Biotechnol Lett (2017) 39:227–234
DOI 10.1007/s10529-016-2235-8

ORIGINAL RESEARCH PAPER

Phosphoenolpyruvate-supply module in *Escherichia coli* improves *N*-acetyl-D-neuraminic acid biocatalysis

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Received: 20 September 2016 / Accepted: 3 October 2016 / Published online: 13 October 2016

Abstract
Objectives *N*-Acetyl-D-neuraminic acid (Neu5Ac) is often synthesized from exogenous *N*-acetylglucosa mine (GlcNAc) and excess pyruvate. We have previously constructed a recombinant *Escherichia coli* strain for Neu5Ac production using GlcNAc and intracellular phosphoenolpyruvate (PEP) as substrates (Zhu et al. Biotechnol Lett 38:1–9, 2016).
Results PEP synthesis-related genes, *pck* and *ppsA,* were overexpressed within different modes to construct PEP-supply modules, and their effects on Neu5Ac production were investigated. All the PEP-supply modules enhanced Neu5Ac production. For the best module, pCDF-pck-ppsA increased Neu5Ac production to 8.6 ± 0.15 g l^{-1}, compared with 3.6 ± 0.15 g l^{-1} of the original strain. Neu5Ac production was further increased to 15 ± 0.33 g l^{-1} in a 1 l fermenter.
Conclusions The PEP-supply module can improve the intracellular PEP supply and enhance Neu5Ac production, which benefited industrial Neu5Ac production.

Keywords Biocatalysis · *N* · *pck* · *ppsA* · Phosphoenolpyruvate-supply

Introduction

Sialic acid (SA) is a family of nine-carbon amino sugars, which are widespread in nature and found in both the deuterostome lineage of animals and in certain bacteria. SA usually occupies the terminal positions on macromolecules and cell membranes and thus plays vital roles in intercellular adhesion, signaling, and microbial attachment (Varki 2008). More than 50 types of SA have been reported (Inoue and Kitajima 2006) with the most ubiquitous being *N*-acetyl-D-neuraminic acid (Neu5Ac), which is considered valuable as medical precursor and food additive. Accordingly, an economic process for large-scale Neu5Ac production is required.

We have previously constructed a recombinant *Escherichia coli* strain for Neu5Ac production, in which GlcNAc 2-epimerase from *Anabaena* sp. CH1 (bAGE, EC 5.3.1.8) and Neu5Ac synthase from *Campylobacter jejuni* (cNeuB, EC 2.5.1.56, formerly EC 4.1.3.19) are coexpressed (Zhu et al. 2016). The yield of Neu5Ac was increased to 3.7 ± 0.04 g l^{-1} by 40 % when the GlcNAc-specific phosphotransferase system (PTS) was eliminated, indicating that the intracellular PEP supply is probably insufficient and plays a vital role in Neu5Ac production.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-016-2235-8) contains supplementary material, which is available to authorized users.

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In general, the pool of intracellular PEP is stable in bacteria; PEP is synthesized under the catalysis of PEP synthase (EC: 2.7.9.2, coded by *ppsA*) and PEP carboxykinase (EC: 4.1.1.49, coded by *pck*). To improve the target product with PEP as precursor, the metabolic flux of PEP synthesis should be strengthened. We hypothesized that strengthening the PEP synthesis pathway may improve Neu5Ac biosynthesis; thus, in the current study, different PEP supply modules were constructed by overexpressing *ppsA* and *pck* to investigate their effects on Neu5Ac production (Fig. 1). Results showed that Neu5Ac production significantly improved because of the extra PEP biosynthesis pathway.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. Three Duet vectors were employed as expression vectors, in which pACYC-Duet-1 was used as low copy vector with a copy number of 10–12, pCDFDuet-1 and pRSFDuet-1 were used as middle copy vector and high copy vector with copy number of 20–40 and >100, respectively. *E. coli* strains were cultured at 37 °C with 200 rpm shaking in LB medium (yeast extract 5 g l^{-1}, tryptone 10 g l^{-1}, NaCl 10 g l^{-1}, pH 7). Various combinations of ampicillin (100 mg l^{-1}), kanamycin (50 mg l^{-1}), streptomycin (40 mg l^{-1}), and chloramphenicol (25 mg l^{-1}) were added to the cultures of plasmid-bearing *E. coli* strains.

