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Correspondence and requests for materials should be addressed to P.X. (pingxu@sjtu.edu.cn)

* These authors contributed equally to this work.

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

Fei Tao[1,2]*, Yinan Zhang[2]*, Cuiqing Ma[2] & Ping Xu[1]

[1]State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China, [2]State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, People's Republic of China.

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[1,2]. *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[3–5]. 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[1,6,7]. 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[8], enzymatic conversion[9–12], or chemical synthesis[13,14]. 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[9–12,15], because of the high efficiency of the process and cheap substrates used (pyruvate and GlcNAc)[16,17]. 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[18–20].

Neu5Ac production using coupled whole-cell catalysts has been reported, but the product amount and reaction rate are much lower than enzymatic catalysis[21–24]. 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 catalyses and has hindered their industrial applications[19]. 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[21–24]. 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[19]. The more

unwanted side reactions have become a crucial drawback of whole-cell biocatalysis which may seriously hamper product purification and reduce the overall yield[19,25]. 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 source[26]. 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 mineralization[26]. 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 expression[27]. 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 undesirable[27,28]. 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 operation[27,28]. 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 work[24].

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/pBVN[24], and *E. coli* K12/pBVS[24] 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 of 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 report[24]. However, this phenomenon cannot be observed in reactions A and B.

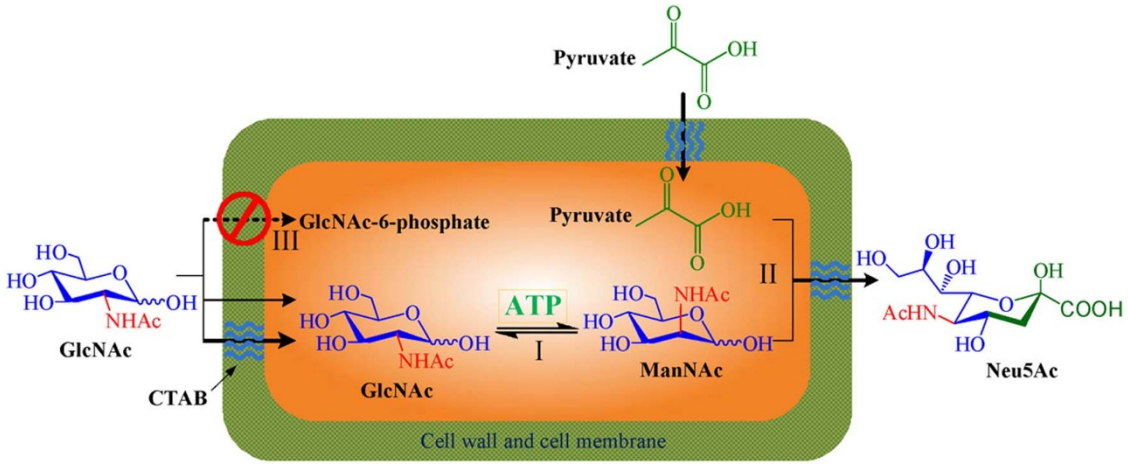

Figure 1 | One-pot bio-synthesis scheme of Neu5Ac production by the constructed whole-cell catalyst. The rounded rectangle indicates a cell of the engineered *E. coli* DT26, whose GlcNAc-specific PTS was eliminated to reduce side reactions about GlcNAc. The enzymes GlcNAc 2-epimerase (I) and Neu5Ac aldolase (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). ▨: Membrane affected by cetyltrimethylammonium bromide (CTAB) that was used to reduce the mass transfer resistance caused by membrane. ⊘: The indicated function, GlcNAc-specific PTS (III), was eliminated, which would reduce side reactions about GlcNAc. ATP (adenosine triphosphate) was supplied by the cell.

