liu et al., *Applied Microbiology and Biotechnology*. 2013. 97: 6149-6158.
- Liu et al., *Applied Microbiology and Biotechnology*. 2013. 97: 6113-6127.


Thanks for your attention.