Analytical methods

Cell concentration was determined from the OD_{600} value (1 OD_{600} unit = 0.382 g dry cell weight l^{-1}). Neu5Ac concentration was measured using HPLC equipped with a Bio-Rad Aminex HPX-87H column and a UV detector. Protein was determined using the method of Bradford with bovine serum albumin as standard. The expression of the recombination enzymes was verified by 12 % SDS-PAGE.

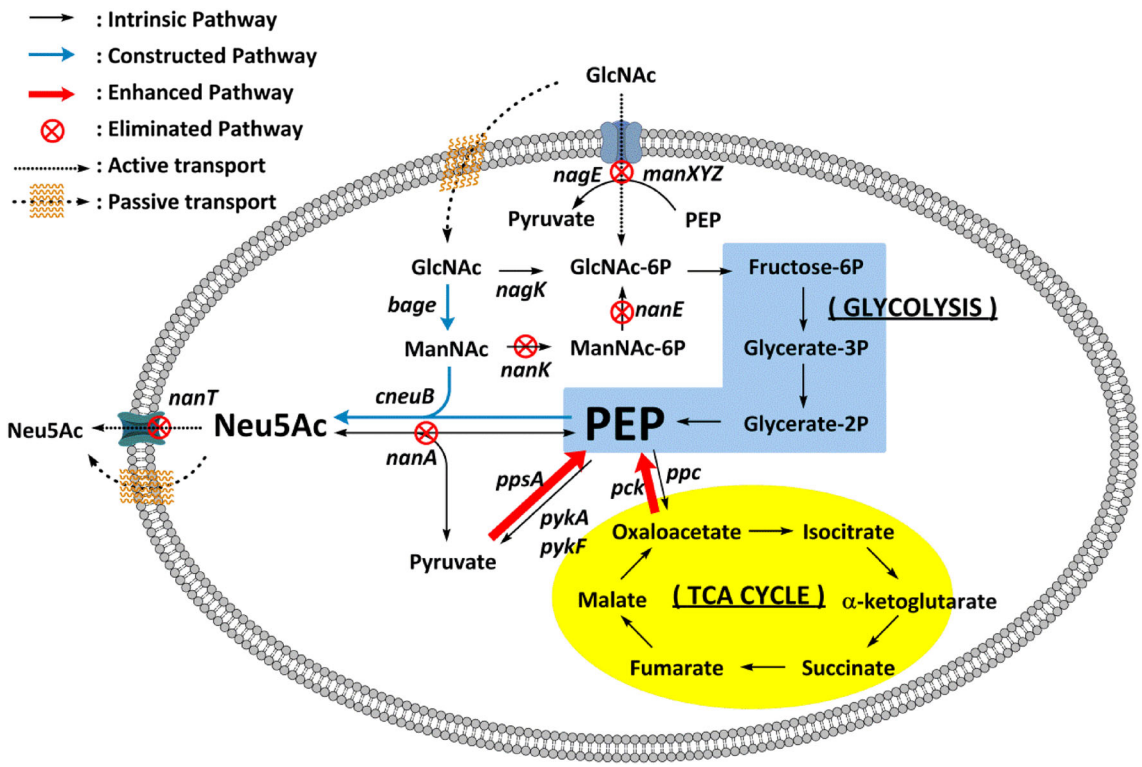

Fig. 1 Metabolic engineering of PEP-centered pathway for Neu5Ac production in *E. coli*. PEP participated in the synthesis of Neu5Ac, and was generated from glycolysis and TCA cycle via PEP synthase (coded by *ppsA* gene) and PEP carboxykinase (coded by *pck* gene)

DE3 lysogenization of host *Escherichia coli*

Lysogenization of the host strain was carried out with λ DE3 Lysogenization Kit (Merck, Germany) with *E. coli* SA-04 as the original strain. First, *E. coli* SA-04 was cultured in lysogeny broth (LB) supplemented with 0.2 % maltose, and 10 mM $MgSO_4$ at 37 °C to an OD_{600} of 0.5. Then, 1 μl pfu λ DE3, 3.3 μl pfu helper phage, and 1 μl pfu selection phage were mixed with 1 μl host cells, and the mixture was incubated at 37 °C for 30 min to allow absorption of the phages by the host cells. Afterwards, 100 μl LB medium was added to the host/phage mixture, and 10 μl of the resulting mixture was pipetted onto a LB plate and spread evenly. After incubation at 37 °C overnight, λ DE3 lysogens were verified by colony PCR.

