One-pot bio-synthesis: *N*-acetyl-D-neuraminic acid production by a powerful engineered whole-cell catalyst

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Whole cell biocatalysis is an important tool for pharmaceutical intermediates synthesis, although it is hindered by some shortcomings, such as high cost and toxicity of inducer, mass transfer resistance caused by cell membrane and side reactions. Whole-cell catalysis using *N*-acetyl-D-glucosamine 2-epimerase (EC 5.1.3.8) and *N*-acetyl-D-neuraminic acid (Neu5Ac) aldolase (EC 4.1.3.3) is a promising approach for the production of Neu5Ac, a potential precursor of many anti-viral drugs. A powerful catalyst was developed by packaging the enzymes in an engineered bacterium and using a safe temperature-induced vector. Since the mass transfer resistance and the side reactions were substantially reduced, a high Neu5Ac amount (191 mM) was achieved. An efficient method was also presented, which allows one-pot synthesis of Neu5Ac with a safe and economic manner. The results highlight the promise of large-scale Neu5Ac synthesis and point at a potential of our approach as a general strategy to improve whole-cell biocatalysis.

In light of the current pandemic threat and the emergence of oseltamivir resistance, development of effective anti-influenza drugs and related precursors is crucial. *N*-Acetyl-D-neuraminic acid (Neu5Ac) and its derivatives play significant roles in many physiological and pathological processes, such as cellular recognition and communication, bacterial and viral infections, and tumour metastasis. Therefore they are potentially useful pharmaceutical molecules. For example, zanamivir (Neu5Ac2en, 2-deoxy Neu5Ac, Biota/Glaxo), a commercial derivative of Neu5Ac, has been used to inhibit the neuraminidases of influenza viruses A and B in clinical settings. This compound prevents influenza virus infections, such as those caused by the avian influenza virus H5N1 and the current H1N1. However, the high price of Neu5Ac caused by its conventional method of production has impeded its industrial application.

Neu5Ac can be prepared from natural sources by hydrolysis, enzymatic conversion, or chemical synthesis. Among these methods, enzymatic conversion catalyzed by *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase (EC 5.1.3.8) and Neu5Ac aldolase (EC 4.1.3.3) is preferred, because of the high efficiency of the process and cheap substrates used (pyruvate and GlcNAc). However, the enzymes must be partially or completely purified, and ATP is required to activate GlcNAc 2-epimerase; consequently the process is complicated and costly. In comparison with isolated enzymes, whole-cell catalysts can be more readily and inexpensively prepared and are particularly useful for multi-enzyme reactions or those that require cofactor regeneration.

Neu5Ac production using coupled whole-cell catalysts has been reported, but the product amount and reaction rate are much lower than enzymatic catalysis. This disadvantage of coupled whole-cell catalysts is largely due to the mass transfer resistance caused by cell membrane, which also exists in other previously reported whole-cell catalysts and has hindered their industrial applications. It is notable that the reactions catalyzed by GlcNAc 2-epimerase and Neu5Ac aldolase were separately packaged in two types of cells in all the previous reports, where the reaction intermediate (*N*-acetyl-D-mannosamine, ManNAc) needs to penetrate between cells. Therefore, single cell packaging of the reactions would prevent the penetration of ManNAc and then largely reduce the mass transfer resistance. Moreover, packaging the reactions in a single cell would also simplify the operation.

The existence of side reactions is another factor leading to low efficiency of whole-cell catalyzed Neu5Ac production. Because living cells still have certain metabolic capabilities, the side reactions that occur during the whole-cell catalytic processes are generally more serious than those occur during enzymatic synthesis. The more
unwanted side reactions may become a crucial drawback of whole-cell biocatalysis which may seriously hamper product purification and reduce the overall yield20,23,24. In whole-cell catalyzed synthesis of Neu5Ac, both the substrates pyruvate and GlcNAc can be utilized by the cells, where pyruvate is the central branch point and GlcNAc serves as the carbon and nitrogen source23,24. Of the two substrates required for Neu5Ac synthesis, GlcNAc is relatively more expensive and is mainly transported into cells by the active transporter of the phosphotransferase system (PTS) with phosphorylation followed by mineralization25. Therefore, the elimination of PTS for GlcNAc should be effective in reducing the GlcNAc-related side reactions and the cost.

