Bioresource Technology 130 (2013) 23–29

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Construction and expression of a polycistronic plasmid encoding *N*-acetylglucosamine 2-epimerase and *N*-acetylneuraminic acid lyase simultaneously for production of *N*-acetylneuraminic acid

Wujin Sun, Wenyan Ji, Nan Li, Peng Tong, Jian Cheng, Ying He, Yong Chen, Xiaochun Chen, Jinglan Wu, Pingkai Ouyang, Jingjing Xie *, Hanjie Ying *

State Key Laboratory of Materials-Oriented Chemical Engineering, National Engineering Technique Research Center for Biotechnology, College of Biotechnology and Pharmaceutical Engineering, Nanjing University of Technology, Nanjing, China

HIGHLIGHTS

- Encoding enzymes for two reactions in one plasmid simplified fermentation process.
- Activities of two types of *N*-acetylglucosamine 2-epimerase were compared.
- Intact *E. coli* cells avoid adding ATP to activate *N*-acetylglucosamine 2-epimerase.
- Lowering *N*-acetylglucosamine 2-epimerase expression level improved its solubility.
- High production of 61.3 g/l *N*-acetylneuraminic acid.

ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised form 4 December 2012
Accepted 7 December 2012
Available online 19 December 2012

Keywords:
Biocatalysis
N-acetylneuraminic acid
Inclusion bodies
N-acetylneuraminic acid lyase
N-acetylglucosamine 2-epimerase

ABSTRACT

Synthesis of *N*-acetylneuraminic acid (Neu5Ac) from *N*-acetylglucosamine (GlcNAc) and pyruvate was carried out by constructing and expressing a polycistronic plasmid encoding an *N*-acetylglucosamine 2-epimerase (AGE) gene and an *N*-acetylneuraminic acid lyase (Nal) gene simultaneously. Nal from *Escherichia coli* K12 and AGEs from *Synechocystis* sp. PCC 6803 (snAGE) and *Anabaena* sp. CH1 (anAGE) were used. And four polycistronic plasmids were constructed in which the positions of AGE gene differed with respect to Nal gene. Among these plasmids, pET-28a-Nal-anAGE with anAGE gene located next to Nal gene caused the production of the highest amount of Neu5Ac, generating 61.3 g/L in 60 h by whole-cell catalysis without the addition of ATP as AGE activator. And pET-28a-Nal-anAGE lowered anAGE's expression level, allowing it to fold properly. Thus, an inclusion-body-free *E. coli* strain capable of producing Neu5Ac by whole-cell catalysis with high yield and low cost was constructed in the present study.

1. Introduction

Mammalian cells are covered with sugar chains which are terminated by a family of 9-carbon amino sugars called sialic acids. These sugar derivatives are often part of the recognition sites where pathogens attach (Varki and Varki, 2007). Sialic acids are involved in the modulation of various biological processes, such as virus invasion, cell differentiation, fertilization, cell adhesion, inflammation, and tumorigenesis (Hu et al., 2010). More than 40 types of sialic acids have been discovered in nature, and Neu5Ac is the most ubiquitous and the biosynthetic precursor for all other sialic acids (Maru et al., 2002; Ogura, 2011). Neu5Ac is a potential raw material in the synthesis of zanamivir, which prevents both influenza type A and B infections (Tao et al., 2010). Neu5Ac is also an important additive in dairy products, as it is able to strengthen the immunity of infants (Oriquat et al., 2011). In addition, Neu5Ac is of great diagnostic value as an important indicator for many diseases (Gopaul and Crook, 2006).

Traditionally, Neu5Ac has been prepared by extraction from natural sources (such as milk or eggs), or by hydrolyzing capsular

Abbreviations: Nal, *N*-acetylneuraminic acid lyase; AGE, *N*-acetylglucosamine 2-epimerase; snAGE, *N*-acetylglucosamine 2-epimerase from *Synechocystis* sp. PCC 6803; anAGE, *N*-acetylglucosamine 2-epimerase from *Anabaena* sp. CH1; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetyl-D-mannosamine; Neu5Ac, *N*-acetylneuraminic acid.