Cloning and modification of *pck* and *ppsA* from *Escherichia coli*

Gene *pck* was obtained via PCR using *E. coli* MG1655 chromosomal DNA as template and pck-1-f/r as primers. The PCR product was cloned into pMD-T-simple to generate pMD-pck. Plasmid pMD-ppsA was obtained in the same manner.

The nonsense mutation of *Nco*I site in *ppsA* gene was carried out by inverse PCR using ppsA-M-f/r as primers that were composed of homologous sequences before and after the target site.

Construction of various PEP-supply modules

For the single-gene expression vector for the individual gene, both pMD-pck and pCDFDuet-1 were digested by *Nco*I and *Hin*dIII. Afterwards, the digested *pck* fragment was inserted into the corresponding site of pCDFDuet-1, generating pCDF-pck. The plasmid pCDF-ppsA was constructed in the same manner.

To construct the coexpression vector of *pck* and *ppsA* genes, the *pck* fragment with *Nde*I and *Kpn*I tails was obtained by PCR with pck-2-f/r as primers. PCR products and plasmid pCDF-ppsA were both digested by *Nde*I and *Kpn*I, followed by purification and ligation to generate the coexpression vector pCDF-ppsA-pck. Similarly, pCDF-pck-ppsA was constructed. Then, both pCDF-pck-ppsA and pRSFDuet-1 were digested by *Nco*I and *Kpn*I. After purification, the products were ligated to generate the high copy coexpression vector pRSF-pck-ppsA. Similarly, pRSF-ppsA-pck, pACYC-pck-ppsA, and pACYC-ppsA-pck were constructed.

Preparation of recombinant cells and the biocatalysis process of Neu5Ac

E. coli SA-05 cells harboring both pDTrc-AB and PEP-supply module vectors were cultured in LB medium. When an OD_{600} of 1.2 was reached, 0.2 mM IPTG was added, and culturing was continued for 5 h to induce protein expression. To assay the enzyme activity, the induced cells were then collected and lysed by ultrasonication in an ice bath. After centrifugation, the supernatant was collected as the crude enzyme solution.

For the biocatalysis of Neu5Ac, the induced cells were collected by centrifugation (4 °C, 8000×*g*, 20 min), washed twice by ice-cold 0.9 % NaCl and resuspended in the reaction mixture containing 110 mM PBS (pH 7.0), 10 mM $MgSO_4$, 0.4 M glucose, and 0.4 M GlcNAc in 50 ml (OD_{600nm} = 30). Biocatalysis process was performed in 500 ml Erlenmeyer flasks at 37 °C with shaking at 200 rpm.

When the biocatalysis process was carried out in a 1 l fermenter (KLF 2000, Bioengineering AG, Switzerland), 15 mM PBS (pH 7.0) was used instead of 110 mM PBS, and pH was adjusted to 7 with 1 M NaOH/HCl. The fermenter was operated at 37 °C, with ventilatory capacity of 1.5 vvm and stirring at300 rpm.

Assay of enzymatic activity

AGE activity is much higher than NeuB activity (Zhu et al. 2016). NeuB activity was the probable bottleneck for Neu5Ac production. Therefore, the NeuB activity was measured.

The activity of NeuB was quantified by measuring the conversion rate of ManNAc to Neu5Ac. The reaction mixture (1 ml) consisted of Tris/HCl (0.1 M, pH 7), 10 mM $MnSO_4$, 50 mM ManNAc, 50 mM PEP, and 20 μl crude enzyme solution. After incubation at 37 °C for 20 min, the mixture was boiled for 10 min to terminate the reaction. One unit of enzyme activity was defined as 1 nmol Neu5Ac formed per min.

Reverse transcription-quantitative PCR (RT-qPCR)

To conduct transcriptional analysis of the target genes, the total RNA was extracted from the cells after being

induced with 0.2 mM IPTG for 5 h in LB. RT-qPCR was carried out as described by Kang et al. (2012). The 16 s RNA housekeeping gene was assigned as control for normalization. All primers used are listed in Supplementary Table 2.