Chemical induction systems such as isopropyl β-D-1-thiogalactopyranoside (IPTG) induced system are frequently used for driving gene expression26. However, for applications such as development of organisms for use in food products and development of biocatalysts for therapeutic products, supplement of the toxic and expensive inducer for induction is undesirable27,28. Temperature induction is considered as a good alternative of chemical induction for scaled-up production of therapeutic products using biocatalysis because of its safety, low cost, and ease of operation29,30. Therefore, developing a biocatalyst based on temperature induction expression system would be beneficial to the industrialization of whole-cell catalyzed Neu5Ac production. Actually, a temperature induction vector named pBV220 has been successfully used in Neu5Ac production by coupled whole cells in our previous work31.

With these considerations in mind, we constructed a whole-cell catalyst by genetic engineering. At first, a mutant of Escherichia coli K12 was constructed by elimination of the PTS for GlcNAc. Then, the two reactions catalyzed by GlcNAc 2-epimerase and Neu5Ac aldolase were packaged in the single cell of the mutant by co-expressing the slr1975 gene from Synechocystis sp. PCC6803 and the nanA gene from E. coli K12. A novel one-pot reaction method for Neu5Ac production was also developed with the catalyst (Fig. 1). A series of whole-cell reactions were performed to investigate the advantages of the constructed catalyst and developed method.

Results

Construction of E. coli DT26/pBVNsS. To coexpress the slr1975 gene encoding GlcNAc 2-epimerase and the nanA gene encoding Neu5Ac aldolase in the same cell, pBVNsS was constructed using a temperature-induced expression vector pBV220 as described in the method (Fig. 2A, Fig. S1). The construction was verified by restriction digest. To determine whether genes slr1975 and nanA could be co-expressed in E. coli cells, the constructed plasmid pBVNsS was introduced into E. coli K12 to form E. coli K12/pBVNsS. E. coli K12/pBVNsS was then cultured in lysogenic broth (LB) and induced at 42°C, followed by SDS-PAGE analysis (Fig. 2B). As controls, strains E. coli K12/pBV220 (E. coli K12 harbouring plasmid pBV220), E. coli K12/pBVNS, and E. coli K12/pBVS were used. Figure 2B shows that the two proteins were coexpressed in the recombinant strain, which indicated that the construction of pBVNsS was successful.

To eliminate the catabolism of GlcNAc, the nagE gene was disrupted using homologous recombination as described in the method (Fig. 2C, Fig. S2). PCR was used to verify the disruption event of nagE gene using primer set PnagE-u plus PnagE-d. The result in Fig. 2D shows that the PCR using the primer set generated products of the expected sizes. The resulting mutant was named DT26. The desired strain E. coli DT26/pBVNsS was then constructed by transforming E. coli DT26 with pBVNsS (Fig. 1).

Reaction catalyzed by E. coli DT26/pBVNsS. To demonstrate the advantages of E. coli DT26/pBVNsS, various reaction solutions were prepared for Neu5Ac production (reaction A, E. coli DT26/pBVNsS; reaction B, E. coli K12/pBVNsS; and reaction C, E. coli K12/pBVN and E. coli K12/pBVS). Using 1 M pyruvate and 200 mM GlcNAc, Neu5Ac was formed. No ATP was added in any of the reactions. The Neu5Ac amount, average reaction rates, and yields were compared (Table 3). After induction, the enzyme activities of the different induced whole cells were also calculated and listed (Table 3). It was showed that E. coli DT26/pBVNsS produced 59.8 mM Neu5Ac in 48 h while E. coli K12/pBVNsS and the coupled whole cells (E. coli K12/pBVN and E. coli K12/pBVS) produced 36.8 and 25.1 mM Neu5Ac, respectively.