* Corresponding author. Tel.: +86 25 86990001; fax: +86 25 58133398.
E-mail addresses: xiej@njut.edu.cn (J. Xie), yinghanjie@njut.edu.cn, cxc_1981@sohu.com (H. Ying).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.12.042

polysaccharides from *Escherichia coli* (Hu et al., 2010; Tabata et al., 2002). Current enzymatic production methods are based on the *E. coli* K1 sialic acid synthesis pathway (Ferrero and Aparicio, 2010; Plumbridge and Vimr, 1999) (Fig. 1). *N*-acetylneuraminic acid lyase (Nal, NanA, EC 4.1.3.3) from *E. coli* (Hu et al., 2010; Mahmoudian et al., 1997; Maru et al., 1998; Wang et al., 2009; Zhang et al., 2010) and many other organisms (Krüger et al., 2001; Li et al., 2008; Nahalka et al., 2008; Sanchez-Carron et al., 2011) are utilized to condense *N*-acetyl-D-mannosamine (ManNAc) and pyruvate into Neu5Ac through a reversible reaction. *N*-acetylneuraminic acid synthases (NeuB, EC 4.1.3.19) from *E. coli* (Ishikawa and Koizumi, 2010; Tabata et al., 2002) and other sources (Hao et al., 2005) are used to condense ManNAc and phosphoenolpyruvate (PEP) into Neu5Ac by an irreversible reaction.

However, ManNAc is too expensive to be applied on an industrial scale (Hu et al., 2010). It is possible to synthesize *E. coli* K1 ManNAc from UDP-GlcNAc by UDP-GlcNAc 2-epimerase (NeuC, EC 5.1.3.14); or via a 3-step reaction with GlcNAc as a substrate that involves the phosphotransferase system (PTS), ManNAc-6P epimerase (NanE) and a proposed phosphatase (Ferrero and Aparicio, 2010; Plumbridge and Vimr, 1999). However, ManNAc could be utilized as an intermediate for Neu5Ac synthesis. *N*-acetylglucosamine 2-epimerase (AGE, EC 5.1.3.8), identified in mammals as a renin binding protein, catalyzes a single-step reaction to produce ManNAc from inexpensive GlcNAc in a reversible process (Maru et al., 1996). However, AGE is not prevalent in nature and no functional AGE has been reported in *E. coli*. The hypothetical protein YihS from *E. coli*, which has significant structural similarity to AGE, shows no AGE activity (Itoh et al., 2008). AGEs have been discovered in mammals, such as humans (Lee et al., 2004), pigs (Itoh et al., 2000) and rats (Van Rinsum et al., 1983); in cyanobacteria, such as *Synechocystis* sp. PCC 6803 (Tabata et al., 2002) and *Anabaena* sp. CH1 (Lee et al., 2007a); and recently in *Bacteroides ovatus* ATCC 8483 (Sola-Carvajal et al., 2012).

A simple and efficient two-step enzymatic catalytic strategy involving AGE and Nal has been adopted to produce Neu5Ac (Fig. 2) (Hu et al., 2010; Maru et al., 1998; Wang et al., 2009; Zhang et al., 2010). While Nal from *E. coli* is soluble when cloned and over-expressed in *E. coli*, AGE tends to form inclusion bodies when expressed heterogeneously (Chien et al., 2007; Lee et al., 2007a). When recombinant plasmids inducible at 42 °C were constructed to produce Neu5Ac (Zhang et al., 2010), the increased temperature negatively impacted AGE expression (Hu et al., 2010). Therefore, chemically induced expression would be preferable. Most studies were designed to carry out the two-step reaction in two separate cells, which requires laborious fermentation of two distinct strains (Hu et al., 2010; Maru et al., 1998; Wang et al., 2009; Zhang et al., 2010). Over-expressing these two enzymes in one single strain could simplify the fermentation process as well as reduce production costs. In addition, AGE is dependent on adenosine triphosphate (ATP) as an activator (Takahashi et al., 2001). Adding ATP as a cofactor into a cell-free system significantly increases production costs, while intact cells contain sufficient ATP to activate the reaction (Ishikawa and Koizumi, 2010).