Statistical analysis

All the experiments were performed for three times, and the statistical analysis was carried out with Student's *t* test. P values of <0.05 were considered statistically significant. The specific Neu5Ac production rate was calculated by Origin software (Version 8.6, OriginLab Corp., USA).

Results and discussion

Cloning and expression of *ppsA* and *pck* in *Escherichia coli*

With *E. coli* MG1655 chromosomal DNA as template, two ORFs of 1623 and 2379 bp for *pck* and *ppsA* were amplified, respectively. The nonsense mutation by inverse PCR was introduced to mutate C (777 bp in *ppsA* sequence) to G to eliminate the *Nco*I cutting site in *ppsA* gene. The authenticities of these fragments were identified by dideoxy DNA sequencing (Sangon, Shanghai, China).

The Duet vectors are designed to coexpress two target proteins in *E. coli*, and they carry compatible replicons and antibiotic resistance markers (Ajikumar et al. 2010); certain combinations of Duet vectors and pTrc99a vectors are also compatible for coexpression. These advantages make Duet vectors ideal as the overexpression vectors for *ppsA* and *pck* even in the presence of pDTrc-AB in *E. coli*. The expression vectors pCDF-pck and pCDF-ppsA were constructed using the method mentioned above and verified by dideoxy DNA sequencing, respectively.

The lysogenization of host strain was verified by colony PCR with DE3-f/r as primers and was named as *E. coli* SA-05. When *E. coli* SA-05/pCDF-pck was induced, the target protein PEP carboxykinase was expressed well, whereas the target protein was not observed in the control strain *E. coli* SA-04/pCDF-pck lacking T7 polymerase (Fig. 2).

Construction of PEP-supply module with different expression vectors

Copy numbers of vectors and the gene order sequence of the two genes in the vectors affected gene expression. Therefore, to obtain the optimal PEP-supply module, vectors with different copy numbers were used: pACYCDuet-1 (low copy, copy number 10–12), pCDFDuet-1 (medium copy, copy number 20–40), and pRSFDuet-1 (high copy, copy number >100). For each vector, two expression vectors with different gene order sequences were constructed (Fig. 2). The expression vectors were verified by dideoxy DNA sequencing.

Single gene overexpression of *pck* and *ppsA* for the biocatalysis of Neu5Ac

For the biocatalysis of Neu5Ac, pCDF-pck and pCDF-ppsA were separately transformed into Neu5Ac producing strain *E. coli* SA-05/pDTrc-AB. Compared with the original strain, the PEP supply modules with the single-gene overexpression of *pck* and *ppsA* increased the yield of Neu5Ac by 61 and 96.4 %, reaching 5.8 ± 0.16 and 7.1 ± 0.24 g l^{-1}, respectively (Fig. 2A–C).

The above figures showed that the overexpression of *ppsA* gene induced more promotional effects on Neu5Ac production, indicating that PEP synthase could promote the supply of intracellular PEP more directly. The overexpression of *pck* could also increase PEP supply; however, the substrates for PEP regeneration could be limited by the TCA cycle and the anaplerotic regulation of PEP carboxylase. Meanwhile, the glyoxylate shunt can also reduce PEP regeneration (Yang et al. 2003). As shown in Fig. 2B, C, the strains with pCDF-pck and pCDF-ppsA both showed enhanced transcription levels of corresponding genes, thereby increasing the activities of intracellular PEP carboxykinase and PEP synthase, as well as the related metabolic flux. Therefore, intracellular PEP supply and Neu5Ac production were improved. However, as PEP occupies a key branch node in the metabolites of carbohydrate with complex feedback regulation system (Tabe-Bordbar and Marashi 2013; Yang et al. 2003), when the single-gene overexpression of *pck* or *ppsA* was conducted in *E. coli*, the transcription level of the other gene was slightly reduced.