Time courses of Neu5Ac production in the three reaction solutions were also investigated and described in Fig. 3. Figure 3 shows that the reaction rate of reaction A (1.4 mmol l⁻¹ h⁻¹) is much higher than that of reaction B (1.0 mmol l⁻¹ h⁻¹) and C (1.0 mmol l⁻¹ h⁻¹). It is also showed that the time of reactions A and B (24 h) is longer than that of reaction C (42 h) when the concentration of Neu5Ac increased. In reaction C, it appears as if Neu5Ac production is biphasic, with an early rapid rate and a later slower rate, which is consistent with our previous report31. However, this phenomenon cannot be observed in reactions A and B.

Figure 1 | One-pot bio-synthesis scheme of Neu5Ac production by the constructed whole-cell catalyst. The rounded rectangle indicates a cell of the engineered E. coli DT26, whose GlcNAc-specific PTS was eliminated to reduce side reactions about GlcNAc. The enzymes GlcNAc 2-epimerase (I) and Neu5Ac aldolase (II) were coexpressed in E. coli DT26 with a safe temperature-induced vector. The mass transfer resistance was mainly caused by the membrane permeation of substrate and product (the membrane penetration by the intermediate ManNAc does not exist). ManNAc: Membrane affected by cetyltrimethylammonium bromide (CTAB) that was used to reduce the mass transfer resistance caused by membrane. ManNAc: The indicated function, GlcNAc-specific PTS (III), was eliminated, which would reduce side reactions about GlcNAc. ATP (adenosine triphosphate) was supplied by the cell.
Effect caused by *nagE* disruption. To investigate the effect of disruption of *nagE*, 1 M GlcNAc (almost the saturated concentration) was used in Neu5Ac production reactions. Figure 4 shows the time courses of Neu5Ac production by different types of reactions. *E. coli* DT26/pBVNsS synthesized 86.8 mM Neu5Ac in 60 h while *E. coli* K12/pBVNsS produced 38.0 mM Neu5Ac and coupled cells (*E. coli* K12/pBVN + *E. coli* K12/pBVS) produced 21.3 mM Neu5Ac.

Effect of surfactant on Neu5Ac production. To enhance the mass transfer of substrates and product, different surfactants were supplemented in Neu5Ac production reaction solutions, respectively. The result showed that the surfactant cetyltrimethylammonium bromide (CTAB) has the best effect on improving Neu5Ac production (Fig. S5). Time courses of Neu5Ac producing reactions with/without CTAB are showed in Fig. 5. It is showed that strain *E. coli* DT26/pBVNsS could produce 191.9 mM Neu5Ac in reaction mixture containing CTAB, while only 75.2 mM Neu5Ac was synthesized in mixture without CTAB.

![Figure 2](https://www.nature.com/srep/20142/f2.jpg)  
**Figure 2** | Construction of *E. coli* DT26/pBVNsS. (A) Map of pBVNsS. P_LP_R, promoters; MCS, multiple cloning site; *rrnBT1T2*, transcriptional terminator; *Amp'*, ampicillin resistance gene; *ori*, origin of plasmid replication; *clbs857*, temperature sensitive gene. (B) SDS-PAGE analysis of cells after heat induction. Lane 220: *E. coli* K12/pBV220 bacterial protein; Lane N: *E. coli* K12/pBVN bacterial protein; Lane S: *E. coli* K12/pBVS bacterial protein; Lane NS: *E. coli* K12/pBVNsS bacterial protein; Lane M: molecular mass standard. (C) diagram illustrating the sequence analysis of the disruption of the *nagE*. (D) Analysis of PCR fragments to confirm *nagE* disruption. Lane M: molecular mass standard (∝DNA/HindIII); Lane 1-4: product amplified with mutant genomic DNA as the template; Lane 5: product amplified with wild-type *E. coli* genomic DNA as the template. The PCRs were performed with primers PnagE-u and PnagE-d.

![Figure 3](https://www.nature.com/srep/20142/f3.png)  
**Figure 3** | Time courses of Neu5Ac production in the three reaction solutions. ▲, reaction A; ■, reaction B; ○, reaction C. Error bars indicate s.d. (n=3).