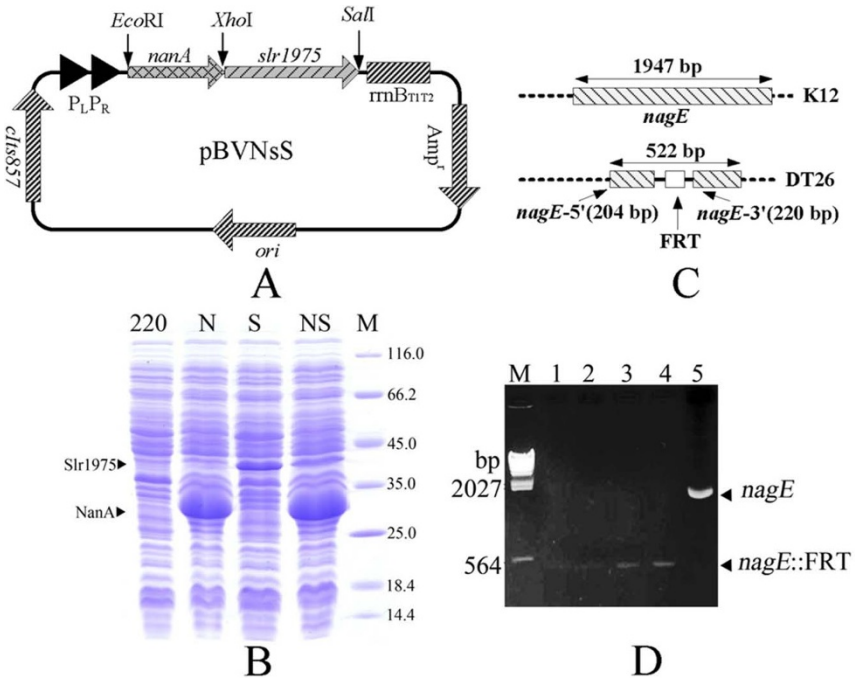

Figure 2 | Construction of *E. coli* DT26/pBVNsS. (A) Map of pBVNsS. P_LP_R, promoters; MCS, multiple cloning site; *rrn*B_{T1T2}, transcriptional terminator; Amp^r, ampicillin resistance gene; *ori*, origin of plasmid replication; *cIts857*, temperature sensitive gene. (B) SDS-PAGE analysis of cells 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 (λ DNA/*Hin*dIII); 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.

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.

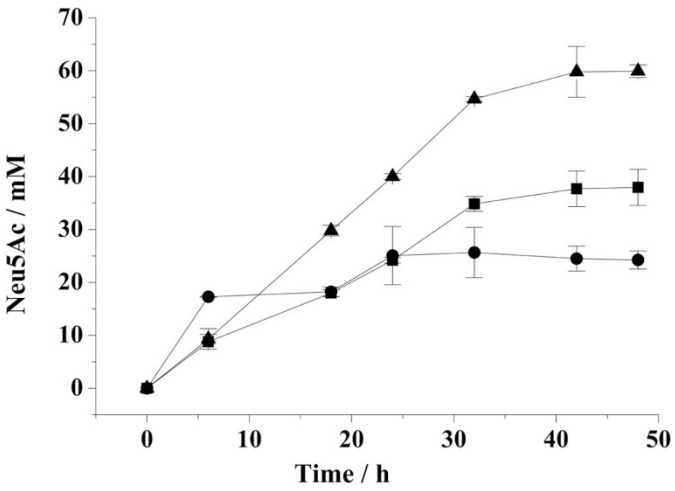

Figure 3 | Time courses of Neu5Ac production in the three reaction solutions. ▲, reaction A; ■, reaction B; ●, reaction C. Error bars indicate s.d. (n=3).

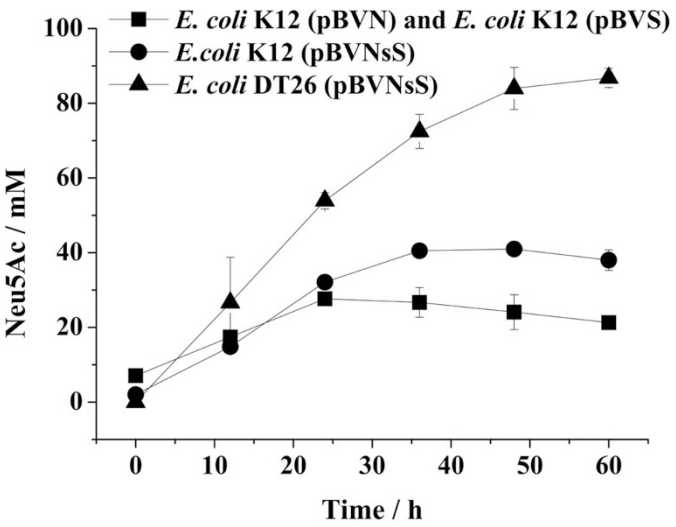

Figure 4 | Comparison of the reactions catalyzed by different whole cells with high concentrations of GlcNAc. The Neu5Ac concentration in reaction solutions with an *E. coli* K12 /pBVN and *E. coli* K12 /pBVS cells mixture (■), *E. coli* K12 /pBVNsS cells (●), or *E. coli* DT26 /pBVNsS cells (▲) as the catalysts. The initial pyruvate concentration is 500 mM. The performance was analyzed at 0, 12, 24, 36, 48 and 60 h.