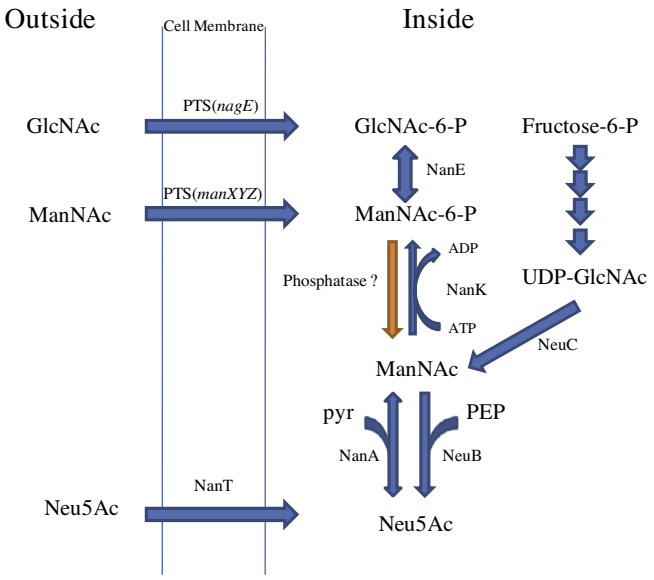

Fig. 1. Sialic acid synthesis pathway in *E. coli* K1 (Ferrero and Aparicio, 2010; Plumbridge and Vimr, 1999). Pyr: pyruvate, PEP: phosphoenolpyruvate, NanT, *E. coli* Neu5Ac transporter, NeuC, *E. coli* UDP-GlcNAc 2-epimerase, NeuB, *E. coli* Neu5Ac synthase, NanA, Neu5Ac aldolase, NanK, ManNAc kinase, NanE, ManNAc-6P epimerase, PTS, phosphotransferase system.

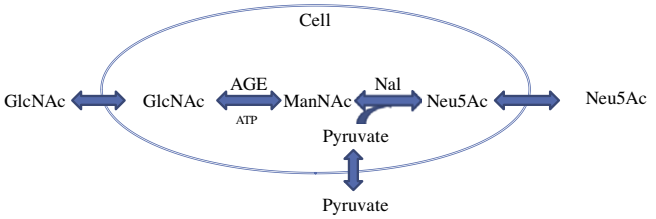

Fig. 2. Diagram of the two-step reaction of this study. GlcNAc was transported inside the cells through the cell membrane, and it was converted into ManNAc by AGE. The resulting ManNAc was condensed with pyruvate by Nal to produce Neu5Ac.

In the present study, AGEs from *Synechocystis* sp. PCC 6803 and *Anabaena* sp. CH1 were used to convert GlcNAc into ManNAc. Nal from *E. coli* K12 was chosen to condense ManNAc and pyruvate into Neu5Ac because pyruvate is economically more competitive than PEP. Four chemically-induced polycistronic plasmids co-expressing both genes were introduced into *E. coli* Rosetta (DE3) pLysS to produce Neu5Ac from GlcNAc. The different positions of these genes on the expression plasmid lead to different expression levels and catalysis efficiency.

2. Methods

2.1. Chemicals, enzymes and strains

Restriction enzymes, T4 DNA ligase, Taq DNA polymerase, DNA markers and protein molecular weight markers were obtained from Takara Biotechnology Co. Ltd (Dalian, China). *E. coli* DH5α and *E. coli* Rosetta (DE3) pLysS (Merck, Germany) were used as cloning and expression hosts, respectively. Vectors pMD18-T simple and pET-28a (+) were used as cloning and expression vectors. GlcNAc, ManNAc and Neu5AC were purchased from Sigma Aldrich (Shanghai, China). Oligonucleotide primers were synthesized by Genescript (Nanjing, China). Whole-gene synthesis was done by Generay (Shanghai, China). All other chemicals were of reagent grade and obtained from commercial sources.

2.2. Construction of polycistronic plasmids co-expressing two genes

The temperature-inducible vectors, pBV220 containing AGE from *Synechocystis* sp. PCC 6803 (pBVS) and Nal from *E. coli* K12 (pBVN), were gifts from Dr. P. Xu (Zhang et al., 2010). The AGE gene from *Anabaena* sp. CH1 was optimized according to its nucelotide sequence (Genbank accession number DQ661858 (Lee et al., 2007a)) and synthesized chemically by Generay. The synthesized gene was ligated into the *EcoRI*/*NotI* restriction sites of pPIC9K (pPIC9k-anAGE).