![Figure 4](https://www.nature.com/srep/20142/f4.png)  
**Figure 4** | Comparison of the reactions catalyzed by different whole cells with high concentrations of GlcNAc. The Neu5Ac concentration in reaction solutions with an *E. coli* K12/pBVN and *E. coli* K12/pBVS cells mixture (■), *E. coli* K12/pBVNsS cells (○), or *E. coli* DT26/pBVNsS cells (▲) as the catalysts. The initial pyruvate concentration is 500 mM. The performance was analyzed at 0, 12, 24, 36, 48 and 60 h.
Figure 5 | Time courses of Neu5Ac production by E. coli DT26/pBVNsS with and without CTAB. ●, Neu5Ac (without CTAB); ■, Neu5Ac (with 0.03% CTAB). Error bars indicate s.d. (n=3).

Reaction kinetics. The kinetics of Neu5Ac production was also investigated to further understand the reasons leading to the above advantages. The apparent kinetic parameters were determined using Lineweaver-Burk plots as described in the method (Fig S1)\cite{29,30}. The $K_{m}$ values of reactions catalyzed by E. coli K12/pBVNsS, E. coli DT26/pBVNsS and E. coli DT26/pBVNsS supplemented with CTAB were 2.2, 3.3 and 4.5 times lower than that with the coupled cells, while the $V_{max}$ values were increased by 1.7, 1.2 and 2.3 times, respectively (Table 4). These results imply that a higher Neu5Ac production rate can be achieved using lower concentrations of substrates by packaging Neu5Ac aldolase and GlcNAc 2-epimerase in one cell, disrupting nagE and supplying the surfactant, which may weaken mass transfer resistance and eliminate side reactions. When we calculate the overall reaction efficiency, $V_{max}/K_{m}$, the reaction with E. coli DT26/pBVNsS (supplemented with CTAB) exhibited significantly enhanced bioconversion efficiency, with a 10-fold increase compared to the method using coupled cells.

Discussion

Use of whole-cell biocatalysts for the industrial synthesis of chemicals has been attracting much attention as an environment-friendly synthetic method\cite{31}. However, there are still some problems should be taken into consideration (i.e. permeation of substrates across the cell membrane, side reactions causing degradation of products, accumulation of byproducts, etc.), which hinder the industrialization of whole-cell biocatalysis\cite{32}. Neu5Ac is an important precursor of therapeutic chemicals, which can be produced by whole-cell biocatalysis\cite{33}. The two problems about substrate permeation and side reactions have seriously impeded the application of whole-cell biocatalysis in Neu5Ac production\cite{34}. Therefore, the development of method to solve the problems is very important. Here, the permeation resistance was reduced by coexpressing slr1795 and nana, which should avoid the transfer of the intermediate ManNAc. The nagE gene of E. coli K12 encoding the GlcNAC-specific PTS transporter (EIICBANag) was also successfully knocked out to create the strain E. coli DT26 whose PTS mediated metabolism of GlcNAC was blocked (Fig. 2CD, Fig. S2). The strain should be advantageous as a host strain for Neu5Ac production from GlcNAC for the less side reactions about GlcNAC. Since Neu5Ac is a drug precursor, we also put safety as a key consideration. It was reported that the use of temperature induced expression vector was safe comparing with chemical-induced systems\cite{35}. Induction by temperature shift is also superior to that by chemical stimulation when the biocatalytic production of therapeutic products is scaled-up, because the former is cheaper and easier to operate\cite{36}. E. coli K12 is a nonpathogenic strain whose genetic background has been studied extensively, and its genome has been sequenced\cite{37,38}. Many methods to removing the endotoxin, which can cause pyrogenic and endotoxic shock reactions in mammals, were also developed\cite{39}. Therefore the temperature induced vector pBV220 and E. coli K12 were used in this work.