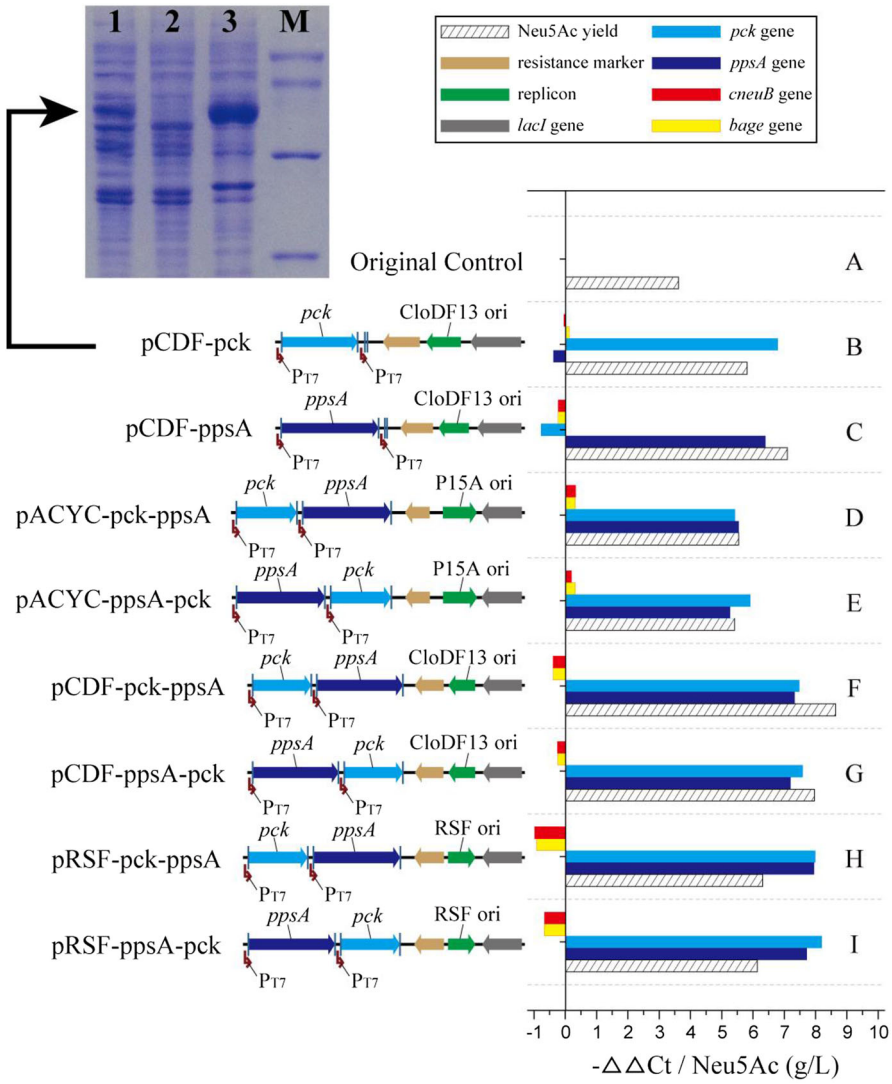

Fig. 2 Various PEP-supply modules and their effects on transcription levels and Neu5Ac production. For SDS-PAGE, target protein PEP carboxykinase (59.6 kDa), *Line 1 E. coli* SA-05/pCDF-pck, *Line 2 E. coli* SA-04/pCDF-pck, *Line 3 E. coli* BL21 (DE3)/pCDF-pck, *Line M* Protein Ladder (97.2-66.4-44.3-29.0 kDa)

Co-overexpression of *pck* and *ppsA* for the biocatalysis of Neu5Ac

Effects of vector copy number

As shown in Fig. 2D–I, co-overexpression of both *pck* and *ppsA* genes eliminated the feedback inhibition mentioned above and the transcription levels of the two genes increased simultaneously. Theoretically, the increase in vector copy number can benefit the gene-transcription level. However, an excessively large copy number may increase the growth burden of host strains (Bentley et al. 2009; Ramírez et al. 2016; Song et al. 2016). Considering that low copy vectors could minimize the effects on host cells, they were used to construct engineered bacteria in some cases (Jones et al. 2000). Therefore, a vector with an appropriate copy number is essential to develop a highly efficient PEP-supply module.