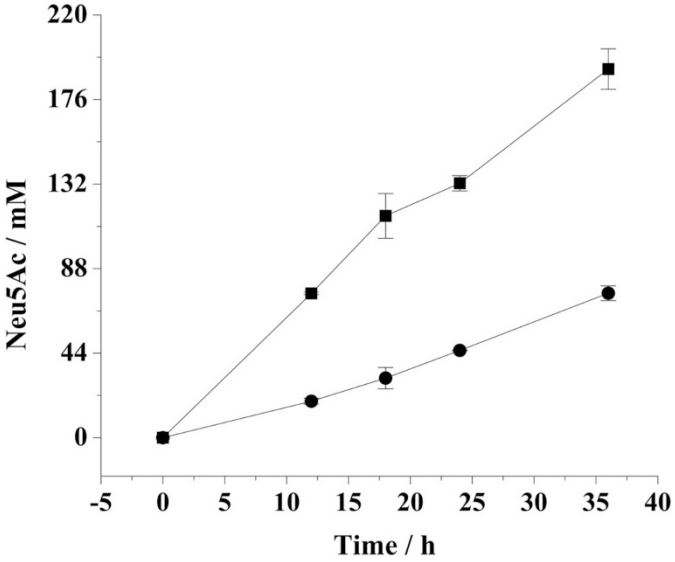

Figure 5 | Time courses of Neu5Ac production by *E. coli* DT26/pBVNsS with and without CTAB. ●, Neu5Ac (without CTAB); ■, Neu5Ac (with 0.03% CTAB). Error bars indicate s.d. (n=3).

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)[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[19]. 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[19]. Neu5Ac is an important precursor of therapeutic chemicals, which can be produced by whole-cell biocatalysis[25]. The two problems about substrate permeation and side reactions have seriously impeded the application of whole-cell biocatalysis in Neu5Ac production[25]. Therefore, the development of method to solve the problems is very important. Here, the permeation resistance was reduced by coexpressing *slr1975* and *nanA*, which should avoid the transfer of the intermediate ManNAc. The *nagE* gene of *E. coli* K12 encoding the GlcNAc-specific PTS transporter ($EIICBA^{Nag}$) 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[31]. 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[28]. *E. coli* K12 is a nonpathogenic strain whose genetic background has been studied extensively, and its genome has been sequenced[32,33]. Many methods to removing the endotoxin, which can cause pyrogenic and endotoxic shock reactions in mammals, were also developed[34]. 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[35]. 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 $EIICBA^{Nag}$ 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[24]. 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[10,11]. 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[10,36], 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[35]. It was reported that permeabilizing the membrane using certain compounds such as surfactants and organic solvents could enhance the Neu5Ac producing rate[21,23]. The

Table 1 | Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
E. coli strain		
DH5α	F⁻ Φ80 *lacZΔM15Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *gal-phoA supE44* λ^- *thi-1 gyrA96 relA1*	Collection in our lab
K12	wild type	ATCC 25404
DT12	K12, *ΔnagE::FRT-tet-FRT*	This study
DT26	K12, *ΔnagE::FRT*	This study
K12/pBVN	pBV220 harboring *nanA*	[24]
K12/PBVS	pBV220 harboring *slr1975*	[24]
Synechocystis sp. PCC6803		FACHB-898 (Wuhan, China)
Plasmid		
pET15b-nanA	pET15b harboring *nanA* (Neu5Ac aldolase; Amp^r)	[23]
pMD18-T	*bla* TA cloning vector (Amp^r)	TaKaRa
pMD18-T4639	pMD18-T harboring *nagE* (Amp^r)	This study
pMD18-T4740	pMD18-T harboring *nagE::FRT-tet-FRT* (Amp^r)	This study
pBV220	*bla*, pUC8 origin, cI857^{ts}, P_RP_L promoter (Amp^r)	Collection in our lab
pBVNsS	pBV220 harboring *slr1975*-SD-*nanA* (GlcNAc 2-epimerase; Neu5Ac aldolase; Amp^r)	This study
pLOI2065	*bla*, *FRT-tet-FRT*, colE1 (Amp^r)	[39]
pKD46	*bla*, *λ*, *β*, *exo*, pSC101 replicon^{ts} (red recombinase; Amp^r)	[39]
pFT-A	*bla*, *flp*, pSC101 replicon^{ts} (FLP recombinase; Amp^r)	[39]

presence of surfactants at a concentration below the critical micelle concentration (CMC) can enhance the permeability of the cell membrane without destroying it[35,37]. 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 ± 3.8 mM to 191 ± 10.5 mM) and 1.5 times (from 2.1 mM h^{-1} to 5.3 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 cell membrane is indeed a major obstacle of whole-cell biocatalytic Neu5Ac production.