As we expect, strain E. coli DT26/pBVNsS (reaction A) showed significant advantages compared to coupled cells (reaction C) and E. coli K12/pBVNsS (reaction B). In reaction B, an increase (of about 46.8%) in Neu5Ac amount compared to reaction C (Table 3 and Fig. 1). Both the Neu5Ac amount and Neu5Ac yield were greatly increased in reaction A compared to reaction B (about 1.5 fold; Table 3). The average rates of reactions B and A were 1.0 and 1.4 mmol l$^{-1}$ h$^{-1}$, respectively, during the phase in which the yield increased (Table 3). It is reported that productivity of whole-cell biocatalysis is often severely compromised by cell membrane, and can be significantly enhanced by treating the cell membrane\cite{40}. Therefore, it is logical to suggest that the elimination of membrane penetration by the intermediate might have accelerated the mass transfer and contributed to the increase of Neu5Ac amount. In addition, the nagE knockout destroyed the EIICBANag so that the amount of GlcNAc being phosphorylated and then consumed by the cells decreased. Thus, the amount of GlcNAC transported without phosphorylation, which serves as the substrate for GlcNAc 2-epimerase, increased. The relatively high substrate concentration should result in higher reaction rates and final amounts of Neu5Ac. The apparently improved Neu5Ac yield in the reaction A, where E. coli DT26/pBVNsS is used, might be due to the reduced consumption of GlcNAc.

We have previously reported that Neu5Ac production is likely biphasic by coupled cells (reaction C), with an early rapid rate and a later slower rate\cite{41}. Here, we did not observe the phenomenon in reaction A or B. We initially speculated that this phenomenon was caused by cell lysis, and performed experiments (analyzed the supernatant obtained by centrifuging reaction mixture) to demonstrate this. However, the result showed that there was no obvious protein release which should be caused by cell lysis. As a phenomenon that existed in Neu5Ac production using coupled cells, it is logical to propose that this is related to the mass transfer of the intermediate ManNAc, and the elimination of mass transfer resistance of the intermediate ManNAc have led to the elimination of the later phase of slower rate.

It is considered that the use of a high concentration of pyruvate to drive Neu5Ac production is not ideal, because it is difficult to isolate Neu5Ac from reaction mixtures containing high concentrations of pyruvate\cite{42,43}. By eliminating the side reactions related to GlcNAc, it is expected that the substrate GlcNAc, which can be separated from Neu5Ac by a simple operation\cite{44,45}, could be used to drive Neu5Ac production. In addition, compared with pyruvate, the high GlcNAc concentration did not seriously inhibit the enzyme activities (Fig. S3). Therefore, 1 M GlcNAc was used in the following reactions. As expected, the reaction with a high GlcNAc concentration and E. coli DT26/pBVNsS as the catalyst showed a higher yield and a higher Neu5Ac amount than other two reactions (Fig. 4). The initial pyruvate concentration was also optimized based on the molar ratio of Neu5Ac to pyruvate when the reaction reached equilibrium for subsequent product isolation. Based on the result, 500 mM pyruvate was selected as the optimal concentration (Fig. S4).

For small hydrophilic molecules (molecular mass $< 600$ Da), such as pyruvate and GlcNAc, the inner membrane of the cell is the primary barrier to mass transfer\cite{46}. It was reported that permeabilizing the membrane using certain compounds such as surfactants and organic solvents could enhance the Neu5Ac producing rate\cite{47,48}. The
presence of surfactants at a concentration below the critical micelle concentration (CMC) can enhance the permeability of the cell membrane without destroying it35,37. After investigating the effects of different surfactants at different concentrations, 0.03% CTAB was used for whole-cell catalysis (Fig. S5). In comparison with the reaction without CTAB, the Neu5Ac amount and reaction rate increased by 154.7% (from 75 μM to 191 μM) and 1.5 times (from 2.1 mM h−1 to 3.8 mM h−1), respectively (Fig. 5). This result shows that elimination of mass transfer can efficiently enhance the Neu5Ac production, and indicates that the mass transfer resistance caused by membrane is a ubiquitous reason which leads to low reaction rates by membrane is a ubiquitous reason which leads to low reaction rates of whole cell catalysts35, it is reasonable to suggest that our method is also useful in other similar biocatalyses, especially that referring multiple-step reactions.