The transcription level was positively correlated with the copy number of expression vectors, whereas the variation in Neu5Ac production differed (shown in Supplementary Fig. 1), the strain with medium copy vector pCDFDuet-1 observed a maximum Neu5Ac yield (8.6 ± 0.15 g l^{-1}), compared with the 6.1–6.3 g l^{-1} from the strain with the high copy vector pRSFDuet-1. As shown in Fig. 3, the specific Neu5Ac production rate with pCDF-pck-ppsA and pCDF-

ppsA-pck PEP-supply modules were much higher than those with other PEP-supply modules. In general, the transcription level of target genes was important but was not the sole factor. In the case of high copy vectors, the replication of vectors and the overexpression of exogenous genes consumed large amounts of metabolic intermediates and energy, which may cause low-efficiency intracellular energy metabolism (Nam et al. 2013; Wu et al. 2016). Unexpectedly, the Neu5Ac yield with low copy co-overexpression vectors was even slightly lower than that with single-gene overexpression vectors.

Therefore, the benefit of low copy vector was masked by its deficiency in expressing the target genes, whereas the high copy coexpression vector increased the transcription level of target genes but failed to enhance Neu5Ac production. The highest Neu5Ac yield was obtained with the PEP-supply module of medium copy number vector.

Effects of different gene order sequences

The Duet vectors have two T7 promoters and two MCS regions but only a single T7 terminator for the cloning and expression of two target genes. Therefore, as shown in Fig. 2D–I, the transcription level of the distal gene was higher than the proximal one, which was due to the fact that the distal gene was promoted by two T7 promoters, whereas the proximal gene was influenced only by the first T7 promoter. The highest Neu5Ac yield was achieved with the PEP-supply module of pCDF-pck-ppsA (Fig. 2F), in which *ppsA* gene was inserted into the distal MCS and the Neu5Ac yield was 8.4 % higher than that of pCDF-ppsA-pck.

Transcriptional analysis of *bage* and *cneuB* genes

As shown in Fig. 2, RT-qPCR results revealed that when the low copy vector pACYCDuet-1 was used, the transcription levels of *cneuB* and *bage* genes only slightly varied. Conversely, the transcription levels of *cneuB* and *bage* genes were significantly affected (negative) when the medium copy vector pCDFDuet-1 and high copy plasmid pRSFDuet-1 were used. The high copy vector may have significantly burdened the physiological activities of the host bacteria and thus negatively affected the expression of exogenous genes. However, although the transcription levels of *cneuB* and *bage* genes were reduced when medium and high copy plasmids were used, this did not reduce Neu5Ac production, which indicates that neither cNeuB activity nor bAGE activity was the bottleneck for the Neu5Ac production.

Neu5Ac production in fermenter

The above shake flask experiment data showed that pCDF-pck-ppsA was the optimum PEP-supply module with the highest efficiency. In a bench scale, Neu5Ac production by biocatalysis was carried out in a 1 l fermenter with induced *E. coli* SA-05/pDTrc-AB/pCDF- pck-ppsA. As shown in Fig. 4, the final yield of Neu5Ac reached 15 ± 0.33 g l^{-1}, which was 74 % higher than that obtained in shake-flasks. Biomass

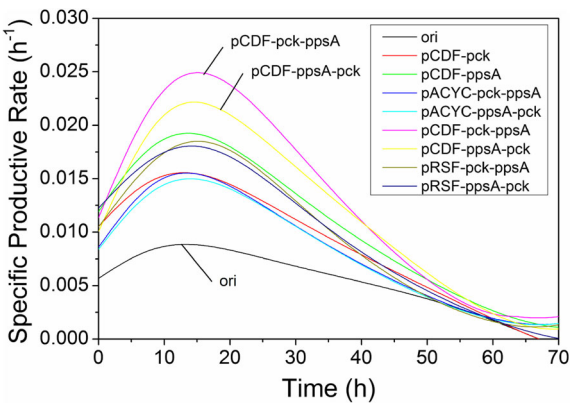

Fig. 3 Effects of different PEP-supply modules on specific production rates of Neu5Ac

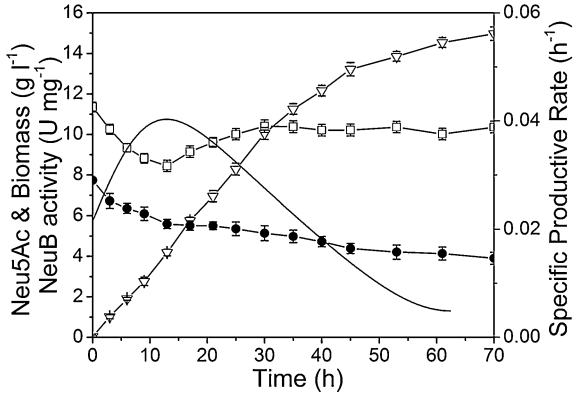

Fig. 4 Time course of *E. coli* SA-05/pDTrc-AB/pCDF-pck-ppsA in a 1 l fermenter. *White triangle* Neu5Ac concentration, *black circle* NeuB activity, *white square* Biomass