It is notable that the effect of CTAB is similar to that caused by packaging the two enzymes in a single cell. As shown in Table 4, both the methods can reduce the K_m' and increase the V_{max}', which suggest that similar factors were involved in them. It was reported that the apparent K_m values of immobilized enzymes increased in most cases for the immobilizations increased the diffusion limitations[38]. Therefore, it is logical to suggest that reduction of diffusion limitations would reduce the apparent K_m of enzymes. It was also reported that addition of surfactant can reduce the diffusion limitations caused by membrane[35]. Moreover, the penetration of ManNAc can be avoided by packaging the two enzymes in a single cell. It is not difficult to conclude that both the methods enhanced the reaction by improving the mass transfer.

In summary, a powerful engineered whole-cell catalyst (*E. coli* DT26/pBVNsS) was constructed in which Neu5Ac aldolase and GlcNAc 2-epimerase were coexpressed from a temperature-induced expression vector in a GlcNAc-specific PTS eliminated system. Using this catalyst, we developed an efficient process for the one-pot synthesis of Neu5Ac. The side reactions and mass transfer limitations due to the membrane barrier were greatly reduced in this process. In comparison with whole-cell catalysis using coupled cells, our process resulted in a much higher Neu5Ac amount (191 ± 10.5 mM, 59.0 ± 3.2 g l^{-1}) and a greatly enhanced bioconversion efficiency (10 times greater than coupled cells). Since the expensive activator ATP, which is necessary in the free enzyme process, could be omitted, our process is highly attractive in terms of convenience, efficiency, and economy. Moreover, as mass transfer resistance caused by membrane is a ubiquitous reason which leads to low reaction rates of whole cell catalyses[35], 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-nanA[23]. Plasmid pBV220 was used in construction of recombinant plasmids[24]. All the genes used were initially cloned into pMD18-T (Takara Co., Ltd., Dalian, China) for sequencing. Plasmids pLOI2065, pKD46, and pFT-A were used in gene disruption[39]. LB medium (tryptone 1%, yeast extract 0.5%, NaCl 1%, pH 7.5) was used for growing *E. coli* cells[40] where ampicillin (0.1 g l^{-1}) was added when necessary. *E. coli* strains were grown at 37°C if not indicated.

Genetic methods. Standard methods were used for restriction digestions, agarose gel electrophoresis, and isolation of plasmid and genomic DNA[40,41]. Primers listed in Table 2 were used to amplify corresponding genes. Chromosomal integration of mutated genes was facilitated by pKD46 containing an arabinose-inducible Red recombinase[42]. Integrants were selected by using antibiotic resistance, screened for

Table 2 | Primers.

Name	Sequence	Restriction site and other element[a]
RecA	5′-ggCgAATTCATggCAACgAATTTACgTggC-3′	*Eco*RI
RecB	5′-TTATCTCCCTCgAgTATTCACCCgCgCTCTTgCA-3′	*Xho*I,SD
RecC	5′-ATACTCgAgggAgATAAATgATTgCCCATCgCCgT-3′	*Xho*I
RecD	5′-ggCgTCgACTTAACTAACCggAAgTTggAgA-3′	*Sal*I
PnagE-u	5′-ATgAATATTTTAggTTTTTCC-3′	
PnagE-d	5′-TTACTTTTTgATTTCATACAgC-3′	
Ptet-u	5′-gggTACCgAgCTCgAATT-3′	
Ptet-d	5′-ggggAgCTTgTCgACAAT-3′	

[a]SD, Shine-Dalgarno sequence.