Methods

Bacterial strains, plasmids and culture conditions. All the bacterial strains and plasmids used are listed in Table 1. E. coli DH5α was used as host in general genetic manipulation. E. coli K12 from American Type Culture Collection (ATCC 25404) was used as the host strain for constructed recombinant plasmids. Synechocystis sp. PCC6803 was used as the source of gene slr1975, which encodes GlcNAc-2-epimerase. The gene encoding Neu5Ac aldolase was from plasmid pET15b-nanA23. Plasmid pBV220 was used in construction of recombinant plasmids24. All the genes used were aSD, Shine-Dalgarno sequence.

Table 1 | Bacterial strains and plasmids.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| E. coli strain | DH5α | F- φ80 lacZΔM15Δ1(lacZYA-argF)U169 degr recA1 endA1 hsdR17 (rK'/mK') gal-phaA supE44 Δ (thr-1) gyrA96 relA1 | Collection in our lab |
| K12 | wild type | ATCC 25404 |
| DT12 | K12, ΔnanE::FRT-tet-FRT | This study |
| DT26 | K12, ΔnanE::FRT | This study |
| K12/pBVN | pBV220 harboring nanA | 24 |
| K12/PBV5 | pBV220 harboring slr1975 | 24 |
| Synechocystis sp. PCC6803 | | FACHB-898 (Wuhan, China) |
| Plasmid | pET15b-nanA | bla TA cloning vector (Amp') |
| | pMD18-T | bla TA cloning vector (Amp') |
| | pMD18-T4639 | pMD18-T harboring nanE(Amp') |
| | pMD18-T4740 | pMD18-T harboring nanE::FRT-tet-FRT (Amp') |
| | pBV220 | bla, pUC8 origin, cl857(+), PpP; promoter (Amp') |
| | pBVNS5 | pBV220 harboring slr1975SD-nanA (GlcNAc-2-epimerase; Neu5Ac aldolase; Amp') |
| | pLOI2065 | bla, FRT-tet-FRT, colE1 (Amp') |
| | pKD46 | bla, flp, pSC101 replicon (red recombinase; Amp') |
| | pFT-A | bla, flp, pSC101 replicon (FLP recombinase; Amp') |

Table 2 | Primers.

| Name | Sequence | Restriction site and other element* |
|------|----------|-----------------------------------|
| RecA | 5′-gggCgAATTCACTggCAACgAATTTCgTggC3′ | EcoRI |
| RecB | 5′-TTATCTCCCTCgAgtATATCACCgCgCTTgCA3′ | XhoI, SD |
| RecC | 5′-ATATCgAgtgggAggATAAATgATTgCCCATCgCGT3′ | XhoI |
| PnaE-u | 5′-AgtgAAATATTgTTgggTTTTTCC3′ | SalI |
| PnaE-d | 5′-TTATCTTTTATTCTACAgCgC3′ | |
| Pteu | 5′-gggTACCgAgtCTgAATT3′ | |
| Pted | 5′-gggAgCTTgTcgAAT3′ | |

*SD, Shine-Dalgarno sequence.
Amplified DNA was integrated into producing rate. Since all the reactions showed Michael-Menten kinetic patterns, was 1 M, and the reaction rates were determined by calculating the Neu5Ac pro-

Whole-cell reactions were assayed according to previous reports. One unit of enzyme activity of Neu5Ac aldolase was designated as the amount that produces 1 µmol of pyruvate per minute at 37°C. One unit of enzymatic activity of GlcNAc 2-epimerase was defined as the amount that produces 1 µmol of ManNAc per min at 32°C. The Bradford protein assay was used to determine the amount of total protein. The concentrations of Neu5Ac, ManNAc, GlcNAc, pyruvate, and all the carbon sources were measured by high-performance liquid chromatography (HPLC, Agilent 1100 series, Hewlett-Packard), equipped with a Bio-Rad Aminex HPX-87H column (300×7.8 mm) and a refractive index detector. Analysis was performed with a mobile phase of 10 mM H2SO4 at a flow rate of 0.4 mL min-1 and at 55°C.