Table 1 Neu5Ac production by different strategies

Strains/matrix	Enzymes	Substrates	Yield ($g\ l^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)	Reference
E. coli	AGE + NanA	GlcNAc/pyruvate	59	1.68	Tao et al. (2011)
E. coli	AGE + NanA	GlcNAc/pyruvate	74.2	4.64	Lin et al. (2013)
E. coli	AGE + GlcNAc-6P acetyltransferase	Glucose	7.85	0.082	Kang et al. (2012)
E. coli	BeuBC + GlmS	Glucose	1.7	0.024	Lundgren and Boddy (2007)
E. coli	AGE + NeuB	Glucose/GlcNAc	7.15	0.1	Zhu et al. (2016)
E. coli	AGE + NeuB	Glucose/GlcNAc	15	0.21	This study

slightly decreased from the beginning because of cell lysis in the new environment and then recovered until remaining stable. Intracellular NeuB activity showed no variation. With pH control, low titer of PBS (15 mM) in the fermenter setup, and better mass transfer in the fermenter, this new recombinant *E. coli* could produce more Neu5Ac as expected.

To date, different strategies have been used for Neu5Ac production (listed in Table 1). A fermentation strategy for producing Neu5Ac was developed with a yield of 7.85 $g\ l^{-1}$. The Neu5Ac production strategy by biocatalysis involved an epimerization reaction to generate ManNAc from GlcNAc with AGE and an aldolization reaction to generate Neu5Ac with NeuNAc aldolase (NanA, EC 4.1.3.3). Such strategy is adopted in different catalytic processes, including whole-cell catalysis, immobilized enzymatic catalysis (Hu et al. 2010), and one-pot biosynthesis (Tao et al. 2011). Although this strategy is considered as the most economical method of industrial-scale Neu5Ac production, a significant drawback exists and could not be neglected. This drawback is that the excess pyruvate (usually fivefold) is indispensable to shift the reaction equilibrium toward Neu5Ac synthesis (Lin et al. 2013), which causes great waste of pyruvate and increases the environmental cost. The strategy adopted in this current study did not require pyruvate as substrate by using NeuB instead of NanA, and the biocatalysis of Neu5Ac was promoted by the consumption of PEP high-energy phosphate bond instead of excess pyruvate addition.

Conclusion

As a precursor of Neu5Ac synthesis, the supply of intracellular PEP is important for Neu5Ac production by biocatalysis. To construct an effective PEP-supply module, *pck* and *ppsA* genes of *E. coli* were single- and co-overexpressed. At the same time, expression vectors with different copy numbers were used, and the gene order sequence was also adjusted. Vector pCDF-pck-ppsA was the optimal PEP-supply module, resulting in Neu5Ac yield of $8.63 \pm 0.15\ g\ l^{-1}$ from *E. coli* SA-05/pDTrc-AB/pCDF-pck-ppsA, which was enha nced by 139 % as compared with that from the original strain. Neu5Ac yield was further increased to $15 \pm 0.33\ g\ l^{-1}$ in a 1 l fermenter. Consequently, our efficient PEP-supply module can improve the intracellular PEP supply and thus benefit Neu5Ac production.

Acknowledgments This research was supported by the National Natural Science Foundation of China No. 31171640, the Program of Introducing Talents of Discipline to Universities (111-2-06), the Fundamental Research Funds for the Central Universities (JUSRP51504, JUSRP51632A), Key Technology Research and Development Program of Wuxi (CLE01N1208), and Program for the Wuxi Bio-Agriculture Entrepreneurial Leader (130 Plan).

Supporting information Supplementary Table 1—Strains and plasmids used in this study.

Supplementary Table 2—Primers used in this study.

Supplementary Fig. 1—Time courses of *E. coli* SA-05/pDTrc-AB harboring various PEP-supply modules.

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