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

Reaction[a]	Biocatalyst	Specific enzymatic activity (U mg total protein^{-1})		Neu5Ac (mM)	Yield (%)[b]
		Neu5Ac aldolase	GlcNAc 2-epimerase		
A	*E. coli* DT26/pBVNsS	5.321	0.486	59.8 ± 4.8	29.9
B	*E. coli* K12/pBVNsS	3.547	0.327	36.8 ± 2.2	18.4
C	*E. coli* K12/pBVN	7.746	Not Detected (ND)	25.1 ± 5.5	12.6
	E. coli K12/pBVS	ND	1.966		

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

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

Reaction[a]	K_m' (mM)	V_{max}' (mM h^{-1})	V_{max}'/K_m' (h^{-1})
Coupled cells	315.9	3.2	0.01027
K12/pBVNsS	147.1	5.5	0.03722
DT26/pBVNsS	96.2	3.8	0.03950
DT26/pBVNsS+CTAB	70.7	7.2	0.10272

[a]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).

appropriate antibiotic resistance markers and phenotypic traits, and verified by analyses of PCR products and fermentation profiles. *FRT* (FLP recognition target)-flanked antibiotic resistance genes were deleted by using FLP recombinase[39,42].

Construction of pBVNsS. Recombinant PCR[43] was performed using primers RecA, RecB, RecC, and RecD. In the first round, the gene *nanA* (fragment AB) was amplified by PCR with primers RecA and RecB at an annealing temperature of 60°C using pET15b-nanA as the template. Similarly, the gene *slr1975* (fragment CD) was obtained using primers RecC and RecD and pBVS as the template. After purification by gel extraction, a mixture of fragments AB and CD was used as the templates for the second round of PCR with primers RecA and RecD to obtain fragment AD (DNA fragment contain both *nanA* and *slr1975*). Both AD and pBV220 were digested with the restriction enzymes *Eco*RI and *Sal*I. Ligation of the AD and pBV220 resulted in the co-expression plasmid pBVNsS (Fig. 2A, Fig. S1).

Disruption of *nagE*. The *nagE* gene of *E. coli* K12 encoding the GlcNAc-specific PTS transporter (EIICBA^{Nag}) was knocked out to eliminate GlcNAc metabolism by using a method described by Causey et al.[39]. 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 *Aor*51HI sites of *nagE* was replaced by the *Sma*I fragment from pLOI2065 containing a *tet* gene flanked by FRT sites to produce pMD18-T-4740. Plasmid pMD18-T-4740 served as a template for the amplification of *nagE*::FRT-tet-FRT (3.4 kbp) with primers PnagE-u and PnagE-d. Amplified DNA was integrated into *E. coli* K12/pKD46 as described[39] to produce DT12. The *tet* gene was removed by using FLP recombinase, and the resulting strain was designated DT26 (Fig. 2C, Fig. S2).

Whole-cell catalyst preparation. One percent inoculum of the overnight culture of *E. coli* strain K12 containing plasmid pBVNsS, pBVN, or pBVS was inoculated in LB medium (containing 100 mg l^{-1} ampicillin), and incubated at 30°C with shaking for 2 h. The temperature was then increased to 42°C to induce the cells, and incubation was continued for 5 h with shaking. The cells were collected by centrifugation at 8,000 rpm for 5 min and then washed twice with 0.85% NaCl solution.

Whole-cell reactions. Erlenmeyer flasks (50 mL) were used in all the reactions in which Neu5Ac was produced. The reaction mixture (8 mL) contained 60 mM potassium phosphate buffer (PKB, pH 7.4), 10 mM $MgCl_2$. It was incubated in a rotary shaker at 200 rpm and 30°C. Appropriate concentrations (below the critical micelle concentrations) of surfactants (Triton X-100, CHAPS, CTAB, and SDS) were added when necessary. Neu5Ac, ManNAc, GlcNAc, and pyruvate were analyzed as reported earlier[23].

Kinetics for different types of biocatalytic reactions. Whole-cell biocatalytic reactions were performed with various substrate (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* K12/pBVNsS as catalyst, and reaction with *E. coli* DT26/pBVNsS 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 (K_m', V_{max}', and V_{max}'/K_m') which were analogous to those (K_m, V_{max}, and V_{max}/K_m) for enzymatic reactions.

Analytical methods. The activities of Neu5Ac aldolase and GlcNAc 2-epimerase were assayed according to previous reports[12,21]. One unit of enzyme activity of Neu5Ac aldolase was defined 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[44]. 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 H_2SO_4 at a flow rate of 0.4 ml min^{-1} and at 55°C.

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Author contributions

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

Additional information

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Competing financial interests The authors declare no competing financial interests.

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