### Table 3 | Enzymatic activities and the reactions catalyzed by three different reaction solutions

| Reaction | Biocatalyst | Specific enzymatic activity (U mg total protein-1) | GlcNAc 2-epimerase | Neu5Ac (mM) | Yield [%]
|----------|-------------|-----------------------------------------------|--------------------|-------------|---------|
| A        | E. coli DT26/pBVNS | 3.521 | 0.486 | 59.8 ± 4.8 | 29.9 |
| B        | E. coli K12/pBVNS | 3.547 | 0.327 | 36.8 ± 2.2 | 18.4 |
| C        | E. coli K12/pBVN | 7.746 | Not Detected (ND) | | |
|          | E. coli K12/pBVS | | 1.966 | | |

*Neu5Ac aldolase/GlcNAc 2-epimerase in the three reaction solutions was equal in quantity. *Yield of Neu5Ac based on GlcNAc used.

Disruption of nagE. The nagE gene of E. coli K12 encoding the GlcNAc-specific PTS transporter (EIICBAnag) was knocked out to eliminate GlcNAc metabolism by using a method described by Causey et al. A single derivative of pMD18-T was selected in which the amplified nagE gene was oriented in the same direction as the lac promoter (pMD18-T-4639). The fragment between two restriction enzymes SalI and SmaI was knocked out to eliminate GlcNAc metabolism by using a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP-sialic acid synthetases. J. Enzyme. Med. 12, 6427–6435 (2004).

Table 4 | Apparent kinetic parameters of Neu5Ac production using different whole-cell biocatalysts.

| Reaction | Km (mM) | Vmax (mM h-1) | Vmax/Km (h-1) |
|----------|---------|---------------|---------------|
| Coupled cells | 147.1 | 5.5 | 0.03722 |
| K12/pBVNS | 147.1 | 5.5 | 0.03722 |
| DT26/pBVNS | 96.2 | 3.8 | 0.03950 |
| DT26/pBVNS+CTAB | 70.7 | 7.2 | 0.10272 |

The volume of each reaction mixture was 8 ml, and Neu5Ac aldolase and GlcNAc 2-epimerase in the three reaction solutions was equal in quantity, respectively (167.5 U of Neu5Ac aldolase and 31.8 U of GlcNAc 2-epimerase).

### Kinetics for different types of biocatalytic reactions

Whole-cell biocatalytic reactions were performed using different substrates (GlcNAc) concentrations (100 mM, 200 mM, 300 mM, 400 mM, and 500 mM) in order to compare reaction kinetics for the three types of reaction systems (reaction with coupled cells as catalyst, reaction with E. coli DT26/pBVNS as catalyst, and reaction with E. coli DT26/pBVNS as catalyst and containing 0.03% CTAB). In all the reactions, pyruvate concentration was 1 M, and the reaction rates were determined by calculating the Neu5Ac producing rate. Since all the reactions showed Michael-Menten kinetic patterns, Lineweaver-Burk plot analysis was performed for determining the apparent kinetic parameters (Km', Vmax', and Vmax'/Km') which were analogous to those (Km, Vmax, and Vmax/Km) for enzymatic reactions.

### Analytical methods

The activities of Neu5Ac aldolase and GlcNAc 2-epimerase were measured using a standard assay described by Wang et al. A single derivative of pMD18-T was selected in which the amplified nagE gene was oriented in the same direction as the lac promoter (pMD18-T-4639). The fragment between two restriction enzymes SalI and SmaI was knocked out to eliminate GlcNAc metabolism by using a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP-sialic acid synthetases. Bioorg. Med. Chem. 12, 6427–6435 (2004).

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Author contributions
P.X., Y.Z., F.T., and C.M., conceived and designed the experiments. F.T., Y.Z. performed the experiments. P.X., Y.Z., F.T., and C.M., analyzed the data. F.T., Y.Z., and P.X. wrote the paper.

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